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(54) Titre : THERAPIES ANTI-CD20, THERAPIES ANTI-CD22, ET POLYTHERAPIES COMPRENANT UNE CELLULE EXPRIMANT LE RECEPTEUR ANTIGENIQUE CHIMERIQUE (CAR) DIRIGE CONTRE LE CD19

(54) Title: CD20 THERAPIES, CD22 THERAPIES, AND COMBINATION THERAPIES WITH A CD19 CHIMERIC ANTIGEN RECEPTOR (CAR)- EXPRESSING CELL

(57) Abrégé/Abstract:
The invention provides compositions and methods for treating diseases associated with expression of CD19, e.g., by administering a recombinant T cell comprising the CD19 CAR as described herein, in combination with one or more B-cell inhibitors, e.g., inhibitors of one or more of CD1O, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. The disclosure additionally features novel antigen binding domains and CAR molecules directed to CD20 and CD22, and uses, e.g., as monotherapies or in combination therapies. The invention also provides kits and compositions described herein.

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(54) **Title**: CD20 THERAPIES, CD22 THERAPIES, AND COMBINATION THERAPIES WITH A CD19 CHIMERIC ANTIGEN RECEPTOR (CAR) - EXPRESSING CELL

(57) **Abstract**: The invention provides compositions and methods for treating diseases associated with expression of CD19, e.g., by administering a recombinant T cell comprising the CD19 CAR as described herein, in combination with one or more B-cell inhibitors, e.g., inhibitors of one or more of CD1O, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. The disclosure additionally features novel antigen binding domains and CAR molecules directed to CD20 and CD22, and uses, e.g., as monotherapies or in combination therapies. The invention also provides kits and compositions described herein.

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CD20 THERAPIES, CD22 THERAPIES, AND COMBINATION THERAPIES WITH A CD19 CHIMERIC ANTIGEN RECEPTOR (CAR)- EXPRESSING CELL

This application claims priority to U.S. Serial No. 62/144,615 filed April 8, 2015, U.S. Serial No. 62/144,497 filed April 8, 2015 U.S. Serial No. 62/144,639 filed April 8, 2015, U.S. Serial No. 62/207,255 filed August 19, 2015, U.S. Serial No. 62/263,423 filed December 4, 2015, the contents of which are incorporated herein by reference in their entireties.

FIELD OF THE INVENTION

The present invention relates generally to the use of T cells engineered to express a Chimeric Antigen Receptor (CAR), optionally in combination with a B cell inhibitor, e.g., one or more inhibitors of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a to treat a disease associated with expression of the Cluster of Differentiation 19 protein (CD19).

BACKGROUND OF THE INVENTION

Many patients with B cell malignancies are incurable with standard therapy. In addition, traditional treatment options often have serious side effects. Attempts have been made in cancer immunotherapy, however, several obstacles render this a very difficult goal to achieve clinical effectiveness. Although hundreds of so-called tumor antigens have been identified, these are generally derived from self and thus are poorly immunogenic. Furthermore, tumors use several mechanisms to render themselves hostile to the initiation and propagation of immune attack.

Recent developments using chimeric antigen receptor (CAR) modified autologous T cell (CART) therapy, which relies on redirecting T cells to a suitable cell-surface molecule on cancer cells such as B cell malignancies, show promising results in harnessing the power of the immune system to treat B cell malignancies and other cancers (see, e.g., Sadelain et al., Cancer Discovery 3:388-398 (2013)). The clinical results of the murine derived CART19 (i.e., "CTL019") have shown promise in establishing complete remissions in patients suffering with CLL as well as in childhood ALL (see, e.g., Kalos et al., Sci Transl Med 3:95ra73 (2011), Porter et al., NEJM 365:725-733 (2011), Grupp et al., NEJM 368:1509-1518 (2013)). Besides the

ability for the chimeric antigen receptor on the genetically modified T cells to recognize and destroy the targeted cells, a successful therapeutic T cell therapy needs to have the ability to proliferate and persist over time, in order to survey for leukemic relapse. The variable quality of T cells, resulting from anergy, suppression, or exhaustion, will have effects on CAR-transformed T cells' performance, over which skilled practitioners have limited control at this time. To be effective, CAR transformed patient T cells need to persist and maintain the ability to proliferate in response to the cognate antigen. It has been shown that ALL patient T cells perform can do this with CART19 comprising a murine scFv (see, e.g., Grupp et al., NEJM 368:1509-1518 (2013)).

SUMMARY OF THE INVENTION

The disclosure features, at least in part, a method of treating a disorder associated with expression of the Cluster of Differentiation 19 protein (CD19) (e.g., OMIM Acc. No. 107265, Swiss Prot. Acc No. P15391). In certain embodiments, the disorder is a cancer, e.g., a hematological cancer. In some embodiments, the method comprises administering a Chimeric Antigen Receptor (CAR) molecule that binds CD19 in combination with a B-cell inhibitor, for example, one or more (e.g., one, two, three or more) B-cell inhibitors. In some embodiments, the B-cell inhibitor is chosen from an inhibitor of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, or ROR1, or a combination thereof. In some embodiments, the combination maintains or has better clinical effectiveness as compared to either therapy alone. In some embodiments, the methods herein involve the use of engineered cells, e.g., T cells, to express a CAR molecule that binds CD19, in combination with a B-cell inhibitor (e.g., an antibody (e.g., a mono- or bispecific antibody) to a second B target, e.g., CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, or ROR1) or a CAR-expressing cell e.g., a CAR-expressing immune effector cell, that binds to the second B cell target, or a combination thereof) to treat the disorder associated with expression of CD19. The disclosure additionally features novel antigen binding domains and CAR molecules directed to CD20 and CD22, and uses, e.g., as monotherapies or in combination therapies.

Accordingly, in one aspect, the invention pertains to a method of treating a subject (e.g., a mammal) having a disease associated with expression of CD19. The method comprises

administering to the subject a CD19 inhibitor, e.g., a CAR molecule that binds CD19 described herein, in combination with a B-cell inhibitor. For instance, the method comprises administering to the subject an effective number of one or more cells that express a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein (e.g., a wild-type or mutant CD19), in combination with a B-cell inhibitor. In certain embodiments, the B-cell inhibitor is chosen from a CD10 inhibitor, e.g., one or more CD10 inhibitors described herein; a CD20 inhibitor, e.g., one or more CD20 inhibitor described herein; a CD22 inhibitor, e.g., one or more CD22 inhibitors described herein; a CD34 inhibitor, e.g., one or more CD34 inhibitors described herein; a CD123 inhibitor, e.g., one or more CD123 inhibitor described herein; a FLT-3 inhibitor, e.g., one or more FLT-3 inhibitors described herein; an ROR1 inhibitor, e.g., one or more ROR1 inhibitor described herein; a CD79b inhibitor, e.g., one or more CD79b inhibitor described herein; a CD179b inhibitor, e.g., one or more CD179b inhibitor described herein; a CD79a inhibitor, e.g., one or more CD79a inhibitor described herein or any combination thereof. In certain aspects, a method of treating a subject having a B-cell leukemia or B-cell lymphoma, comprising administering to the subject an effective number of one or more cells that express a CAR molecule that binds CD19, in combination with one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a is disclosed.

In a related aspect, the present disclosure provides a method of reducing the proliferation of CD19-expressing cells, e.g., by administering to a subject, e.g., a patient in need thereof, a combination therapy as described herein, e.g., a CD19 inhibitor in combination with a B-cell inhibitor, e.g., one or more B-cell inhibitors as described herein. In another aspect, the present disclosure provides a method of selectively killing CD19-expressing cells, e.g., by administering to a subject, e.g., a patient in need thereof, a combination therapy as described herein, e.g., a CD19 inhibitor in combination with a B-cell inhibitor, e.g., one or more B-cell inhibitors as described herein. In certain aspects, the disclosure provides a method of providing an anti-tumor immunity in a subject, e.g., a mammal, comprising administering to the mammal an effective amount of a combination (e.g., one or more CAR-expressing cells) as described herein.

In an aspect, the disclosure provides a method of preventing a CD19-negative relapse in a mammal, comprising administering to the mammal one or more B-cell inhibitors, wherein the B-cell inhibitor comprises an inhibitor of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

In another aspect, the disclosure provides a method of treating a subject having a disease associated with expression of CD19, e.g., DLBCL (e.g. primary DLBCL). The method comprises administering to the subject an effective number of one or more cells that express a CAR molecule that binds CD19, e.g., a CD19 CAR, optionally in combination with a PD1 inhibitor. Optionally, the subject has, or is identified as having, at least 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of cancer cells, e.g., DLBCL cells, which are CD3+/PD1+.

In an aspect, the disclosure provides a method of treating a subject having a disease associated with expression of CD19, e.g., DLBCL. The method comprises administering to the subject an effective number of one or more cells that express a CAR molecule that binds CD19, e.g., a CD19 CAR, in combination with a PD-L1 inhibitor. Optionally, the subject has, or is identified as having, less than 20%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% of cells in the cancer, e.g., cancer microenvironment, are double positive for CD19 and PD-L1.

In an aspect, the disclosure provides one or more B-cell inhibitors, wherein the B-cell inhibitor comprises an inhibitor of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a, for use in the treatment of a subject having a disease associated with expression of CD19, and wherein said subject has received, is receiving or is about to receive a cell that expresses a CAR molecule that binds CD19, e.g., a CD19 CAR.

TIMING AND DOSAGE OF THE COMBINATION ADMINISTRATION

The one or more therapies described herein can be administered to the subject substantially at the same time or in any order. For instance, a CD19 inhibitor, e.g., a CD19 CAR-expressing cell described herein, the one or more B-cell inhibitor, and/or optionally the at least one additional therapeutic agent can be administered simultaneously, in the same or in separate compositions, or sequentially.

For sequential administration, the CAR-expressing cell described herein (e.g., a CD19 CAR-expressing cell, a CD20 CAR-expressing cell, or a CD22 CAR-expressing cell) can be administered first, and the additional agent can be administered second, or the order of administration can be reversed. In some embodiments, the first therapy (e.g., a CAR-expressing cell such as a CD19 CART cell, CD20 CART cell, or CD22 CART cell) is continued when the second therapy is introduced, and in other embodiments the first therapy is withdrawn before,

after, or at the same time as the second therapy is introduced. In instances of sequential administration, in some embodiments, the second therapy is initiated after a predetermined amount of time, or after the subject displays one or more indications that relapse has occurred or is likely to occur. The indication can be, e.g., the presence of cancer cells having a disturbance in the target of the first therapy, e.g., CD19, CD20, or CD22. The disturbance may be, e.g., a frameshift mutation and/or a premature stop codon.

In other embodiments, the two or more therapies (e.g., a CD19 CAR-expressing cell and a B-cell inhibitor) are administered simultaneously. Without being bound by theory, in some embodiments, simultaneous administration of the therapies can reduce the likelihood of relapse and/or delay relapse.

When administered in combination, the first therapy (e.g., CAR therapy, e.g., CAR-expressing cell directed against CD19, CD20, or CD22) and the additional agent (e.g., second or third agent, e.g., a B-cell inhibitor), or all, can be administered in an amount or dose that is higher, lower, or the same as the amount or dosage of each agent used individually, e.g., as a monotherapy. In certain embodiments, the administered amount or dosage of the first therapy, second therapy, optionally a third therapy, or all, is lower (e.g., at least 20%, at least 30%, at least 40%, or at least 50%) than the amount or dosage of each agent used individually, e.g., as a monotherapy. In other embodiments, the amount or dosage of the first therapy, second therapy, optionally a third therapy, or all, that results in a desired effect (e.g., treatment of cancer) is lower (e.g., at least 20%, at least 30%, at least 40%, or at least 50% lower) than the amount or dosage of each agent used individually, e.g., as a monotherapy, required to achieve the same therapeutic effect. In certain embodiments, the lower dose results in reduced side effects compared to those seen when the regular (monotherapy) dose is administered.

In an embodiment, the therapy comprises a population of cells. In embodiments, the cells are immune effector cells, e.g., CAR-expressing cells.

Alternatively, or in combination with the methods described herein, methods are disclosed that comprise a diagnostic step or a patient selection step, for instance as described below.

In one aspect, the invention provides a method of evaluating a subject, e.g., a patient, for relapser status (e.g. a relapser or a non-relapser after a CAR-therapy). In one embodiment, the

method identifies a subject, e.g., a patient, who has relapsed ("relapser") or who is are likely to relapse, or who has not relapsed ("non-relapser") or who is likely not to relapse, after treatment with a CAR therapy (e.g., a CD19 CART therapy, e.g., described herein, e.g., a CTL019 therapy). In an embodiment, relapser status (e.g. relapser or non-relapser after a CART therapy) is determined by assaying for one or more characteristics of CD19.

In one embodiment, the one or more characteristics of CD19 include an alteration in a nucleic acid sequence (e.g., a mutation such as an insertion, a deletion, or a substitution, or a combination thereof), an alteration in a nucleic acid level, an alteration in a protein sequence, or an alteration in a protein level, or a combination thereof. In one embodiment, a relapser has one or more mutations in CD19, e.g., one or more mutations (e.g. insertions or deletions) in exon 2 of CD19. In an embodiment, a relapser has one or more mutations in exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, or exon 7 of CD19. In an embodiment, the mutation produces a premature stop codon, e.g., by an insertion or deletion leading to a frameshift, e.g., in exon 2 of CD19. In an embodiment, the mutation is a mutation of **Table 31**.

In an embodiment, the characteristic of CD19 is compared to a reference characteristic. For example, when the characteristic is a sequence (e.g., protein or nucleic acid sequence from a biological sample), the reference characteristic can be a wild-type sequence (e.g., protein or nucleic acid sequence) of CD19. The characteristic may be the percent of cells in the sample having a mutant sequence. When the characteristic is a level (e.g., protein or nucleic acid level), the reference characteristic can be a wild-type level (e.g., protein or nucleic acid level) of CD19. The characteristic may be the level of protein or nucleic acid in the sample. The characteristic may be the percentage of cells in the sample that have a level of protein or nucleic acid that is above a given threshold.

In an embodiment, methods are provided for identifying a subject having cancer, e.g., a hematological cancer, such as, e.g., CLL or ALL, as being a relapser or non-relapser after a treatment that comprises a CAR therapy, e.g., a CD19 CART therapy. The method comprises: (1) acquiring a sample from the subject (e.g., an apheresis sample obtained from the blood of the subject; and/ or e.g., a manufactured product sample, e.g., genetically engineered T cells obtained from the blood of the subject); (2) determining a characteristic of CD19, e.g., a sequence or level as described herein; and (3) (optionally) comparing the determined characteristic of CD19 to a reference characteristic; wherein the difference, e.g., statistically

significant difference, between the determined characteristic compared to the reference characteristic is predictive of relapse to the CAR therapy; and (4) identifying the subject as a relapser or non-relapser to the CAR therapy, e.g., based on the determined characteristic of CD19. In one embodiment, the presence or absence of the characteristic of CD19 is the presence or absence of a premature stop codon, e.g., by an insertion or deletion leading to a frameshift. In an embodiment, the presence of the characteristic of CD19 is a mutation of **Table 31**.

In an embodiment, the provided methods comprise (1) acquiring a sample from the subject (e.g., an apheresis sample obtained from the blood of the subject; and/ or, e.g., a manufactured product sample, e.g., genetically engineered T cells obtained from the blood of the subject, e.g., a manufactured CART19 product); (2) determining a characteristic of CD19, e.g., a sequence or level as described herein; and (3) (optionally) comparing the determined characteristic of CD19 to a reference characteristic; wherein the presence of the characteristic of CD19 (e.g., the difference, e.g., a statistically significant difference, between the determined characteristic compared to the reference characteristic) is predictive of relapse to the CAR therapy. In one embodiment, the presence of the characteristic of CD19 is the presence of a premature stop codon, e.g., by an insertion or deletion leading to a frameshift. In an embodiment, the presence of the characteristic of CD19 is a mutation of **Table 31**.

In an embodiment, methods are provided for determining the relapse of a subject having cancer, e.g., a hematological cancer such as CLL or ALL, after a treatment comprising a CAR therapy, e.g., a CD19 CAR therapy as described herein. The method comprises determining a characteristic of CD19 in a sample obtained prior to relapse. In an embodiment, the presence of the characteristic of CD19 (e.g., the difference, e.g., a statistically significant difference, between the determined characteristic compared to the reference characteristic) is indicative of relapse after CAR therapy. In one embodiment, the presence of the characteristic of CD19 is the presence of a premature stop codon, e.g., by an insertion or deletion leading to a frameshift. In an embodiment, the presence of the characteristic of CD19 is a mutation of **Table 31**.

In an embodiment, methods are provided for evaluating a subject having cancer, e.g., a hematological cancer such as CLL or ALL. The method comprises acquiring a value of relapser status for the subject that comprises a measure of one or characteristics of CD19, e.g., one or more of the characteristics of CD19 as described herein, thereby evaluating the subject.

In an embodiment, methods are provided for evaluating or monitoring the effectiveness of a CAR therapy, e.g., a CD19 CART therapy, in a subject having cancer comprising acquiring a value of relapser status for the subject that comprises a measure of one or more characteristic of CD19, e.g., one or more of the characteristics of CD19 as described herein, thereby evaluating or monitoring the effectiveness of the CAR therapy in the subject

In an embodiment, methods are provided for providing a prediction for success rate of a CAR therapy, e.g., a CD19 CART therapy, e.g., described herein, in a subject having cancer, said method comprising steps of providing a biological sample from the subject; determining one or more characteristic of CD19, e.g., one or more of the characteristics of CD19 as described herein; and based on the characteristic determined, providing a prognosis to the subject.

In some aspects, the present disclosure provides, e.g., a method of, or assay for, identifying a subject having cancer as having an increased or decreased likelihood to respond to a treatment that comprises a chimeric antigen receptor (CAR) therapy, the method comprising:

(1) acquiring a sample from the subject;

(2) determining a value for one or more of:

(i) a level of one or more markers listed in **Table 29** in the sample;

(ii) a characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or

(iii) a level or activity of T_{REG} cells; and

(3) (optionally) comparing the determined value, e.g., the level, activity or characteristic of (i), (ii) or (iii) or a combination thereof, to a reference value, wherein the difference, e.g., a statistically significant difference, between the determined value compared to the reference value, is predictive of the subject's responsiveness to the CAR therapy; and

(4) identifying the subject as a complete responder, partial responder or non-responder, or relapse or non-relapser to the CAR therapy based on the determined value.

In certain embodiments, any of the aforesaid methods can further include the following:

(i) administering to the subject a therapeutically effective dose of a CAR therapy, e.g., a therapy comprising a CD19-expressing cell, if no difference, e.g., no statistically

significant difference, is detected in the value for one, two or more (all) of (i) the level or activity of one or more markers listed in **Table 29**; (ii) the characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) the level of T_{REG} cells in a biological sample;

(ii) administering to the subject a therapeutically effective dose of a CAR therapy, e.g., a therapy comprising a CD19-expressing cell and one or more B-cell inhibitor (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1 as described herein), if a difference, e.g., a statistically significant difference, is detected in the value for one, two or more (all) of (i) the level or activity of one or more markers listed in **Table 29**; (ii) the characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) the level of T_{REG} cells in a biological sample; or

(iii) discontinuing a first therapy, e.g., a therapy comprising a CD19-expressing cell, and administering a second therapy, e.g., one or more B-cell inhibitor (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1 as described herein), if a difference, e.g., a statistically significant difference, is detected in the value for one, two or more (or all) of (i) the level or activity of one or more markers listed in **Table 29**; (ii) the characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) the level of T_{REG} cells in a biological sample.

The administration steps (i)-(iii) can be performed before or after the patient evaluation steps, as described in exemplary embodiments below.

In certain aspects, a method for treating a subject having cancer is disclosed. The method comprises:

(a) acquiring, e.g., determining, if the subject has a value for one, two or more (all) of:

(i) a level of one or more markers listed in **Table 29**;

(ii) a characteristic of CD19, e.g., a mutation causing a frameshift or a premature stop codon or both, or

(iii) a level or activity of T_{REG} cells in a biological sample, and

(b) responsive to said value, further include the following:

(i) administering to the subject a therapeutically effective dose of a CAR therapy,

e.g., a therapy comprising a CD19-expressing cell, if no difference, e.g., no statistically significant difference, is detected in one, two or more (or all) of (i) the level or activity of one or more markers listed in **Table 29**; (ii) the characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) the level of T_{REG} cells in a biological sample;

(ii) administering to the subject a therapeutically effective dose of a CAR therapy, e.g., a therapy comprising a CD19-expressing cell and one or more B-cell inhibitor (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1 as described herein), if a difference, e.g., a statistically significant difference, is detected in one, two or more (or all) of (i) the level or activity of one or more markers listed in **Table 29**; (ii) the characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) the level of T_{REG} cells in a biological sample; or

(iii) discontinuing a first therapy, e.g., a therapy comprising a CD19-expressing cell, and administering a second therapy, e.g., one or more B-cell inhibitor (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1 as described herein), if a difference, e.g., a statistically significant difference, is detected in one or more of (i) the level or activity of one or more markers listed in **Table 29**; (ii) the characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) the level of T_{REG} cells in a biological sample.

In another aspect, a method for treating a subject having cancer is provided. The method includes:

(a) administering to a subject a therapeutically effective dose of a CAR therapy, e.g., a therapy comprising a CD19-expressing cell,

(b) acquiring a value for (e.g., determining if the subject has), one, two or more (all) of:

(I) a level of one or more markers listed in **Table 29**;

(II) a characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or

(III) a level or activity of T_{REG} cells in a biological sample, and

(c) in response to the value or determination in step (b) (I-III), performing one or more of the

following:

(i) administering to the subject a therapeutically effective dose of a CAR therapy, e.g., a therapy comprising a CD19-expressing cell, if no difference, e.g., no statistically significant difference, is detected in one or more of (I) the level or activity of one or more markers listed in **Table 29**; (II) the characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (III) the level of T_{REG} cells in a biological sample;

(ii) administering to the subject a therapeutically effective dose of a CAR therapy, e.g., a therapy comprising a CD19-expressing cell and one or more B-cell inhibitor (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1 as described herein), if a difference, e.g., a statistically significant difference, is detected in one or more of (I) the level or activity of one or more markers listed in **Table 29**; (II) the characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (III) the level of T_{REG} cells in a biological sample; or

(iii) discontinuing a first therapy, e.g., a therapy comprising a CD19-expressing cell, and administering a second therapy, e.g., one or more B-cell inhibitor (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1 as described herein), if a difference, e.g., a statistically significant difference, is detected in one or more of (I) the level or activity of one or more markers listed in **Table 29**; (II) the characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (III) the level of T_{REG} cells in a biological sample.

In some embodiments of any of the aforesaid methods, the sample is a biological sample selected from a blood, plasma, or a serum sample. In a particular embodiment, a biological sample is a blood sample. In one embodiment, the sample is an apheresis sample, e.g., T cells obtained from the blood of the subject. In an embodiment, the sample is a manufactured product sample, e.g. genetically engineered T cells obtained from the blood of the subject, e.g., a manufactured CAR product, e.g., a manufactured CART19 product.

In an embodiment, the methods herein can be used to determine if a patient is likely to respond to CAR therapy (e.g., CD19 CART), e.g., if a patient who has not received CAR therapy is likely to respond to CAR therapy, or if a patient who has received CAR therapy is

likely to respond to continued CAR therapy. In general, the same CD19 characteristics that predict relapse predict that a patient is less likely to respond to a CD19 CAR therapy. A patient who is identified as less likely to respond to a CD19 CAR therapy can be administered a different type of therapy, such as B-cell inhibitor (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a as described herein).

In another aspect, a method for treating a subject having cancer, e.g., a hematological cancer, is provided. In an embodiment, the method includes determining if a subject has a difference, e.g., statistically significant difference, in a characteristic of CD19 relative to a reference characteristic, and if there is a difference, e.g., statistically significant difference between the determined characteristic and reference characteristic, administering to the subject a therapeutically effective dose of a CAR therapy, e.g., CART, thereby treating the subject. In an embodiment, the characteristic is CD19 sequence, e.g., protein or nucleic acid sequence. In an embodiment, the method comprises assaying for the presence or absence of frameshifted CD19, e.g., CD19 comprising a premature stop codon.

In embodiments of any of the aforesaid methods, the treatment comprises administering a CD19 CAR-expressing cell, optionally in combination with one or more B-cell inhibitors. In an embodiment, the CD19 CAR therapy is administered simultaneously with one or more B-cell inhibitors (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a as described herein). In an embodiment, the CD19 CAR therapy is administered before the one or more of B-cell inhibitors. In an embodiment, the CD19 CAR therapy is administered after the one or more of B-cell inhibitors.

In an embodiment, wherein there is a difference between the determined characteristic and reference characteristic, the method comprises modifying the CAR product prior to infusion into the subject. In an embodiment, wherein there is a different between the determined characteristic and the reference characteristic, the method comprises modifying the manufacture of a CAR product prior to infusion into the subject. In an embodiment, if there is a difference between the determined characteristic and reference characteristic the method comprises adjusting the CAR infusion dose to achieve an anticancer effect.

In an embodiment, the methods of treatment comprise determining if a subject has an increased likelihood to respond to a CAR therapy, e.g., a CD19 CART therapy, e.g., a CD19

CART therapy described herein, by comparing a characteristic of CD19 in a sample from the subject relative to a reference characteristic, wherein a difference in the characteristic relative to the reference characteristic is indicative of an increased likelihood of response; and administering to the subject a therapeutically effective dose of a CAR therapy, thereby treating the subject.

In an embodiment, the methods of treatment comprise obtaining a sample from a subject; determining a characteristic of CD19 (e.g., the presence or absence of a frameshift or premature stop codon), relative to a reference characteristic; and administering a therapeutically effective dose of a CAR expressing cell, if the subject is identified as having a statistically significant difference between the CD19 characteristic of the sample and a reference characteristic in the sample.

The CD19 characteristic can be used to design a treatment for the patient. For example, in an embodiment, when a patient sample comprises wild-type CD19, the patient is administered a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CD19 CART. In an embodiment, when a patient sample comprises mutant CD19, e.g., frameshifted CD19, e.g., CD19 comprising a premature stop codon, the patient is administered a therapy other than a CD19 inhibitor, e.g., the patient is administered another B-cell inhibitor. In an embodiment, when a patient sample comprises at least normal levels of CD19, the patient is administered a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CD19 CART. In an embodiment, when a patient sample comprises lower than normal levels of CD19, the patient is administered a therapy other than a CD19 inhibitor, e.g., the patient is administered another B-cell inhibitor (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a as described herein).

In an embodiment, the methods of treatment comprise acquiring a value of relapser status for the subject that comprises a measure of a CD19 characteristic, and responsive to a determination of relapser status, performing one, two, three four or more of: (1) identifying the subject as a relapse or non-relapser; (2) administering a CAR therapy; (3) selecting or altering a dosing of a CAR therapy; (4) selecting or altering the schedule or time course of a CAR therapy; (5) administering, e.g., to a relapser, an additional agent in combination with the CAR therapy, e.g., administering one or more B-cell inhibitors; or a checkpoint inhibitor, e.g., a checkpoint inhibitor described herein, or a kinase inhibitor, e.g., a kinase inhibitor described herein; (6)

administering to a relapser a therapy that increases the number of naïve T cells in the subject prior to treatment with a CAR therapy; modifying a manufacturing process of a CAR therapy, e.g., enrich for naïve T cells prior to introducing a nucleic acid encoding a CAR, e.g., for a subject identified as a relapser; or (7) selecting an alternative therapy, e.g., a standard of care for a particular cancer (e.g., as described herein), e.g., for a relapser; thereby treating cancer in the subject.

In some embodiments, the method comprises administering one, two, three or more B-cell inhibitors (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1 as described herein). For instance, in an embodiment, the method includes administering a CD19 inhibitor, e.g., a cell expressing a CD19 CAR, in combination with a CD10 inhibitor, or any combination of a CD10 inhibitor and an inhibitor of CD20, CD22, CD34, CD123, FLT-3, or ROR1 as described herein. In another embodiment, the method includes administering a CD19 inhibitor, e.g., a cell expressing a CD19 CAR, in combination with a CD20 inhibitor, or any combination of a CD20 inhibitor and an inhibitor of CD10, CD22, CD34, CD123, FLT-3, or ROR1 as described herein. In another embodiment, the method includes administering a CD19 inhibitor, e.g., a cell expressing a CD19 CAR, in combination with a CD22 inhibitor, or any combination of a CD22 inhibitor and an inhibitor of CD10, CD20, CD34, CD123, FLT-3, or ROR1 as described herein. In another embodiment, the method includes administering a CD19 inhibitor, e.g., a cell expressing a CD19 CAR, in combination with a CD34 inhibitor, or any combination of a CD34 inhibitor and an inhibitor of CD10, CD20, CD22, CD123, FLT-3, or ROR1 as described herein. In another embodiment, the method includes administering a CD19 inhibitor, e.g., a cell expressing a CD19 CAR in combination with a CD123 inhibitor, or any combination of a CD123 inhibitor and an inhibitor of CD10, CD20, CD34, CD22, FLT-3, or ROR1 as described herein. In another embodiment, the method includes administering a CD19 inhibitor, e.g., a cell expressing a CD19 CAR, in combination with a FLT-3 inhibitor, or any combination of a FLT-3 inhibitor and an inhibitor of CD10, CD20, CD34, CD123, or ROR1 as described herein. In another embodiment, the method includes administering a CD19 inhibitor, e.g., a cell expressing a CD19 CAR, in combination with a ROR1 inhibitor, or any combination of a ROR1 inhibitor and an inhibitor of CD10, CD20, CD34, CD123, or FLT-3, as described herein. In some embodiments, the method comprises administering one, two, three or more B-cell inhibitors (e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, or

ROR1, CD79b, CD179b, or CD79a as described herein).

In some embodiments, the methods of treatment described herein further comprise one or both of: determining a level of an immune checkpoint molecule (e.g., PD-L1, PD1, LAG3, or TIM3) in a patient sample; and administering an immune checkpoint inhibitor (e.g., an inhibitor of one or more of PD-L1, PD1, LAG3, and TIM3) to the patient. For example, the method can comprise treating a patient with one or more CAR-expressing cells described herein (e.g., CD19 CAR in combination with a B-cell inhibitor, CD20 CAR, or CD22 CAR) and determining the level of an immune checkpoint molecule in the patient before or after the treatment. In some embodiments, the method comprises administering the immune checkpoint inhibitor to a patient that has elevated levels of the immune checkpoint molecule compared to a reference level, e.g., administering a PD-L1 inhibitor in response to elevated PD-L1 levels, administering a PD1 inhibitor in response to elevated PD1 levels, administering a LAG3 inhibitor in response to elevated LAG3 levels, or administering a TIM3 inhibitor in response to elevated TIM3 levels. In some embodiments, the method comprises administering an immune checkpoint inhibitor to a patient who has received, is receiving, or is about to receive therapy with one or more CAR-expressing cells described herein (e.g., CD19 CAR in combination with a B-cell inhibitor, CD20 CAR, or CD22 CAR), wherein the patient has, or is identified as having, elevated levels of the immune checkpoint molecule compared to a reference level.

COMPOSITIONS

In some aspects, the present disclosure provides, e.g., a composition comprising: (i) one or more cells that express a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein, e.g., a CD19 CAR, and (ii) a B-cell inhibitor, e.g., one or more inhibitors of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1. In embodiments, (i) and (ii) are provided separately, and in embodiments, (i) and (ii) are admixed.

In some aspects, the present disclosure provides, e.g., a nucleic acid encoding: (i) a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein, e.g., a CD19 CAR, and (ii) one or more B-cell inhibitors, e.g., inhibitors of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1. In some aspects, the present disclosure provides, e.g., a nucleic acid encoding: (i) a CAR molecule that binds CD19, e.g., a CAR molecule that binds

CD19 described herein, e.g., a CD19 CAR, and (ii) a CAR molecule that binds a B-cell antigen, e.g., one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1. In embodiments, the nucleic acid comprises RNA or DNA.

In some aspects, the present disclosure provides, e.g., a nucleic acid encoding: (i) a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein, e.g., a CD19 CAR, and (ii) a CAR molecule that binds a B-cell antigen, e.g., one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. In embodiments, the nucleic acid comprises RNA or DNA. In embodiments, the nucleic acid sequences encoding (i) and (ii) are situated in the same orientation, e.g., transcription of the nucleic acid sequences encoding (i) and (ii) proceeds in the same direction. In embodiments, the nucleic acid sequences encoding (i) and (ii) are situated in different orientations. In embodiments, a single promoter controls expression of the nucleic acid sequences encoding (i) and (ii). In embodiments, a nucleic acid encoding a protease cleavage site (such as a T2A, P2A, E2A, or F2A cleavage site) is situated between the nucleic acid sequences encoding (i) and (ii). In embodiments, the protease cleavage site is placed such that a cell can express a fusion protein comprising (i) and (ii), which protein is subsequently processed into two peptides by proteolytic cleavage. In some embodiments, the nucleic acid sequences encoding (i) is upstream of the nucleic acid sequences encoding (ii), or the nucleic acid sequences encoding (ii) is upstream of the nucleic acid sequences encoding (i). In embodiments, a first promoter controls expression of the nucleic acid sequence encoding (i) and a second promoter controls expression of the nucleic acid sequence encoding (ii). In embodiments, the nucleic acid is a plasmid. In embodiments, the nucleic acid comprises a viral packaging element. In some aspects, the present disclosure provides a cell, e.g., an immune effector cell, comprising the nucleic acid described herein, e.g., a nucleic acid comprising (i) and (ii) as described above. The cell may comprise a protease (e.g., endogenous or exogenous) that cleaves a T2A, P2A, E2A, or F2A cleavage site.

In some aspects, the present disclosure provides, e.g., a composition comprising: (i) a first nucleic acid encoding a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein, e.g., a CD19 CAR, and (ii) a second nucleic acid encoding one or more B-cell inhibitors, e.g., inhibitors of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1. In some aspects, the present disclosure provides, e.g., a composition comprising: (i) a first nucleic acid encoding a CAR molecule that binds CD19, e.g., a CAR molecule that binds

CD19 described herein, e.g., a CD19 CAR, and (ii) a CAR molecule that binds a B-cell antigen, e.g., one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1. In embodiments, the first nucleic acid and second nucleic acid each comprises RNA or DNA.

In some aspects, the present disclosure provides, e.g., a vector comprising a nucleic acid or nucleic acids as described herein. The present disclosure also provides, in certain aspects, a cell comprising a vector or nucleic acid as described herein.

This disclosure also provides, in certain aspects, a composition comprising one or more immune effector cells and: (i) a first nucleic acid encoding, or a first polypeptide comprising, a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein, e.g., a CD19 CAR, and (ii) a second nucleic acid encoding, or a second polypeptide comprising, a CAR molecule that binds a B-cell antigen, e.g., one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. In embodiments, the first nucleic acid or first polypeptide and the second nucleic acid or second polypeptide are each contained within, e.g., expressed by, a first immune effector cell. In embodiments, the composition comprises a first immune effector cell containing e.g., expressing the first nucleic acid or first polypeptide and a second immune effector cell containing e.g., expressing the second nucleic acid or second polypeptide. In embodiments, the composition does not comprise a cell containing, e.g., expressing, both of the first nucleic acid or first polypeptide and the second nucleic acid or second polypeptide.

MANUFACTURING

In certain aspects, the disclosure provides a method of making a cell, comprising transducing an immune effector cell, e.g., a T cell or NK cell, with a vector as described herein, e.g., a vector encoding a CAR. In certain aspects, the disclosure provides a method of making a cell, comprising introducing a nucleic acid as described herein (e.g., a nucleic acid encoding a CAR) into an immune effector cell, e.g., a T cell or NK cell. In certain aspects, the disclosure provides a method of generating a population of RNA-engineered cells comprising introducing an in vitro transcribed RNA or synthetic RNA into a cell, where the RNA comprises a nucleic acid as described herein, e.g., a nucleic acid encoding a CAR.

In some embodiments, the methods of making disclosed herein further comprise

contacting the population of cells, (e.g., CD19 CAR-expressing cells, CD20 CAR-expressing cells, CD22 CAR-expressing cells, B-cell inhibitor cells, or both of CD19 CAR-expressing cells and B-cell inhibitor cells), with a nucleic acid encoding a telomerase subunit, e.g., hTERT. The nucleic acid encoding the telomerase subunit can be DNA.

In some embodiments, the method of making disclosed herein further comprises culturing the population of cells, (e.g., CD19 CAR-expressing cells, CD20 CAR-expressing cells, CD22 CAR-expressing cells, B-cell inhibitor cells, or both of CD19 CAR-expressing cells and B-cell inhibitor cells), in serum comprising 2% hAB serum.

INDICATIONS

In one embodiment, the disease associated with CD19 expression is selected from a proliferative disease such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia, or is a non-cancer related indication associated with expression of CD19. In one embodiment, the disease is a solid or a liquid tumor. In one embodiment, the cancer is a pancreatic cancer. In one embodiment, the disease is a hematologic cancer. In one embodiment, the hematologic cancer is a leukemia. In one embodiment, the cancer is selected from the group consisting of one or more acute leukemias including but not limited to B-cell acute lymphoid leukemia (BALL), T-cell acute lymphoid leukemia (TALL), small lymphocytic leukemia (SLL), acute lymphoid leukemia (ALL) (e.g., relapsing and refractory ALL); one or more chronic leukemias including but not limited to chronic myelogenous leukemia (CML), and chronic lymphocytic leukemia (CLL). Additional hematologic cancers or conditions include, but are not limited to mantle cell lymphoma (MCL), B cell prolymphocytic leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma, follicular lymphoma, hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions, MALT lymphoma, Marginal zone lymphoma, multiple myeloma, myelodysplasia and myelodysplastic syndrome, non-Hodgkin lymphoma, Hodgkin lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, and "preleukemia." Preleukemia encompasses a diverse collection of hematological conditions united by ineffective production (or dysplasia) of myeloid blood cells In embodiments, a disease associated with CD19 expression include, but not limited to atypical and/or non-classical

cancers, malignancies, precancerous conditions or proliferative diseases expressing CD19; and any combination thereof.

In one embodiment, the disease associated with expression of CD19 is a lymphoma, e.g., MCL or Hodgkin lymphoma. In one embodiment, the disease associated with expression of CD19 is leukemia, e.g., SLL, CLL and/or ALL.

In one embodiment, the disease associated with a tumor antigen, e.g., a tumor antigen described herein, is selected from a proliferative disease such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia, or is a non-cancer related indication associated with expression of a tumor antigen described herein. In an embodiment, the disease associated with a tumor antigen described herein is a solid tumor, e.g., a solid tumor described herein, e.g., prostatic, colorectal, pancreatic, cervical, gastric, ovarian, head, or lung cancer.

In an embodiment, the cancer is chosen from AML, ALL, B-ALL, T-ALL, B-cell prolymphocytic leukemia, chronic lymphocytic leukemia, CML, hairy cell leukemia, Hodgkin lymphoma, mast cell disorder, myelodysplastic syndrome, myeloproliferative neoplasm, plasma cell myeloma, plasmacytoid dendritic cell neoplasm, or a combination thereof.

In an embodiment, the subject (e.g., a subject to be treated with a CD19 CAR, optionally in combination with a second agent such as a PD1 inhibitor or PD-L1 inhibitor) has, or is identified as having, at least 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of cancer cells, e.g., DLBCL cells, which are CD3+/PD1+.

In an embodiment, the subject has relapsed or is identified as having relapsed after treatment with the one or more cells that express a CAR molecule that binds CD19, e.g., a CD19 CAR. In an embodiment, the subject has relapsed or is identified as having relapsed based on one or more of reappearance of blasts in the blood, bone marrow (> 5%), or any extramedullary site, after a complete response. In an embodiment, the subject has relapsed or is identified as having relapsed based on detection of CD19- blasts above a predetermined threshold, e.g., over 1%, 2%, 3%, 4%, 5%, or 10%.

CAR THERAPIES

In certain embodiments, the method of treatment comprises a CAR therapy, e.g.,

administration of one or more cells that express one or more CAR molecules. A cell expressing one or more CAR molecules can be an immune effector cell, e.g., a T cell or NK cell. In an embodiment, the subject is a human.

In one embodiment, the cell expressing the CAR molecule comprises a vector that includes a nucleic acid sequence encoding the CAR molecule. In one embodiment, the vector is selected from the group consisting of a DNA, an RNA, a plasmid, a lentivirus vector, adenoviral vector, or a retrovirus vector. In one embodiment, the vector is a lentivirus vector. In one embodiment, the vector further comprises a promoter. In one embodiment, the promoter is an EF-1 promoter. In one embodiment, the EF-1 promoter comprises a sequence of SEQ ID NO: 100. In one embodiment, the vector is an *in vitro* transcribed vector, e.g., a vector that transcribes RNA of a nucleic acid molecule described herein. In one embodiment, the nucleic acid sequence in the *in vitro* vector further comprises a poly(A) tail, e.g., a poly A tail described herein, e.g., comprising about 150 adenosine bases. In one embodiment, the nucleic acid sequence in the *in vitro* vector further comprises a 3'UTR, e.g., a 3' UTR described herein, e.g., comprising at least one repeat of a 3'UTR derived from human beta-globulin. In one embodiment, the nucleic acid sequence in the *in vitro* vector further comprises promoter. In one embodiment, the nucleic acid sequence comprises a T2A sequence.

In one embodiment, the cell expressing the CAR molecule is a cell described herein, e.g., a human T cell or a human NK cell, e.g., a human T cell described herein or a human NK cell described herein. In one embodiment, the human T cell is a CD8+ T cell. In one embodiment, the human T cell is a CD4+ T cell. In one embodiment, the human T cell is a CD4+/CD8+ T cell. In one embodiment the human T cell is a mixture of CD8+ and CD4+ T cells. In one embodiment, the cell is an autologous T cell. In one embodiment, the cell is an allogeneic T cell. In one embodiment, the cell is a T cell and the T cell is diacylglycerol kinase (DGK) deficient. In one embodiment, the cell is a T cell and the T cell is Ikaros deficient. In one embodiment, the cell is a T cell and the T cell is both DGK and Ikaros deficient.

In another embodiment, the cell expressing the CAR molecule, e.g., as described herein, can further express another agent, e.g., an agent which enhances the activity of a CAR-expressing cell.

In one embodiment, the method includes administering a cell expressing the CAR

molecule, as described herein, in combination with an agent which enhances the activity of a CAR-expressing cell, wherein the agent is a cytokine, e.g., IL-7, IL-15, IL-21, or a combination thereof. The cytokine can be delivered in combination with, e.g., simultaneously or shortly after, administration of the CAR-expressing cell. Alternatively, the cytokine can be delivered after a prolonged period of time after administration of the CAR-expressing cell, e.g., after assessment of the subject's response to the CAR-expressing cell.

For example, in one embodiment, the agent that enhances the activity of a CAR-expressing cell can be an agent which inhibits an immune inhibitory molecule. Examples of immune inhibitory molecules include PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 and TGFR beta. In one embodiment, the agent that inhibits an immune inhibitory molecule comprises a first polypeptide, e.g., an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, e.g., an intracellular signaling domain described herein. In one embodiment, the agent comprises a first polypeptide, e.g., of an immune inhibitory molecule such as PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 or TGFR beta, or a fragment of any of these (e.g., at least a portion of the extracellular domain of any of these), and a second polypeptide which is an intracellular signaling domain described herein (e.g., comprising a costimulatory domain (e.g., 41BB, CD27 or CD28, e.g., as described herein) and/or a primary signaling domain (e.g., a CD3 zeta signaling domain described herein). In one embodiment, the agent comprises a first polypeptide of PD1 or a fragment thereof (e.g., at least a portion of the extracellular domain of PD1), and a second polypeptide of an intracellular signaling domain described herein (e.g., a CD28 signaling domain described herein and/or a CD3 zeta signaling domain described herein).

In one embodiment, lymphocyte infusion, for example allogeneic lymphocyte infusion, is used in the treatment of the cancer, wherein the lymphocyte infusion comprises at least one CD19 CAR-expressing cell described herein and optionally at least one cell expressing a CAR directed against a B-cell antigen. In one embodiment, autologous lymphocyte infusion is used in the treatment of the cancer, wherein the autologous lymphocyte infusion comprises at least one CD19-expressing cell and optionally at least one cell expressing a CAR directed against a B-cell antigen.

In one embodiment, the CAR expressing cell, e.g., T cell, is administered to a subject that has received a previous stem cell transplantation, e.g., autologous stem cell transplantation, or a subject that has received a previous dose of melphalan.

In one embodiment, the cell expressing the CAR molecule, e.g., a CAR molecule described herein, is administered in combination with an agent that ameliorates one or more side effect associated with administration of a cell expressing a CAR molecule or with administration of the B-cell inhibitor, e.g., an agent described herein.

In one embodiment, the cell expressing the CAR molecule, e.g., a CD19 CAR molecule described herein, and the B-cell inhibitor are administered in combination with an additional agent that treats the disease associated with CD19, e.g., an additional agent described herein.

In one embodiment, the cells expressing a CAR molecule, e.g., a CAR molecule described herein, are administered at a dose and/or dosing schedule described herein.

In one embodiment, the CAR molecule is introduced into T cells, e.g., using *in vitro* transcription, and the subject (e.g., human) receives an initial administration of cells comprising a CAR molecule, and one or more subsequent administrations of cells comprising a CAR molecule, wherein the one or more subsequent administrations are administered less than 15 days, e.g., 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 days after the previous administration. In one embodiment, more than one administration of cells comprising a CAR molecule are administered to the subject (e.g., human) per week, e.g., 2, 3, or 4 administrations of cells comprising a CAR molecule are administered per week. In one embodiment, the subject (e.g., human subject) receives more than one administration of cells comprising a CAR molecule per week (e.g., 2, 3 or 4 administrations per week) (also referred to herein as a cycle), followed by a week of no administration of cells comprising a CAR molecule, and then one or more additional administration of cells comprising a CAR molecule (e.g., more than one administration of the cells comprising a CAR molecule per week) is administered to the subject. In another embodiment, the subject (e.g., human subject) receives more than one cycle of cells comprising a CAR molecule, and the time between each cycle is less than 10, 9, 8, 7, 6, 5, 4, or 3 days. In one embodiment, the cells comprising a CAR molecule are administered every other day for 3 administrations per week. In one embodiment, the cells comprising a CAR molecule are administered for at least two, three, four, five, six, seven, eight or more weeks.

In one embodiment, the therapy described herein (e.g., a CD20 CAR therapy, a CD22 CAR therapy, or a combination of the B-cell inhibitor and the cells expressing a CD19 CAR molecule, e.g., a CD19 CAR molecule described herein) are administered as a first line treatment for the disease, e.g., the cancer, e.g., the cancer described herein. In another embodiment, the therapy described herein (e.g., a CD20 CAR therapy, a CD22 CAR therapy, or a combination of the B-cell inhibitor and the cells expressing a CD19 CAR molecule, e.g., a CD19 CAR molecule described herein) are administered as a second, third, fourth line treatment for the disease, e.g., the cancer, e.g., the cancer described herein.

In one embodiment, a population of cells described herein is administered. In some embodiments the population of cells is isolated or purified.

In one embodiment, the method includes administering a population of cells, a plurality of which comprise a CAR molecule described herein. In some embodiments, the population of CAR-expressing cells comprises a mixture of cells expressing different CARs. For example, in one embodiment, the population of CAR-expressing cells can include a first cell expressing a CAR having an anti-CD19 binding domain described herein, and a second cell expressing a CAR having a different B-cell antigen binding domain. In embodiments, the first and second cell populations are T cells. In embodiments, the first and second populations of T cells are the same isotype, e.g., are both CD4+ T cells, or are both CD8+ T cells. In other embodiments, the first and second populations of T cells are different isotypes, e.g., the first population comprises CD4+ T cells and the second population comprises CD8+ T cells. In embodiments, the first and second populations of T cells are cell types described in WO2012/129514, which is herein incorporated by reference in its entirety. As another example, a population of cells can comprise a single cell type that expresses both a CAR having an anti-CD19 binding domain described herein and a CAR having a different B-cell antigen binding domain. As another example, a population of cells can comprise a single cell type that expresses a CAR having two or more (e.g., 2, 3, 4, or 5) B-cell antigen binding domains, e.g., is a bispecific CAR, e.g., as described herein. As another example, the population of CAR-expressing cells can include a first cell expressing a CAR that includes an anti- CD19 binding domain, e.g., as described herein, and a second cell expressing a CAR that includes an antigen binding domain to a target other than CD19 (e.g., CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, CD79a, or mesothelin). In one embodiment, the population of CAR-expressing cells includes, e.g., a first

cell expressing a CAR that includes a primary intracellular signaling domain, and a second cell expressing a CAR that includes a secondary signaling domain. In one embodiment, the population of CAR-expressing cells includes, e.g., a first cell expressing a CAR that includes a first secondary signaling domain, and a second cell expressing a CAR that includes a secondary signaling domain different from the first secondary signaling domain.

As an example, when the first B-cell inhibitor is a CD19 CAR-expressing cell and the second B-cell inhibitor is a CD10 CAR-expressing cell, the first CAR and second CAR may be expressed by the same cell type or different types. For instance, in some embodiments, the cell expressing a CD19 CAR is a CD4+ T cell and the cell expressing a CD10 CAR is a CD8+ T cell, or the cell expressing a CD19 CAR is a CD8+ T cell and the cell expressing a CD10 CAR is a CD4+ T cell. In other embodiments, the cell expressing a CD19 CAR is a T cell and the cell expressing a CD10 CAR is a NK cell, or the cell expressing a CD19 CAR is a NK cell and the cell expressing a CD10 CAR is a T cell. In other embodiments, the cell expressing a CD19 CAR and the cell expressing a CD10 CAR are both NK cells or are both T cells, e.g., are both CD4+ T cells, or are both CD8+ T cells. In yet other embodiments, a single cell expresses the CD19 CAR and CD10 CAR, and this cell is, e.g., a NK cell or a T cell such as a CD4+ T cell or CD8+ T cell. The first CAR and second CAR can comprise the same or different intracellular signaling domains. For instance, in some embodiments the CD19 CAR comprises a CD3 zeta signaling domain and the CD10 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain, while in some embodiments, the CD19 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain and the CD10 CAR comprises a CD3 zeta signaling domain. In other embodiments, each of the CD19 CAR and the CD10 CAR comprises the same type of primary signaling domain, e.g., a CD3 zeta signaling domain, but the CD19 CAR and the CD10 CAR comprise different costimulatory domains, e.g., (1) the CD19 CAR comprises a 41BB costimulatory domain and the CD10 CAR comprises a different costimulatory domain e.g., a CD27 costimulatory domain, (2) the CD19 CAR comprises a CD27 costimulatory domain and the CD10 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (3) the CD19 CAR comprises a 41BB costimulatory domain and the CD10 CAR comprises a CD28 costimulatory domain, (4) the CD19 CAR comprises a CD28 costimulatory domain and the CD10 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (5) the CD19 CAR comprises a CD27

costimulatory domain and the CD10 CAR comprises a CD28 costimulatory domain, or (6) the CD19 CAR comprises a CD28 costimulatory domain and the CD10 CAR comprises a CD27 costimulatory domain. In another embodiment, a cell comprises a CAR that comprises both a CD19 antigen-binding domain and a CD10 antigen-binding domain, e.g., a bispecific antibody.

As another example, when the first B-cell inhibitor is a CD19 CAR-expressing cell and the second B-cell inhibitor is a CD20 CAR-expressing cell, the first CAR and second CAR may be expressed by the same cell type or different types. For instance, in some embodiments, the cell expressing a CD19 CAR is a CD4+ T cell and the cell expressing a CD20 CAR is a CD8+ T cell, or the cell expressing a CD19 CAR is a CD8+ T cell and the cell expressing a CD20 CAR is a CD4+ T cell. In other embodiments, the cell expressing a CD19 CAR is a T cell and the cell expressing a CD20 CAR is a NK cell, or the cell expressing a CD19 CAR is a NK cell and the cell expressing a CD20 CAR is a T cell. In other embodiments, the cell expressing a CD19 CAR and the cell expressing a CD20 CAR are both NK cells or are both T cells, e.g., are both CD4+ T cells, or are both CD8+ T cells. In yet other embodiments, a single cell expresses the CD19 CAR and CD20 CAR, and this cell is, e.g., a NK cell or a T cell such as a CD4+ T cell or CD8+ T cell. The first CAR and second CAR can comprise the same or different intracellular signaling domains. For instance, in some embodiments the CD19 CAR comprises a CD3 zeta signaling domain and the CD20 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain, while in some embodiments, the CD19 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain and the CD20 CAR comprises a CD3 zeta signaling domain. In other embodiments, each of the CD19 CAR and the CD20 CAR comprises the same type of primary signaling domain, e.g., a CD3 zeta signaling domain, but the CD19 CAR and the CD20 CAR comprise different costimulatory domains, e.g., (1) the CD19 CAR comprises a 41BB costimulatory domain and the CD20 CAR comprises a different costimulatory domain e.g., a CD27 costimulatory domain, (2) the CD19 CAR comprises a CD27 costimulatory domain and the CD20 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (3) the CD19 CAR comprises a 41BB costimulatory domain and the CD20 CAR comprises a CD28 costimulatory domain, (4) the CD19 CAR comprises a CD28 costimulatory domain and the CD20 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (5) the CD19 CAR comprises a CD27 costimulatory domain and the CD20 CAR comprises a CD28 costimulatory domain, or (6) the

CD19 CAR comprises a CD28 costimulatory domain and the CD20 CAR comprises a CD27 costimulatory domain. In another embodiment, a cell comprises a CAR that comprises both a CD19 antigen-binding domain and a CD20 antigen-binding domain, e.g., a bispecific antibody.

As another example, when the first B-cell inhibitor is a CD19 CAR-expressing cell and the second B-cell inhibitor is a CD22 CAR-expressing cell, the first CAR and second CAR may be expressed by the same cell type or different types. For instance, in some embodiments, the cell expressing a CD19 CAR is a CD4+ T cell and the cell expressing a CD22 CAR is a CD8+ T cell, or the cell expressing a CD19 CAR is a CD8+ T cell and the cell expressing a CD22 CAR is a CD4+ T cell. In other embodiments, the cell expressing a CD19 CAR is a T cell and the cell expressing a CD22 CAR is a NK cell, or the cell expressing a CD19 CAR is a NK cell and the cell expressing a CD22 CAR is a T cell. In other embodiments, the cell expressing a CD19 CAR and the cell expressing a CD22 CAR are both NK cells or are both T cells, e.g., are both CD4+ T cells, or are both CD8+ T cells. In yet other embodiments, a single cell expresses the CD19 CAR and CD22 CAR, and this cell is, e.g., a NK cell or a T cell such as a CD4+ T cell or CD8+ T cell. The first CAR and second CAR can comprise the same or different intracellular signaling domains. For instance, in some embodiments the CD19 CAR comprises a CD3 zeta signaling domain and the CD22 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain, while in some embodiments, the CD19 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain and the CD22 CAR comprises a CD3 zeta signaling domain. In other embodiments, each of the CD19 CAR and the CD22 CAR comprises the same type of primary signaling domain, e.g., a CD3 zeta signaling domain, but the CD19 CAR and the CD22 CAR comprise different costimulatory domains, e.g., (1) the CD19 CAR comprises a 41BB costimulatory domain and the CD22 CAR comprises a different costimulatory domain e.g., a CD27 costimulatory domain, (2) the CD19 CAR comprises a CD27 costimulatory domain and the CD22 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (3) the CD19 CAR comprises a 41BB costimulatory domain and the CD22 CAR comprises a CD28 costimulatory domain, (4) the CD19 CAR comprises a CD28 costimulatory domain and the CD22 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (5) the CD19 CAR comprises a CD27 costimulatory domain and the CD22 CAR comprises a CD28 costimulatory domain, or (6) the CD19 CAR comprises a CD28 costimulatory domain and the CD22 CAR comprises a CD27

costimulatory domain. In another embodiment, a cell comprises a CAR that comprises both a CD19 antigen-binding domain and a CD22 antigen-binding domain, e.g., a bispecific antibody.

As another example, when the first B-cell inhibitor is a CD19 CAR-expressing cell and the second B-cell inhibitor is a CD34 CAR-expressing cell, the first CAR and second CAR may be expressed by the same cell type or different types. For instance, in some embodiments, the cell expressing a CD19 CAR is a CD4+ T cell and the cell expressing a CD34 CAR is a CD8+ T cell, or the cell expressing a CD19 CAR is a CD8+ T cell and the cell expressing a CD34 CAR is a CD4+ T cell. In other embodiments, the cell expressing a CD19 CAR is a T cell and the cell expressing a CD34 CAR is a NK cell, or the cell expressing a CD19 CAR is a NK cell and the cell expressing a CD34 CAR is a T cell. In other embodiments, the cell expressing a CD19 CAR and the cell expressing a CD34 CAR are both NK cells or are both T cells, e.g., are both CD4+ T cells, or are both CD8+ T cells. In yet other embodiments, a single cell expresses the CD19 CAR and CD34 CAR, and this cell is, e.g., a NK cell or a T cell such as a CD4+ T cell or CD8+ T cell. The first CAR and second CAR can comprise the same or different intracellular signaling domains. For instance, in some embodiments the CD19 CAR comprises a CD3 zeta signaling domain and the CD34 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain, while in some embodiments, the CD19 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain and the CD34 CAR comprises a CD3 zeta signaling domain. In other embodiments, each of the CD19 CAR and the CD34 CAR comprises the same type of primary signaling domain, e.g., a CD3 zeta signaling domain, but the CD19 CAR and the CD34 CAR comprise different costimulatory domains, e.g., (1) the CD19 CAR comprises a 41BB costimulatory domain and the CD34 CAR comprises a different costimulatory domain e.g., a CD27 costimulatory domain, (2) the CD19 CAR comprises a CD27 costimulatory domain and the CD34 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (3) the CD19 CAR comprises a 41BB costimulatory domain and the CD34 CAR comprises a CD28 costimulatory domain, (4) the CD19 CAR comprises a CD28 costimulatory domain and the CD34 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (5) the CD19 CAR comprises a CD27 costimulatory domain and the CD34 CAR comprises a CD28 costimulatory domain, or (6) the CD19 CAR comprises a CD28 costimulatory domain and the CD34 CAR comprises a CD27 costimulatory domain. In another embodiment, a cell comprises a CAR that comprises both a

CD19 antigen-binding domain and a CD34 antigen-binding domain, e.g., a bispecific antibody.

As another example, when the first B-cell inhibitor is a CD19 CAR-expressing cell and the second B-cell inhibitor is a CD123 CAR-expressing cell, the first CAR and second CAR may be expressed by the same cell type or different types. For instance, in some embodiments, the cell expressing a CD19 CAR is a CD4+ T cell and the cell expressing a CD123 CAR is a CD8+ T cell, or the cell expressing a CD19 CAR is a CD8+ T cell and the cell expressing a CD123 CAR is a CD4+ T cell. In other embodiments, the cell expressing a CD19 CAR is a T cell and the cell expressing a CD123 CAR is a NK cell, or the cell expressing a CD19 CAR is a NK cell and the cell expressing a CD123 CAR is a T cell. In other embodiments, the cell expressing a CD19 CAR and the cell expressing a CD123 CAR are both NK cells or are both T cells, e.g., are both CD4+ T cells, or are both CD8+ T cells. In yet other embodiments, a single cell expresses the CD19 CAR and CD123 CAR, and this cell is, e.g., a NK cell or a T cell such as a CD4+ T cell or CD8+ T cell. The first CAR and second CAR can comprise the same or different intracellular signaling domains. For instance, in some embodiments the CD19 CAR comprises a CD3 zeta signaling domain and the CD123 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain, while in some embodiments, the CD19 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain and the CD123 CAR comprises a CD3 zeta signaling domain. In other embodiments, each of the CD19 CAR and the CD123 CAR comprises the same type of primary signaling domain, e.g., a CD3 zeta signaling domain, but the CD19 CAR and the CD123 CAR comprise different costimulatory domains, e.g., (1) the CD19 CAR comprises a 41BB costimulatory domain and the CD123 CAR comprises a different costimulatory domain e.g., a CD27 costimulatory domain, (2) the CD19 CAR comprises a CD27 costimulatory domain and the CD123 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (3) the CD19 CAR comprises a 41BB costimulatory domain and the CD123 CAR comprises a CD28 costimulatory domain, (4) the CD19 CAR comprises a CD28 costimulatory domain and the CD123 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (5) the CD19 CAR comprises a CD27 costimulatory domain and the CD123 CAR comprises a CD28 costimulatory domain, or (6) the CD19 CAR comprises a CD28 costimulatory domain and the CD123 CAR comprises a CD27 costimulatory domain. In another embodiment, a cell comprises a CAR that comprises both a CD19 antigen-binding domain and a CD123 antigen-

binding domain, e.g., a bispecific antibody.

As another example, when the first B-cell inhibitor is a CD19 CAR-expressing cell and the second B-cell inhibitor is a FLT-3 CAR-expressing cell, the first CAR and second CAR may be expressed by the same cell type or different types. For instance, in some embodiments, the cell expressing a CD19 CAR is a CD4+ T cell and the cell expressing a FLT-3 CAR is a CD8+ T cell, or the cell expressing a CD19 CAR is a CD8+ T cell and the cell expressing a FLT-3 CAR is a CD4+ T cell. In other embodiments, the cell expressing a CD19 CAR is a T cell and the cell expressing a FLT-3 CAR is a NK cell, or the cell expressing a CD19 CAR is a NK cell and the cell expressing a FLT-3 CAR is a T cell. In other embodiments, the cell expressing a CD19 CAR and the cell expressing a FLT-3 CAR are both NK cells or are both T cells, e.g., are both CD4+ T cells, or are both CD8+ T cells. In yet other embodiments, a single cell expresses the CD19 CAR and FLT-3 CAR, and this cell is, e.g., a NK cell or a T cell such as a CD4+ T cell or CD8+ T cell. The first CAR and second CAR can comprise the same or different intracellular signaling domains. For instance, in some embodiments the CD19 CAR comprises a CD3 zeta signaling domain and the FLT-3 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain, while in some embodiments, the CD19 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain and the FLT-3 CAR comprises a CD3 zeta signaling domain. In other embodiments, each of the CD19 CAR and the FLT-3 CAR comprises the same type of primary signaling domain, e.g., a CD3 zeta signaling domain, but the CD19 CAR and the FLT-3 CAR comprise different costimulatory domains, e.g., (1) the CD19 CAR comprises a 41BB costimulatory domain and the FLT-3 CAR comprises a different costimulatory domain e.g., a CD27 costimulatory domain, (2) the CD19 CAR comprises a CD27 costimulatory domain and the FLT-3 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (3) the CD19 CAR comprises a 41BB costimulatory domain and the FLT-3 CAR comprises a CD28 costimulatory domain, (4) the CD19 CAR comprises a CD28 costimulatory domain and the FLT-3 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (5) the CD19 CAR comprises a CD27 costimulatory domain and the FLT-3 CAR comprises a CD28 costimulatory domain, or (6) the CD19 CAR comprises a CD28 costimulatory domain and the FLT-3 CAR comprises a CD27 costimulatory domain. In another embodiment, a cell comprises a CAR that comprises both a CD19 antigen-binding domain and a FLT-3 antigen-binding domain, e.g., a bispecific antibody.

As another example, when the first B-cell inhibitor is a CD19 CAR-expressing cell and the second B-cell inhibitor is a ROR1 CAR-expressing cell, the first CAR and second CAR may be expressed by the same cell type or different types. For instance, in some embodiments, the cell expressing a CD19 CAR is a CD4+ T cell and the cell expressing a ROR1 CAR is a CD8+ T cell, or the cell expressing a CD19 CAR is a CD8+ T cell and the cell expressing a ROR1 CAR is a CD4+ T cell. In other embodiments, the cell expressing a CD19 CAR is a T cell and the cell expressing a ROR1 CAR is a NK cell, or the cell expressing a CD19 CAR is a NK cell and the cell expressing a ROR1 CAR is a T cell. In other embodiments, the cell expressing a CD19 CAR and the cell expressing a ROR1 CAR are both NK cells or are both T cells, e.g., are both CD4+ T cells, or are both CD8+ T cells. In yet other embodiments, a single cell expresses the CD19 CAR and ROR1 CAR, and this cell is, e.g., a NK cell or a T cell such as a CD4+ T cell or CD8+ T cell. The first CAR and second CAR can comprise the same or different intracellular signaling domains. For instance, in some embodiments the CD19 CAR comprises a CD3 zeta signaling domain and the ROR1 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain, while in some embodiments, the CD19 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain and the ROR1 CAR comprises a CD3 zeta signaling domain. In other embodiments, each of the CD19 CAR and the ROR1 CAR comprises the same type of primary signaling domain, e.g., a CD3 zeta signaling domain, but the CD19 CAR and the ROR1 CAR comprise different costimulatory domains, e.g., (1) the CD19 CAR comprises a 41BB costimulatory domain and the ROR1 CAR comprises a different costimulatory domain e.g., a CD27 costimulatory domain, (2) the CD19 CAR comprises a CD27 costimulatory domain and the ROR1 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (3) the CD19 CAR comprises a 41BB costimulatory domain and the ROR1 CAR comprises a CD28 costimulatory domain, (4) the CD19 CAR comprises a CD28 costimulatory domain and the ROR1 CAR comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (5) the CD19 CAR comprises a CD27 costimulatory domain and the ROR1 CAR comprises a CD28 costimulatory domain, or (6) the CD19 CAR comprises a CD28 costimulatory domain and the ROR1 CAR comprises a CD27 costimulatory domain. In another embodiment, a cell comprises a CAR that comprises both a CD19 antigen-binding domain and a ROR1 antigen-binding domain, e.g., a bispecific antibody.

More generally, when the first B-cell inhibitor comprises a CD19 CAR and there is a

second B-cell inhibitor e.g., which comprises a second CAR, the first CAR and the second B-cell inhibitor may be expressed by the same cell type or different types. For instance, in some embodiments, the cell expressing a CD19 CAR is a CD4+ T cell and the cell expressing the second B-cell inhibitor is a CD8+ T cell, or the cell expressing a CD19 CAR is a CD8+ T cell and the cell expressing the second B-cell inhibitor is a CD4+ T cell. In other embodiments, the cell expressing a CD19 CAR is a T cell and the cell expressing a second B-cell inhibitor is a NK cell, or the cell expressing a CD19 CAR is a NK cell and the cell expressing a second B-cell inhibitor is a T cell. In other embodiments, the cell expressing a CD19 CAR and the cell expressing a second B-cell inhibitor are both NK cells or are both T cells, e.g., are both CD4+ T cells, or are both CD8+ T cells. In yet other embodiments, a single cell expresses the CD19 CAR and the second B-cell inhibitor, and this cell is, e.g., a NK cell or a T cell such as a CD4+ T cell or CD8+ T cell. The first CAR and second CAR can comprise the same or different intracellular signaling domains. For instance, in some embodiments the CD19 CAR comprises a CD3 zeta signaling domain and second B-cell inhibitor (or CAR), comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain, while in some embodiments, the CD19 CAR comprises a costimulatory domain, e.g., a 41BB, CD27 or CD28 costimulatory domain and the second B-cell inhibitor (or second CAR), comprises a CD3 zeta signaling domain. In other embodiments, each of the CD19 CAR and the second B-cell inhibitor (or second CAR), comprises the same type of primary signaling domain, e.g., a CD3 zeta signaling domain, but the CD19 CAR and the second B-cell inhibitor comprise different costimulatory domains, e.g., (1) the CD19 CAR comprises a 41BB costimulatory domain and the second B-cell inhibitor (or second CAR), comprises a different costimulatory domain e.g., a CD27 costimulatory domain, (2) the CD19 CAR comprises a CD27 costimulatory domain and the second B-cell inhibitor (or second CAR) comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (3) the CD19 CAR comprises a 41BB costimulatory domain and the second B-cell inhibitor (or second CAR), comprises a CD28 costimulatory domain, (4) the CD19 CAR comprises a CD28 costimulatory domain and the second B-cell inhibitor (or second CAR) comprises a different costimulatory domain e.g., a 41BB costimulatory domain, (5) the CD19 CAR comprises a CD27 costimulatory domain and the second B-cell inhibitor (or second CAR), comprises a CD28 costimulatory domain, or (6) the CD19 CAR comprises a CD28 costimulatory domain and the second B-cell inhibitor (or second CAR), comprises a CD27

costimulatory domain. In another embodiment, a cell comprises a CAR that comprises both a CD19 antigen-binding domain and an antigen-binding domain directed to a second antigen, e.g., a bispecific antibody.

In one embodiment, the 4-1BB costimulatory domain comprises a sequence of SEQ ID NO: 16. In one embodiment, the 4-1BB costimulatory domain comprises an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 16, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO:16. In one embodiment, the 4-1BB costimulatory domain is encoded by a nucleic acid sequence of SEQ ID NO:60, or a sequence with 95-99% identity thereof.

In one embodiment, the CD27 costimulatory domain comprises a sequence of SEQ ID NO: 16. In one embodiment, the CD27 costimulatory domain comprises an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 16, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO:16. In one embodiment, the CD27 costimulatory domain is encoded by a nucleic acid sequence of SEQ ID NO:17, or a sequence with 95-99% identity thereof.

In one embodiment, the CD28 costimulatory domain comprises a sequence of SEQ ID NO: 1317. In one embodiment, the CD28 costimulatory domain comprises an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 1317, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO:1317. In one embodiment, the CD28 costimulatory domain is encoded by a nucleic acid sequence of SEQ ID NO:1318, or a sequence with 95-99% identity thereof.

In one embodiment, the wild-type ICOS costimulatory domain comprises a sequence of SEQ ID NO: 1319. In one embodiment, the wild-type ICOS costimulatory domain comprises an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 1319, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 1319. In one embodiment, the wild-type ICOS costimulatory domain is encoded by a nucleic acid sequence of SEQ ID NO: 1320, or a sequence with 95-99% identity thereof.

In one embodiment, the Y to F mutant ICOS costimulatory domain comprises a sequence of SEQ ID NO: 1321. In one embodiment, the Y to F mutant ICOS costimulatory domain comprises an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 1321, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 1321. In one embodiment, the Y to F mutant ICOS costimulatory domain is encoded by a nucleic acid sequence with 95-99% identity to a nucleic acid sequence of SEQ ID NO:1320 (wherein SEQ ID NO: 1320 encodes wild-type ICOS).

In embodiments, the primary signaling domain comprises a functional signaling domain of CD3 zeta. In embodiments, the functional signaling domain of CD3 zeta comprises SEQ ID NO: 17 (mutant CD3 zeta) or SEQ ID NO: 43 (wild-type human CD3 zeta).

In one embodiment, the method includes administering a population of cells wherein at least one cell in the population expresses a CAR, e.g., having an anti- CD19 domain described herein, and an agent which enhances the activity of a CAR-expressing cell, e.g., a second cell expressing the agent which enhances the activity of a CAR-expressing cell. For example, in one embodiment, the agent can be an agent which inhibits an immune inhibitory molecule.

Examples of immune inhibitory molecules include PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 and TGFR beta. In one embodiment, the agent that inhibits an immune inhibitory molecule comprises a first polypeptide, e.g., an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, e.g., an intracellular signaling domain described herein. In one embodiment, the agent comprises a first polypeptide, e.g., of an inhibitory molecule such as PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 or TGFR beta, or a fragment of any of these (e.g., at least a portion of an extracellular domain of any of these), and a second polypeptide which is an intracellular signaling domain described herein (e.g., comprising a costimulatory domain (e.g., 41BB, CD27 or CD28, e.g., as described herein) and/or a primary signaling domain (e.g., a CD3 zeta signaling domain described herein). In one embodiment, the agent comprises a first polypeptide of PD1 or a fragment thereof (e.g., at least a portion of the extracellular domain of PD1), and a second polypeptide of an intracellular signaling domain described herein (e.g., a CD28 signaling domain described herein

and/or a CD3 zeta signaling domain described herein).

In an embodiment, the B-cell inhibitor comprises an inhibitor of one or more of CD10, CD19, CD20, CD22, CD34, FLT-3, or ROR1. In an embodiment, the B-cell inhibitor comprises an effective number of one or more cells that express a CAR molecule that binds one or more of CD10, CD20, CD22, CD34, FLT-3, ROR1, CD79b, CD179b, or CD79a. In an embodiment, the B-cell inhibitor comprises a CD123 CAR. In an embodiment, the B cell inhibitor comprises one or more cells that express a CAR molecule that binds CD123. In an embodiment, the disease is a CD19-negative cancer, e.g., a CD19-negative relapsed cancer. In an embodiment, the CD19 CAR-expressing cell is administered simultaneously with, before, or after the one or more B-cell inhibitor.

In an embodiment, the method further comprises administering a CD19 inhibitor, e.g., a CD19 CAR-expressing cell. In an embodiment, the CD19 inhibitor comprises a CD19 CAR and the B-cell inhibitor comprises a CD123 CAR. In an embodiment, the CD19 CAR or CD123 CAR comprises a split intracellular signaling domain such that full activation of the cell, e.g., the population of immune effector cells, occurs when both the CD19 CAR and CD123 CAR bind to a target cell, e.g., a target CD19+CD123+ cell (e.g., a B-ALL blast cell), compared to activation when the CD19 CAR and CD123 CAR bind to a target cell that expresses one of CD19 or CD123 (e.g., a hematopoietic stem cell). In an embodiment, the CD123CAR comprises a 4-1BB signaling domain and the CD19 CAR comprises a CD3 zeta signaling domain. In an embodiment, the CD123CAR comprises a costimulatory domain, e.g., a 4-1BB signaling domain, and the CD19 CAR comprises a primary signaling domain, e.g., a CD3 zeta signaling domain. In an embodiment, the CD123CAR comprises a primary signaling domain, e.g., a CD3 zeta signaling domain, and the CD19 CAR comprises a costimulatory domain, e.g., a 4-1BB signaling domain. In an embodiment, the B cell inhibitor comprises a CAR (e.g., a CAR directed against CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a) which comprises a costimulatory domain, and the CD19 CAR comprises a primary signaling domain. In an embodiment, the B cell inhibitor comprises a CAR (e.g., a CAR directed against CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a) which comprises a primary signalling domain, and the CD19 CAR comprises a costimulatory domain. In an embodiment, the B-cell inhibitor comprises one or more cells that express a CAR molecule that binds CD123, and wherein a CD19 CAR-expressing cell is

administered simultaneously with the B-cell inhibitor. In an embodiment, the CD123CAR comprises a 4-1BB signaling domain and the CD19 CAR comprises a CD3 zeta signaling domain.

In an embodiment, the method further comprises transplanting a cell, e.g., a hematopoietic stem cell, or a bone marrow, into the mammal.

In another aspect, the invention pertains to a cell expressing a CAR molecule described herein, e.g., a CD19 CAR molecule, for use as a medicament in combination with a B-cell inhibitor, e.g., a B-cell inhibitor described herein. In another aspect, the invention pertains to a B-cell inhibitor described herein for use as a medicament in combination with a cell expressing a CAR molecule, e.g., a CD19 CAR molecule, described herein.

In another aspect, the invention pertains to a cell expressing a CAR molecule described herein, e.g., a CD19 CAR molecule, for use in combination with a B-cell inhibitor, e.g., a B-cell inhibitor described herein, in the treatment of a disease expressing CD19. In another aspect, the invention pertains to a B-cell inhibitor described herein for use in combination with a cell expressing a CAR molecule described herein, e.g., a CD19 CAR molecule, in the treatment of a disease expressing CD19. In another aspect, the invention pertains to a cell expressing a CAR molecule described herein, e.g., a CD19 CAR molecule, for use in combination with a B-cell inhibitor, e.g., a B-cell inhibitor described herein, in the treatment of a cancer, e.g., a cancer described herein.

In one embodiment, the method includes administering a population of cells wherein at least one cell in the population expresses a therapy herein (e.g., a CD20 CAR, a CD22 CAR, or a CAR having an anti- CD19 domain described herein in combination with a B-cell inhibitor) and an agent which enhances the activity of a CAR-expressing cell, wherein the agent is a cytokine, e.g., IL-7, IL-15, IL-21, or a combination thereof. The cytokine can be delivered in combination with, e.g., simultaneously or shortly after, administration of the CAR-expressing cell(s). Alternatively, the cytokine can be delivered after a prolonged period of time after administration of the CAR-expressing cell(s), e.g., after assessment of the subject's response to the CAR-expressing cell(s). Related compositions for use and methods of making a medicament are also provided.

In one embodiment, the cells described herein (e.g., cells expressing a CD20 CAR molecule, cells expressing a CD22 CAR molecule, or cells expressing a CD19 CAR molecule,

e.g., a CD19 CAR molecule described herein, combination with a B-cell inhibitor) are administered in combination with an agent that increases the efficacy of a cell expressing a CAR molecule or one of the inhibitors, e.g., an agent described herein.

In one embodiment, the cells described herein (e.g., cells expressing a CD20 CAR molecule, cells expressing a CD22 CAR molecule, or expressing a CD19 CAR molecule, e.g., a CD19 CAR molecule described herein, in combination with a B-cell inhibitor) are administered in combination with an agent that ameliorates one or more side effect associated with administration of a cell expressing a CAR molecule or one of the inhibitors, e.g., an agent described herein.

In one embodiment, the cells expressing a CD19 CAR molecule, e.g., a CD19 CAR molecule described herein, are administered in combination with a B-cell inhibitor, and an agent that treats Hodgkin lymphoma, e.g., an agent described herein.

In some aspects, the disclosure provides a method of treating a patient who is a non-responder, partial responder, or relapser to a CD19 inhibitor, e.g., a CD19 CAR therapy, comprising administering to the patient a B-cell inhibitor, e.g., a B-cell inhibitor as described herein, e.g., an inhibitor of one or more of (e.g., 2, 3, 4, 5, 6, 7, 8, 9, or all of) CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. In embodiments, the B-cell inhibitor is a CAR-expressing cell (e.g., T cell or NK cell) that is an inhibitor of one or more of (e.g., 2, 3, 4, 5, 6, or all of) CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1. In embodiments, the patient has, or is identified as having, a CD19-negative cancer cell and a cancer cell that is positive for one or more of (e.g., 2, 3, 4, 5, 6, 7, 8, 9, or all of) CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. In embodiments, the method further comprises administering to the patient a B-cell inhibitor for which the cancer cell is positive, e.g., an inhibitor of one or more of (e.g., 2, 3, 4, 5, 6, 7, 8, 9, or all of) the CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a for which the cancer cell is positive. In embodiments, the method further comprises one or both of a step of determining whether the patient comprises a CD19-negative cancer cell, and a step of determining whether the patient comprises a cancer cell that is positive for one or more of (e.g., 2, 3, 4, 5, 6, 7, 8, 9, or all of) CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. In embodiments, the subject has or is identified as having a population of tumor or cancer cells that test negative for CD19 expression as measured by binding to an anti-CD19 antibody, e.g., an

antibody with the same specificity as any of the CAR molecules in **Table 2** or **Table 3**.

In another aspect, the invention features a composition comprising a cell expressing a Chimeric Antigen Receptor (CAR) molecule that binds CD19, in combination with a B-cell inhibitor, e.g., a B-cell inhibitor chosen from an inhibitor of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a, or a combination thereof. The CAR-expressing cell and the B-cell inhibitor can be present in a single dose form, or as two or more dose forms.

In an embodiment, the composition is a pharmaceutically acceptable composition.

In embodiments, the compositions disclosed herein (e.g., nucleic acids, vectors, or cells) are for use as a medicament.

In embodiments, the compositions disclosed herein are use in the treatment of a disease associated with expression of a B-cell antigen (e.g., CD19), e.g., a B-cell leukemia or lymphoma.

CD19 INHIBITORS

In embodiments, the CD19 inhibitor is a small molecule, an antibody, a fragment of an antibody, or a cell therapy.

In some embodiments, the CD19 inhibitor (e.g., a cell therapy or an antibody) is administered in combination with, or is present in a composition together with, a B cell inhibitor, e.g., one or more inhibitors of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

In one embodiment, the cell expresses a CAR molecule comprising an anti-CD19 binding domain (e.g., a murine or humanized antibody or antibody fragment that specifically binds to CD19), a transmembrane domain, and an intracellular signaling domain (e.g., an intracellular signaling domain comprising a costimulatory domain and/or a primary signaling domain). In one embodiment, the CAR comprises an antibody or antibody fragment which includes an anti-CD19 binding domain described herein (e.g., a murine or humanized antibody or antibody fragment that specifically binds to CD19 as described herein), a transmembrane domain described herein, and an intracellular signaling domain described herein (e.g., an

intracellular signaling domain comprising a costimulatory domain and/or a primary signaling domain described herein).

In one embodiment, the CAR molecule comprises an anti-CD19 binding domain comprising one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of an anti-CD19 binding domain described herein, and one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of an anti-CD19 binding domain described herein, e.g., an anti-CD19 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. In one embodiment, the anti-CD19 binding domain comprises one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of an anti-CD19 binding domain described herein, e.g., the anti-CD19 binding domain has two variable heavy chain regions, each comprising a HC CDR1, a HC CDR2 and a HC CDR3 described herein. In one embodiment, the anti-CD19 binding domain comprises a murine light chain variable region described herein (e.g., in **Table 3**) and/or a murine heavy chain variable region described herein (e.g., in **Table 3).** In one embodiment, the anti-CD19 binding domain is a scFv comprising a murine light chain and a murine heavy chain of an amino acid sequence of **Table 3**. In an embodiment, the anti-CD19 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 3**, or a sequence with 95-99% identity with an amino acid sequence of **Table 3**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 3**, or a sequence with 95-99% identity to an amino acid sequence of **Table 3**. In one embodiment, the anti-CD19 binding domain comprises a sequence of SEQ ID NO:59, or a sequence with 95-99% identity thereof. In one embodiment, the anti-CD19 binding domain is a scFv, and a light chain variable region comprising an amino acid

sequence described herein, e.g., in **Table 3**, is attached to a heavy chain variable region comprising an amino acid sequence described herein, e.g., in **Table 3**, via a linker, e.g., a linker described herein. In one embodiment, the anti-CD19 binding domain includes a $(Gly_4\text{-}Ser)n$ linker, wherein n is 1, 2, 3, 4, 5, or 6, e.g., 3 or 4 (SEQ ID NO: 53). The light chain variable region and heavy chain variable region of a scFv can be, e.g., in any of the following orientations: light chain variable region-linker-heavy chain variable region or heavy chain variable region-linker-light chain variable region.

In one embodiment, the CAR molecule comprises a humanized anti-CD19 binding domain that includes one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a humanized anti-CD19 binding domain described herein, and one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a humanized anti-CD19 binding domain described herein, e.g., a humanized anti-CD19 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. In one embodiment, the humanized anti-CD19 binding domain comprises at least HC CDR2. In one embodiment, the humanized anti-CD19 binding domain comprises one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a humanized anti-CD19 binding domain described herein, e.g., the humanized anti-CD19 binding domain has two variable heavy chain regions, each comprising a HC CDR1, a HC CDR2 and a HC CDR3 described herein. In one embodiment, the humanized anti-CD19 binding domain comprises at least HC CDR2. In one embodiment, the light chain variable region comprises one, two, three or all four framework regions of VK3_L25 germline sequence. In one embodiment, the light chain variable region has a modification (e.g., substitution, e.g., a substitution of one or more amino acid found in the corresponding position in the murine light chain variable region of SEQ ID NO: 58, e.g., a substitution at one or more of positions 71 and 87). In one embodiment, the heavy chain variable region comprises one, two, three or all four framework regions of VH4_4-59 germline sequence. In one embodiment, the heavy chain variable region has a modification (e.g., substitution, e.g., a substitution of one or more amino

acid found in the corresponding position in the murine heavy chain variable region of SEQ ID NO: 58, e.g., a substitution at one or more of positions 71, 73 and 78). In one embodiment, the humanized anti-CD19 binding domain comprises a light chain variable region described herein (e.g., in **Table 2**) and/or a heavy chain variable region described herein (e.g., in **Table 2**). In one embodiment, the humanized anti-CD19 binding domain is a scFv comprising a light chain and a heavy chain of an amino acid sequence of **Table 2**. In an embodiment, the humanized anti-CD19 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 2**, or a sequence with 95-99% identity with an amino acid sequence of **Table 2**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 2**, or a sequence with 95-99% identity to an amino acid sequence of **Table 2**. In one embodiment, the humanized anti-CD19 binding domain comprises a sequence selected from a group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12, or a sequence with 95-99% identity thereof. In one embodiment, the humanized anti-CD19 binding domain is a scFv, and a light chain variable region comprising an amino acid sequence described herein, e.g., in **Table 2**, is attached to a heavy chain variable region comprising an amino acid sequence described herein, e.g., in **Table 2**, via a linker, e.g., a linker described herein. In one embodiment, the humanized anti-CD19 binding domain includes a $(Gly_4\text{-}Ser)n$ linker, wherein n is 1, 2, 3, 4, 5, or 6, e.g., 3 or 4 (SEQ ID NO: 53). The light chain variable region and heavy chain variable region of a scFv can be, e.g., in any of the following orientations: light chain variable region-linker-heavy chain variable region or heavy chain variable region-linker-light chain variable region.

In one embodiment, the CAR molecule comprises an anti-CD19 binding domain that includes one or more (e.g., 2, 3, 4, 5, or 6) LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 of a construct of **Table 4 and 5**, e.g., murine_CART19, humanized_CART19 a, humanized_CART19 b, or humanized_CART19 c.

In one embodiment, the CAR molecule comprises a leader sequence, e.g., a leader

sequence described herein, e.g., a leader sequence of SEQ ID NO: 13, or having 95-99% identity thereof; an anti-CD19 binding domain described herein, e.g., an anti-CD19 binding domain comprising a LC CDR1, a LC CDR2, a LC CDR3, a HC CDR1, a HC CDR2 and a HC CDR3 described herein, e.g., a murine anti-CD19 binding domain described in **Table 3**, a humanized anti-CD19 binding domain described in **Table 2**, or a sequence with 95-99% identity thereof; a hinge region, e.g., a hinge region described herein, e.g., a hinge region of SEQ ID NO:14 or having 95-99% identity thereof; a transmembrane domain, e.g., a transmembrane domain described herein, e.g., a transmembrane domain having a sequence of SEQ ID NO:15 or a sequence having 95-99% identity thereof; an intracellular signaling domain, e.g., an intracellular signaling domain described herein (e.g., an intracellular signaling domain comprising a costimulatory domain and/or a primary signaling domain). In one embodiment, the intracellular signaling domain comprises a costimulatory domain, e.g., a costimulatory domain described herein, e.g., a 4-1BB costimulatory domain having a sequence of SEQ ID NO:16 or SEQ ID NO:51, or having 95-99%identity thereof, and/or a primary signaling domain, e.g., a primary signaling domain described herein, e.g., a CD3 zeta stimulatory domain having a sequence of SEQ ID NO:17 or SEQ ID NO:43, or having 95-99% identity thereof.

In one embodiment, the CAR molecule comprises (e.g., consists of) an amino acid sequence of SEQ ID NO:58, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41 or SEQ ID NO:42, or an amino acid sequence having at least one, two, three, four, five, 10, 15, 20 or 30 modifications (e.g., substitutions) but not more than 60, 50 or 40 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO:58, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41 or SEQ ID NO:42, or an amino acid sequence having 85%, 90%, 95%, 96%, 97%, 98% or 99% identity to an amino acid sequence of SEQ ID NO:58, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41 or SEQ ID NO:42.

The present invention relates generally, in some aspects, to the use of cells, e.g., T cells or natural killer (NK) cells, engineered to express a CAR in combination with one or more B-cell inhibitors to treat a disease associated with expression of the Cluster of Differentiation 19

protein (CD19). In some embodiments, the B-cell inhibitor is an inhibitor of one or more of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

In some embodiments, the CD19 inhibitor comprises an antibody molecule having, e.g., an antibody molecule having a CD19-binding sequence as described herein. For instance, the antibody molecule may comprise CDRs or a VH and VL as described in any of **Tables 2, 3, 4, and 5**, or a sequence with homology thereto, e.g., having 95-99% identity thereto. The antibody molecule may comprise a CD19-binding region having a sequence described in this section, e.g., in the context of a CAR.

In embodiments, the B-cell inhibitor is chosen from an inhibitory nucleic acid, a soluble ligand, an antibody or antigen-binding fragment thereof, a CAR, or a CAR-expressing cell that binds to one or more B-cell antigens, e.g., one or more of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

CD20 BINDING DOMAINS AND INHIBITORS

In some aspects, the present disclosure provides a CD20 inhibitor or binding domain, e.g., a CD20 inhibitor or binding domain as described herein. The disclosure also provides a nucleic acid encoding the CD20 binding domain, e.g., encoding a CAR comprising the CD20 binding domain. The composition may also comprise a second agent, e.g., an anti-CD19 CAR-expressing cell or a CD19 binding domain. The agents may be, e.g., encoded by a single nucleic acid or different nucleic acids.

In some aspects, a CD20 inhibitor or binding domain is administered as a monotherapy. In some aspects, the CD20 inhibitor or binding domain is administered in combination with a second agent such as an anti-CD19 CAR-expressing cell.

The CD20 inhibitor may be, e.g., a small molecule, antibody or antigen-binding fragment thereof, a CAR or a CAR-expressing cell. In one embodiment, the CD20 inhibitor is an anti-CD20 antibody or fragment thereof. In an embodiment, the antibody is a monospecific antibody and in another embodiment the antibody is a bispecific antibody. In an embodiment, the CD20 inhibitor is a chimeric mouse/human monoclonal antibody, e.g., rituximab. In an embodiment, the CD20 inhibitor is a human monoclonal antibody such as ofatumumab. In an embodiment,

the CD20 inhibitor is a humanized antibody such as ocrelizumab, veltuzumab, obinutuzumab, ocaratuzumab, or PRO131921 (Genentech). In an embodiment, the CD20 inhibitor is a fusion protein comprising a portion of an anti-CD20 antibody, such as TRU-015 (Trubion Pharmaceuticals).

In one embodiment, the CD20 inhibitor is an anti-CD20 expressing cell, e.g., CD20 CART or CD20-expressing NK cell.

In some embodiments, the CD20-CAR comprises an optional leader sequence (e.g., an optional leader sequence described herein), an extracellular antigen binding domain, a hinge (e.g., hinge described herein), a transmembrane domain (e.g., transmembrane domain described herein), and an intracellular stimulatory domain (e.g., intracellular stimulatory domain described herein). In one embodiment, an exemplary CD20 CAR construct comprises an optional leader sequence (e.g., a leader sequence described herein), an extracellular antigen binding domain, a hinge, a transmembrane domain, an intracellular costimulatory domain (e.g., an intracellular costimulatory domain described herein) and an intracellular stimulatory domain.

In one embodiment, the CD20 binding domain comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a CD20 binding domain described herein, and/or one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a CD20 binding domain described herein, e.g., a CD20 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. These CDRs may be, e.g., those of **Table 12A, 12B,** and/or **Table 13**. In one embodiment, the CD20 binding domain comprises one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a CD20 binding domain described herein, e.g., the CD20 binding domain has two variable heavy chain regions, each comprising a HC CDR1, a HC CDR2 and a HC CDR3 described herein. In one embodiment, the CD20 binding domain comprises a light chain variable region described herein (e.g., in **Table 15A or 15B**) and/or a heavy chain variable region described herein (e.g., in **Table 14A or 14B**). In one embodiment, the CD20 binding domain comprises a heavy chain variable region described

herein (e.g., in **Table 14A or 14B**), e.g., at least two heavy chain variable regions described herein (e.g., in **Table 14A or 14B**). In one embodiment, the CD20 binding domain is a scFv comprising a light chain and a heavy chain of an amino acid sequence of **Table 14A or 14B or 15A or 15B**. In an embodiment, the CD20 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 15A or 15B**, or a sequence with 95-99% identity with an amino acid sequence of **Table 15A or 15B**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 14A or 14B**, or a sequence with 95-99% identity to an amino acid sequence of **Table 14A or 14B**. The CD20 binding domain may be part of, e.g., an antibody molecule or a CAR molecule.

In one embodiment, the CAR molecule comprises an anti-CD20 binding domain that includes one or more (e.g., 2, 3, 4, 5, or 6) LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 of a construct of **Table 12A, 12B, and/or 13**, e.g., CAR20-1, CAR20-2, CAR20-3, CAR20-4, CAR20-5, CAR20-6, CAR20-7, CAR20-8, CAR20-9, CAR20-10, CAR20-11, CAR20-12, CAR20-13, CAR20-14, CAR20-15, or CAR20-16.

In one embodiment, the CAR molecule comprises an anti-CD22 binding domain that includes a VL and/or VH of a construct of **Table 14A or 14B and 15A or 15B**, e.g., CAR20-1, CAR20-2, CAR20-3, CAR20-4, CAR20-5, CAR20-6, CAR20-7, CAR20-8, CAR20-9, CAR20-10, CAR20-11, CAR20-12, CAR20-13, CAR20-14, CAR20-15, or CAR20-16.

The CD20 scFv may be preceded by an optional leader sequence such as provided in SEQ ID NO: 13, and followed by an optional hinge sequence such as provided in SEQ ID NO: 14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49, a transmembrane region such as provided in SEQ ID NO:15, an intracellular signalling domain that includes SEQ ID NO:16 or SEQ ID NO:51 and a CD3 zeta sequence that includes SEQ ID NO:17 or SEQ ID NO:43, e.g., wherein the domains are contiguous with and in the same reading frame to form a single fusion protein.

Further embodiments include a nucleotide sequence that encodes a polypeptide of any of

Tables 11A-15B Further embodiments include a nucleotide sequence that encodes a polypeptide any of **Tables 11A-15B**, and each of the domains of SEQ ID NOS: 13, 14, 15, 16, 17, and optionally 51.

In one embodiment, the CD20 binding domain is characterized by particular functional features or properties of an antibody or antibody fragment. For example, in one embodiment, the portion of a CAR composition of the invention that comprises an antigen binding domain specifically binds human CD20 or a fragment thereof.

In one embodiment, the CD20 binding domain is a fragment, e.g., a single chain variable fragment (scFv). In one embodiments, the CD20 binding domain is a Fv, a Fab, a (Fab')2, or a bi-functional (e.g. bi-specific) hybrid antibody (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)). In one aspect, the antibodies and fragments thereof of the invention binds a CD20 protein or a fragment thereof with wild-type or enhanced affinity. In some instances, a human scFv can be derived from a display library.

In one embodiment, the CD20 binding domain, e.g., scFv comprises at least one mutation such that the mutated scFv confers improved stability to the CART20 construct. In another embodiment, the CD20 binding domain, e.g., scFv comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mutations arising, e.g., from the humanization process, such that the mutated scFv confers improved stability to the CART20 construct.

In some embodiments, the CD20 inhibitor comprises an antibody molecule having, e.g., an antibody molecule having a CD20-binding sequence as described herein. For instance, the antibody molecule may comprise CDRs or a VH and VL as described in any of **Tables 11A-15B**, or a sequence with homology thereto, e.g., having 95-99% identity thereto. The antibody molecule may comprise a CD20-binding region having a sequence described in this section, e.g., in the context of a CAR.

In one aspect, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD20 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD20 CAR.

In some aspects, a binding domain or antibody molecule described herein binds the same (or substantially the same) or an overlapping (or substantially overlapping) epitope with a

second antibody molecule to CD20, wherein the second antibody molecule is an antibody molecule described herein, e.g., an antibody molecule chosen from **Tables 11A-15B**. In some embodiments, a binding domain or antibody molecule described herein competes for binding, and/or binds the same (or substantially the same) or overlapping (or substantially overlapping) epitope, with a second antibody molecule to CD20, wherein the second antibody molecule is an antibody molecule described herein, e.g., an antibody molecule chosen from **Tables 11A-15B**, e.g., as determined by the methods described in Example 25. In some embodiments, a biparatopic CD20 binding domain binds a first epitope, e.g., an epitope bound by an antibody molecule chosen from **Tables 11A-15B**, and the biparatopic binding domain also binds a second epitope, e.g., a second epitope bound by an antibody molecule chosen from **Tables 11A-15B**. In some aspects, the present disclosure provides a method of treatment comprising administering a first CD20 binding domain that binds a first epitope, e.g., an epitope bound by an antibody molecule chosen from **Tables 11A-15B** and a second CD20 binding domain that binds a second epitope, e.g., a second epitope bound by an antibody molecule chosen from **Tables 11A-15B**. In some embodiments, the CD20 binding domains are part of CAR molecules, e.g., expressed by a CAR-expressing cell.

CD22 BINDING DOMAINS AND INHIBITORS

In some aspects, the present disclosure provides a CD22 inhibitor or binding domain, e.g., a CD22 inhibitor or binding domain as described herein. The disclosure also provides a nucleic acid encoding the CD22 binding domain, e.g., encoding a CAR comprising the CD22 binding domain. The composition may also comprise a second agent, e.g., an anti-CD19 CAR-expressing cell or a CD19 binding domain. The agents may be, e.g., encoded by a single nucleic acid or different nucleic acids.

In some aspects, a CD22 inhibitor or binding domain is administered as a monotherapy. In some aspects, the CD22 inhibitor or binding domain is administered in combination with a second agent such as an anti-CD19 CAR-expressing cell.

The CD22 inhibitor may be, e.g., a small molecule, antibody or antigen-binding fragment thereof, a CAR or a CAR-expressing cell. In one embodiment, the CD22 inhibitor is an anti-CD22 antibody or fragment thereof. In an embodiment, the antibody is a monospecific antibody

and in another embodiment the antibody is a bispecific antibody. In an embodiment, the antibody is a monospecific antibody, optionally conjugated to a second agent such as a chemotherapeutic agent. For instance, in an embodiment the antibody is an anti-CD22 monoclonal antibody-MMAE conjugate (e.g., DCDT2980S). In an embodiment, the antibody is an scFv of an anti-CD22 antibody, e.g., an scFv of antibody RFB4. This scFv can be fused to all of or a fragment of Pseudomonas exotoxin-A (e.g., BL22). In an embodiment, the antibody is a humanized anti-CD22 monoclonal antibody (e.g., epratuzumab). In an embodiment, the antibody or fragment thereof comprises the Fv portion of an anti-CD22 antibody, which is optionally covalently fused to all or a fragment or (e.g., a 38 KDa fragment of) Pseudomonas exotoxin-A (e.g., moxetumomab pasudotox). In an embodiment, the anti-CD22 antibody is an anti-CD19/CD22 bispecific antibody, optionally conjugated to a toxin. For instance, in one embodiment, the anti-CD22 antibody comprises an anti-CD19/CD22 bispecific portion, (e.g., two scFv ligands, recognizing human CD19 and CD22) optionally linked to all of or a portion of diphtheria toxin (DT), e.g., first 389 amino acids of diphtheria toxin (DT), DT 390, e.g., a ligand-directed toxin such as DT2219ARL). In another embodiment, the bispecific portion (e.g., anti-CD19/anti-CD22) is linked to a toxin such as deglycosylated ricin A chain (e.g., Combotox).

In one embodiment, the CD22 inhibitor is an anti-CD22 expressing cell, e.g., a CD22 CART or CD22-expressing NK cell.

In one aspect, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD22 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD22 CAR. As another example, the population of CAR T cells can include a single population expressing more than one, e.g., 2, 3, 4, 5, or 6 or more, CARs, e.g., a CD19 CAR and a CD22 CAR.

In some embodiments, the CD22-CAR comprises an optional leader sequence (e.g., an optional leader sequence described herein), an extracellular antigen binding domain, a hinge (e.g., hinge described herein), a transmembrane domain (e.g., transmembrane domain described herein), and an intracellular stimulatory domain (e.g., intracellular stimulatory domain described herein). In one embodiment, an exemplary CD22 CAR construct comprises an optional leader sequence (e.g., a leader sequence described herein), an extracellular antigen binding domain, a

hinge, a transmembrane domain, an intracellular costimulatory domain (e.g., an intracellular costimulatory domain described herein) and an intracellular stimulatory domain.

In one embodiment, the CD22 binding domain comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a CD22 binding domain described herein, and/or one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a CD22 binding domain described herein, e.g., a CD22 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. These CDRs may be, e.g., one or more CDRs of **Table 7A, 7B, 7C, 8A** and/or **8B**. In one embodiment, the CD22 binding domain comprises one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a CD22 binding domain described herein, e.g., the CD22 binding domain has two variable heavy chain regions, each comprising a HC CDR1, a HC CDR2 and a HC CDR3 described herein. In one embodiment, the CD22 binding domain comprises a light chain variable region described herein (e.g., in **Table 10A or 10B**) and/or a heavy chain variable region described herein (e.g., in **Table 9A or 9B**). In one embodiment, the CD22 binding domain comprises a heavy chain variable region described herein (e.g., in **Table 9A or 9B**), e.g., at least two heavy chain variable regions described herein (e.g., in **Table 9A or 9B**). In one embodiment, the CD22 binding domain is a scFv comprising a light chain and a heavy chain of an amino acid sequence of **Table 9A or 9B and 10A or 10B**. In an embodiment, the CD22 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 10A or 10B**, or a sequence with 95-99% identity with an amino acid sequence of **Table 10A or 10B**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 9A or 9B**, or a sequence with 95-99% identity to an amino acid sequence of **Table 9A or**

9B. The CD22 binding domain may be part of, e.g., an antibody molecule or a CAR molecule.

In one embodiment, the CAR molecule comprises an anti-CD22 binding domain that includes one or more (e.g., 2, 3, 4, 5, or 6) LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 of a construct of **Table 7A, 7B, 7C, 8A and/or 8B**, e.g., m971, CAR22-1, CAR22-2, CAR22-3 , CAR22-4 , CAR22-5 , CAR22-6 , CAR22-7, CAR22-8 , CAR22-9 , CAR22-10, CAR22-11, CAR22-12, CAR22-13, CAR22-14, CAR22-15, CAR22-16, CAR22-17, CAR22-18, CAR22-19, CAR22-20, CAR22-21, CAR22-22, CAR22-23, CAR22-24, CAR22-25, CAR22-26, CAR22-27, CAR22-28, CAR22-29, CAR22-30, CAR22-31, CAR22-32, CAR22-33, CAR22-34, CAR22-35, CAR22-36, CAR22-37, or CAR22-38.

In one embodiment, the CAR molecule comprises an anti-CD22 binding domain that includes a VL and/or VH of a construct of **Table 9A, 9B, 10A, and/or 10B**, e.g., m971, CAR22-1, CAR22-2, CAR22-3 , CAR22-4 , CAR22-5 , CAR22-6 , CAR22-7, CAR22-8 , CAR22-9 , CAR22-10, CAR22-11, CAR22-12, CAR22-13, CAR22-14, CAR22-15, CAR22-16, CAR22-17, CAR22-18, CAR22-19, CAR22-20, CAR22-21, CAR22-22, CAR22-23, CAR22-24, CAR22-25, CAR22-26, CAR22-27, CAR22-28, CAR22-29, CAR22-30, CAR22-31, CAR22-32, CAR22-33, CAR22-34, CAR22-35, CAR22-36, CAR22-37, or CAR22-38, or a sequence with 95-99% identity thereto.

The scFv may be preceded by an optional leader sequence such as provided in SEQ ID NO: 13, and followed by an optional hinge sequence such as provided in SEQ ID NO: 14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49, a transmembrane region such as provided in SEQ ID NO:15, an intracellular signalling domain that includes SEQ ID NO:16 or SEQ ID NO:51 and a CD3 zeta sequence that includes SEQ ID NO:17 or SEQ ID NO:43, e.g., wherein the domains are contiguous with and in the same reading frame to form a single fusion protein.

Further embodiments include a nucleotide sequence that encodes a polypeptide of any of **Tables 6A-10B.** Further embodiments include a nucleotide sequence that encodes a polypeptide of any of **Tables 6A-10B**, and each of the domains of SEQ ID NOS: 13, 14, 15, 16, 17, and optionally 51.

In one embodiment, the CD22 binding domain is characterized by particular functional features or properties of an antibody or antibody fragment. For example, in one embodiment, the portion of a CAR composition of the invention that comprises an antigen binding domain

specifically binds human CD22 or a fragment thereof.

In one embodiment, the CD22 binding domain is a fragment, e.g., a single chain variable fragment (scFv). In one embodiments, the CD22 binding domain is a Fv, a Fab, a (Fab')2, or a bi-functional (e.g. bi-specific) hybrid antibody (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)). In one aspect, the antibodies and fragments thereof of the invention binds a CD22 protein or a fragment thereof with wild-type or enhanced affinity. In some instances, a human scFv can be derived from a display library.

In one embodiment, the CD22 binding domain, e.g., scFv comprises at least one mutation such that the mutated scFv confers improved stability to the CART22 construct. In another embodiment, the CD22 binding domain, e.g., scFv comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mutations arising, e.g., from the humanization process such that the mutated scFv confers improved stability to the CART22 construct.

In some embodiments, the CD22 inhibitor comprises an antibody molecule having, e.g., an antibody molecule having a CD22-binding sequence as described herein. For instance, the antibody molecule may comprise CDRs or a VH and VL as described in any of Tables **6A-10B**, or a sequence with homology thereto, e.g., having 95-99% identity thereto. The antibody molecule may comprise a CD22-binding region having a sequence described in this section, e.g., in the context of a CAR.

In one embodiment, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD22 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD22 CAR.

In some aspects, a binding domain or antibody molecule described herein binds the same (or substantially the same) or an overlapping (or substantially overlapping) epitope with a second antibody molecule to CD22, wherein the second antibody molecule is an antibody molecule described herein, e.g., an antibody molecule chosen from **Tables 6A-10B**. In some embodiments, a binding domain or antibody molecule described herein competes for binding, and/or binds the same (or substantially the same) or overlapping (or substantially overlapping) epitope, with a second antibody molecule to CD22, wherein the second antibody molecule is an antibody molecule described herein, e.g., an antibody molecule chosen from **Tables 6A-10B**,

e.g., as determined by the methods described in Example 25. In some embodiments, a biparatopic CD22 binding domain binds a first epitope, e.g., an epitope bound by an antibody molecule chosen from **Tables 6A-10B**, and the biparatopic binding domain also binds a second epitope, e.g., a second epitope bound by an antibody molecule chosen from **Tables 6A-10B**. In some aspects, the present disclosure provides a method of treatment comprising administering a first CD22 binding domain that binds a first epitope, e.g., an epitope bound by an antibody molecule chosen from **Tables 6A-10B** and a second CD22 binding domain that binds a second epitope, e.g., a second epitope bound by an antibody molecule chosen from **Tables 6A-10B**. In some embodiments, the CD22 binding domains are part of CAR molecules, e.g., expressed by a CAR-expressing cell.

In some embodiments, a CD22 binding domain binds to one or more of Ig-like domains 1, 2, 3, 4, 5, 6, or 7 of CD22. In some embodiments, the CD22 binding domain binds to domains 1 and 2; to domains 3 and 4; or to domains 5, 6, and 7.

In some aspects, this disclosure provides a method of treating a CD19-negative cancer, e.g., a leukemia, e.g., an ALL, e.g., B-ALL, comprising administering a CD22 inhibitor, e.g., a CD22 binding domain or CD22 CAR-expressing cell described herein. In some embodiments, the method includes a step of determining whether the cancer is CD19-negative. In some embodiments, the subject has received a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, and is resistant, relapsed, or refractory to the CD19 inhibitor.

ROR1 INHIBITORS

The ROR1 inhibitor may be, e.g., a small molecule, antibody, or fragment thereof. In one embodiment, the ROR1 inhibitor is an anti-ROR1 antibody or fragment thereof. In one embodiment, the anti-ROR1 antibody or fragment thereof is a monoclonal antibody, e.g., cirmtuzumab.

In one embodiment, the ROR1 inhibitor is an anti-ROR1 expressing cell, e.g., ROR1 CART or ROR1-expressing NK cell.

In some embodiments, the ROR1-CAR comprises an optional leader sequence (e.g., an optional leader sequence described herein), an extracellular antigen binding domain, a hinge (e.g., hinge described herein), a transmembrane domain (e.g., transmembrane domain described

herein), and an intracellular stimulatory domain (e.g., intracellular stimulatory domain described herein). In one embodiment, an exemplary ROR1 CAR construct comprises an optional leader sequence (e.g., a leader sequence described herein), an extracellular antigen binding domain, a hinge, a transmembrane domain, an intracellular costimulatory domain (e.g., an intracellular costimulatory domain described herein) and an intracellular stimulatory domain.

In one embodiment the ROR1 binding domain comprises an scFv portion, e.g., a human scFv portion. The scFv the scFv may be preceded by an optional leader sequence such as provided in SEQ ID NO: 13, and followed by an optional hinge sequence such as provided in SEQ ID NO: 14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49, a transmembrane region such as provided in SEQ ID NO:15, an intracellular signalling domain that includes SEQ ID NO:16 or SEQ ID NO:51 and a CD3 zeta sequence that includes SEQ ID NO:17 or SEQ ID NO:43, e.g., wherein the domains are contiguous with and in the same reading frame to form a single fusion protein.

In some embodiments, the present disclosure encompasses a recombinant nucleic acid construct comprising a nucleic acid molecule encoding a ROR1 CAR, wherein the nucleic acid molecule comprises the nucleic acid sequence encoding a ROR1 binding domain, e.g., described herein, e.g., that is contiguous with and in the same reading frame as a nucleic acid sequence encoding an intracellular signaling domain. An exemplary intracellular signaling domain that can be used in the CAR includes, but is not limited to, one or more intracellular signaling domains of, e.g., CD3-zeta, CD28, 4-1BB, and the like. In some instances, the CAR can comprise any combination of CD3-zeta, CD28, 4-1BB, and the like.

In one embodiment, the ROR1 binding domain is characterized by particular functional features or properties of an antibody or antibody fragment. For example, in one embodiment, the portion of a CAR composition of the invention that comprises an antigen binding domain specifically binds human ROR1 or a fragment thereof. In certain embodiments, the scFv is contiguous with and in the same reading frame as a leader sequence. In one aspect the leader sequence is the polypeptide sequence provided as SEQ ID NO:13.

In one embodiment, the ROR1 binding domain is a fragment, e.g., a single chain variable fragment (scFv). In one embodiments, the ROR1 binding domain is a Fv, a Fab, a (Fab')2, or a bi-functional (e.g. bi-specific) hybrid antibody (e.g., Lanzavecchia et al., Eur. J. Immunol. 17,

105 (1987)). In one aspect, the antibodies and fragments thereof of the invention binds a ROR1 protein or a fragment thereof with wild-type or enhanced affinity. In some instances, a human scFv can be derived from a display library.

In one embodiment, the ROR1 binding domain, e.g., scFv comprises at least one mutation such that the mutated scFv confers improved stability to the ROR1 CART construct. In another embodiment, the ROR1 binding domain, e.g., scFv comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mutations arising from the humanization process such that the mutated scFv confers improved stability to the ROR1 CART construct.

In one embodiment, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and ROR1 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a ROR1 CAR.

CD123 INHIBITORS

The CD123 inhibitor may be, e.g., a small molecule, antibody, or fragment thereof (e.g., a monospecific or bispecific antibody or fragment thereof); a recombinant protein, e.g., fusion protein, that binds to CD123; inhibitory nucleic acid; or a cell expressing a CD123 CAR, e.g., a CD123 CART.

In one embodiment, the CD123 inhibitor is a recombinant protein, e.g., comprising the natural ligand (or a fragment) of the CD123 receptor, e.g., SL-401 (also called DT388IL3; University of Texas Southwestern Medical Center).

In another embodiment, the CD123 inhibitor is an anti-CD123 antibody or fragment thereof, e.g., a monoclonal antibody (e.g., a monospecific or bispecific antibody or fragment thereof), such as CSL360 (CSL Limited), CSL362 (CSL Limited), or MGD006 (MacroGenics).

In one embodiment, the CD123 inhibitor is an anti-CD123 CAR expressing cell, e.g., CD123 CART or CD123 CAR-expressing NK cell.

In some embodiments, the CD123-CAR comprises an optional leader sequence (e.g., an optional leader sequence described herein), an extracellular antigen binding domain, a hinge (e.g., hinge described herein), a transmembrane domain (e.g., transmembrane domain described

herein), and an intracellular stimulatory domain (e.g., intracellular stimulatory domain described herein). In one embodiment, an exemplary CD123 CAR construct comprises an optional leader sequence (e.g., a leader sequence described herein), an extracellular antigen binding domain, a hinge, a transmembrane domain, an intracellular costimulatory domain (e.g., an intracellular costimulatory domain described herein) and an intracellular stimulatory domain.

In one embodiment, the CD123 binding domain comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a CD20 binding domain described herein, and/or one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a CD123 binding domain described herein, e.g., a CD123 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. These CDRs may be, e.g., those of any of **Tables 17, 18, 26, or 27**. In one embodiment, the CD123 binding domain comprises one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a CD123 binding domain described herein, e.g., the CD123 binding domain has two variable heavy chain regions, each comprising a HC CDR1, a HC CDR2 and a HC CDR3 described herein. In one embodiment, the CD123 binding domain comprises a light chain variable region described herein and/or a heavy chain variable region described herein. In one embodiment, the CD123 binding domain comprises a heavy chain variable region described herein, e.g., at least two heavy chain variable regions described herein. In one embodiment, the CD123 binding domain is a scFv comprising a light chain and a heavy chain of an amino acid sequence of **Table 16 or 25**. In an embodiment, the CD123 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region in **Table 16 or 25**, or a sequence with 95-99% identity with a light chain variable region in **Table 16 or 25**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain

variable region in **Table 16 or 25**, or a sequence with 95-99% identity to a heavy chain variable region in **Table 16 or 25**.

In one embodiment, the CAR molecule comprises an anti-CD123 binding domain that includes one or more (e.g., 2, 3, 4, 5, or 6) LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 of a construct of **Table 17 and 18**, e.g., CAR123-1, CAR123-2, CAR123-3, or CAR123-4. In one embodiment, the CAR molecule comprises an anti-CD123 binding domain that includes one or more (e.g., 2, 3, 4, 5, or 6) LC CDR1, LC CDR2, LC CDR3, HC CDR1, HC CDR2, and HC CDR3 of a construct of **Table 26 and 27,** e.g., hzCAR123.

The CD123 scFv may be preceded by an optional leader sequence such as provided in SEQ ID NO: 13, and followed by an optional hinge sequence such as provided in SEQ ID NO: 14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49, a transmembrane region such as provided in SEQ ID NO:15, an intracellular signalling domain that includes SEQ ID NO:16 or SEQ ID NO:51 and a CD3 zeta sequence that includes SEQ ID NO:17 or SEQ ID NO:43, e.g., wherein the domains are contiguous with and in the same reading frame to form a single fusion protein.

Further embodiments include a nucleotide sequence that encodes a polypeptide of **any of Tables 16-27.** Further embodiments include a nucleotide sequence that encodes a polypeptide **any of Tables 16-27**, and each of the domains of SEQ ID NOS: 13, 14, 15, 16, 17, and optionally 51.

In one embodiment, the CD123 binding domain is characterized by particular functional features or properties of an antibody or antibody fragment. For example, in one embodiment, the portion of a CAR composition of the invention that comprises an antigen binding domain specifically binds human CD123 or a fragment thereof.

In one embodiment, the CD123 binding domain is a fragment, e.g., a single chain variable fragment (scFv). In one embodiments, the CD123 binding domain is a Fv, a Fab, a (Fab')2, or a bi-functional (e.g. bi-specific) hybrid antibody (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)). In one aspect, the antibodies and fragments thereof of the invention binds a CD123 protein or a fragment thereof with wild-type or enhanced affinity. In some instances, a human scFv can be derived from a display library.

In one embodiment, the CD123 binding domain, e.g., scFv comprises at least one mutation such that the mutated scFv confers improved stability to the CART123 construct. In another embodiment, the CD123 binding domain, e.g., scFv comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mutations arising, e.g., from the humanization process, such that the mutated scFv confers improved stability to the CART123 construct.

In some embodiments, the CD123 inhibitor comprises an antibody molecule, e.g., an antibody molecule having a CD123-binding sequence as described herein. For instance, the antibody molecule may comprise CDRs or a VH and VL as described in any of Tables **16-27**, or a sequence with homology thereto, e.g., having 95-99% identity thereto. The antibody molecule may comprise a CD123-binding region having a sequence described in this section, e.g., in the context of a CAR.

In one embodiment, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD123 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD123 CAR.

CD10 INHIBITORS

The CD10 inhibitor may be, e.g., a small molecule, antibody, or fragment thereof (e.g., a monospecific or bispecific antibody or fragment thereof); a recombinant protein, e.g., fusion protein, that binds to CD10; inhibitory nucleic acid; or a cell expressing a CD10 CAR, e.g., a CD10 CART.

In an embodiment, the CD10 inhibitor comprises a small molecule, such as sacubitril (Novartis), valsartan/sacubritril (Novartis), omapatrilat (Bristol-Myers Squibb), RB-101, UK-414,495 (Pfizer), or a pharmaceutically acceptable salt or a derivative thereof.

In one embodiment, the CD10 inhibitor is an anti-CD10 CAR expressing cell, e.g., CD10 CART or CD10 CAR-expressing NK cell.

In one embodiment, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD10 CARs. For example, in one embodiment, the population of CART cells can include a first cell

expressing a CD19 CAR and a second cell expressing a CD10 CAR.

CD34 INHIBITORS

The CD34 inhibitor may be, e.g., a small molecule, antibody, or fragment thereof (e.g., a monospecific or bispecific antibody or fragment thereof); a recombinant protein, e.g., fusion protein, that binds to CD34; inhibitory nucleic acid; or a cell expressing a CD34 CAR, e.g., a CD34 CART.

In an embodiment, the CD34 inhibitor comprises a monoclonal antibody or fragment thereof that targets CD34 or an immunoliposome comprising an anti-CD34 monoclonal antibody or fragment thereof.

In one embodiment, the CD34 inhibitor is an anti-CD34 CAR-expressing cell, e.g., CD34 CART or CD34 CAR-expressing NK cell.

In one embodiment, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD34 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD34 CAR.

FLT-3 INHIBITORS

The FLT-3 inhibitor may be, e.g., a small molecule, antibody, or fragment thereof (e.g., a monospecific or bispecific antibody or fragment thereof); a recombinant protein, e.g., fusion protein, that binds to FLT-3; inhibitory nucleic acid; or a cell expressing a FLT-3 CAR, e.g., a FLT-3 CART.

In some embodiments, the FLT-3 inhibitor comprises a small molecule, such as quizartinib (Ambit Biosciences), midostaurin (Technische Universitat Dresden), sorafenib (Bayer and Onyx Pharmaceuticals), sunitinib (Pfizer), lestaurtinib (Cephalon), or a pharmaceutically acceptable salt or derivative thereof.

In one embodiment, the FLT-3 inhibitor is an anti-FLT-3 CAR expressing cell, e.g., FLT-3 CART or FLT-3 CAR-expressing NK cell.

In one embodiment, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and FLT-3 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a FLT-3 CAR.

CD79b INHIBITORS

In certain embodiments, the CD19 CAR-expressing cell is administered with a CD79b inhibitor. The CD79b inhibitor can be, e.g., a small molecule, antibody, or fragment thereof (e.g., a monospecific or bispecific antibody or fragment thereof); a recombinant protein, e.g., fusion protein, that binds to CD79b; inhibitory nucleic acid; or a cell expressing a CD79b CAR, e.g., a CD79b CAR-expressing T cell or NK cell. In one embodiment, the CD79b inhibitor is an anti-CD79b CAR expressing cell, e.g., CD79b CART or CD79b CAR-expressing NK cell. Exemplary CD79b inhibitors are described in more detail below.

In an embodiment, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells or CAR-expressing NK cells, comprising a mixture of cells expressing CD19 CARs and CD79b CARs. For example, in one embodiment, the population of CAR-expressing cells includes a first cell expressing a CD19 CAR and a second cell expressing a CD79b CAR.

CD179b INHIBITORS

In certain embodiments, the CD19 CAR-expressing cell is administered with a CD179b inhibitor. The CD179b inhibitor can be, e.g., a small molecule, antibody, or fragment thereof (e.g., a monospecific or bispecific antibody or fragment thereof); a recombinant protein, e.g., fusion protein, that binds to CD179b; inhibitory nucleic acid; or a cell expressing a CD179b CAR, e.g., a CD179b CAR-expressing T cell or NK cell. In one embodiment, the CD79b inhibitor is an anti-CD179b CAR expressing cell, e.g., CD179b CART or CD179b CAR-expressing NK cell. Exemplary CD179b inhibitors are described in more detail below.

In an embodiment, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells or CAR-expressing NK cells, comprising a mixture of cells expressing CD19

CARs and CD179b CARs. For example, in one embodiment, the population of CAR-expressing cells includes a first cell expressing a CD20 CAR and a second cell expressing a CD179b CAR.

C79a INHIBITORS

In certain embodiments, the CD19 CAR-expressing cell is administered with a CD79a inhibitor. The CD79a inhibitor can be, e.g., a small molecule, antibody, or fragment thereof (e.g., a monospecific or bispecific antibody or fragment thereof); a recombinant protein, e.g., fusion protein, that binds to CD79a; inhibitory nucleic acid; or a cell expressing a CD79a CAR, e.g., a CD79a CAR-expressing T cell or NK cell. In one embodiment, the CD79a inhibitor is an anti-CD79a CAR expressing cell, e.g., CD79a CART or CD79a CAR-expressing NK cell. Exemplary CD79a inhibitors are described in more detail below.

In an embodiment, the present disclosure provides a population of CAR-expressing cells, e.g., CART cells or CAR-expressing NK cells, comprising a mixture of cells expressing CD19 CARs and CD79a CARs. For example, in one embodiment, the population of CAR-expressing cells includes a first cell expressing a CD19 CAR and a second cell expressing a CD79a CAR.

CAR MOLECULES

The binding domains described herein (e.g., binding domains against one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a) may further comprise one or more additional amino acid sequences.

In one embodiment, the CAR molecule comprises a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. In one embodiment, the transmembrane domain comprises a sequence of SEQ ID NO: 15. In one embodiment, the transmembrane domain comprises an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 15, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 15.

In one embodiment, the binding domain is connected to the transmembrane domain by a

hinge region, e.g., a hinge region described herein. In one embodiment, the encoded hinge region comprises SEQ ID NO:14 or SEQ ID NO:45, or a sequence with 95-99% identity thereof.

In one embodiment, the CAR molecule further comprises a sequence encoding a costimulatory domain, e.g., a costimulatory domain described herein. In one embodiment, the costimulatory domain comprises a functional signaling domain of a protein selected from the group consisting of OX40, CD2, CD27, CD28, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), and 4-1BB (CD137). In one embodiment, the costimulatory domain comprises a sequence of SEQ ID NO: 16. In one embodiment, the costimulatory domain comprises a sequence of SEQ ID NO:51. In one embodiment, the costimulatory domain comprises an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO: 16 or SEQ ID NO:51, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO: 16 or SEQ ID NO:51. In one embodiment, the costimulatory domain comprises a functional signaling domain of a protein selected from the group consisting of MHC class I molecule, TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83. In embodiments, the costimulatory domain comprises 4-1BB, CD27, CD28, or ICOS.

In one embodiment, the CAR molecule further comprises a sequence encoding an intracellular signaling domain, e.g., an intracellular signaling domain described herein. In one embodiment, the intracellular signaling domain comprises a functional signaling domain of 4-

1BB and/or a functional signaling domain of CD3 zeta. In one embodiment, the intracellular signaling domain comprises the sequence of SEQ ID NO: 16 and/or the sequence of SEQ ID NO:17. In one embodiment, the intracellular signaling domain comprises the sequence of SEQ ID NO:16 and/or the sequence of SEQ ID NO:43. In one embodiment, the intracellular signaling domain comprises a functional signaling domain of CD27 and/or a functional signaling domain of CD3 zeta. In one embodiment, the intracellular signaling domain comprises the sequence of SEQ ID NO: 51 and/or the sequence of SEQ ID NO:17. In one embodiment, the intracellular signaling domain comprises the sequence of SEQ ID NO:51 and/or the sequence of SEQ ID NO:43. In one embodiment, the intracellular signaling domain comprises an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 20, 10 or 5 modifications (e.g., substitutions) of an amino acid sequence of SEQ ID NO:16 or SEQ ID NO:51 and/or an amino acid sequence of SEQ ID NO:17 or SEQ ID NO:43, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO:16 or SEQ ID NO:51 and/or an amino acid sequence of SEQ ID NO:17 or SEQ ID NO:43. In one embodiment, the intracellular signaling domain comprises the sequence of SEQ ID NO:16 or SEQ ID NO:51 and the sequence of SEQ ID NO: 17 or SEQ ID NO:43, wherein the sequences comprising the intracellular signaling domain are expressed in the same frame and as a single polypeptide chain.

In one embodiment, the CAR molecule further comprises a leader sequence, e.g., a leader sequence described herein. In one embodiment, the leader sequence comprises an amino acid sequence of SEQ ID NO: 13, or a sequence with 95-99% identity to an amino acid sequence of SEQ ID NO:13.

In one aspect, the CAR (e.g., a CD19 CAR, a ROR1 CAR, a CD20 CAR, a CD22 CAR, a CD123 CAR, a CD10 CAR, a CD34 CAR, a FLT-3 CAR, a CD79b CAR, a CD179b CAR, or a CD79a CAR) comprises an optional leader sequence (e.g., an optional leader sequence described herein), an extracellular antigen binding domain, a hinge (e.g., hinge described herein), a transmembrane domain (e.g., transmembrane domain described herein), and an intracellular stimulatory domain (e.g., intracellular stimulatory domain described herein). In one aspect an exemplary CAR construct comprises an optional leader sequence (e.g., a leader sequence described herein), an extracellular antigen binding domain, a hinge, a transmembrane domain, an intracellular costimulatory domain (e.g., an intracellular costimulatory domain

described herein) and an intracellular stimulatory domain.

BISPECIFIC ANTIBODIES

A bispecific antibody molecule (which can be, e.g., administered alone or as a portion of a CAR) can comprise two VH regions and two VL regions. In some embodiments, the upstream antibody or portion thereof (e.g. scFv) is arranged with its VH (VH_1) upstream of its VL (VL_1) and the downstream antibody or portion thereof (e.g. scFv) is arranged with its VL (VL_2) upstream of its VH (VH_2), such that the overall bispecific antibody molecule has the arrangement VH_1-VL_1-VL_2-VH_2. In other embodiments, the upstream antibody or portion thereof (e.g. scFv) is arranged with its VL (VL_1) upstream of its VH (VH_1) and the downstream antibody or portion thereof (e.g. scFv) is arranged with its VH (VH_2) upstream of its VL (VL_2), such that the overall bispecific antibody molecule has the arrangement VL_1-VH_1-VH_2-VL_2.

BISPECIFIC CD22/CD19 INHIBITORS

In an embodiment, the B-cell inhibitor comprises a bispecific CAR19/CAR22 antibody molecule. For instance, in some embodiments, the B-cell inhibitor comprises one or more amino acid sequences of **Table 28**, or a sequence having 95-99% identity thereto. Further provided are nucleic acids according to **Table 28**, or a sequence having 95-99% identity thereto. In an embodiment, the B-cell inhibitor comprises a CD19-specific antibody molecule of **Table 2 or 3** (or a sequence having 95-99% identity thereto) and a CD22-specific antibody molecule of **Table 6A or 6B** (or a sequence having 95-99% identity thereto). In an embodiment, the B-cell inhibitor comprises a CD19-specific antibody molecule having one or more CDRs of **Table 4 or 5** (or a sequence having 1, 2, 3, 4, 5, or 6 alterations e.g., substitutions thereto) and a CD22-specific antibody molecule having CDRs of **Table 7A, 7B, 7C, 8A or 8B** (or a sequence having 1, 2, 3, 4, 5, or 6 alterations e.g., substitutions thereto).

MTOR INHIBITORS

In one embodiment, the cells expressing a CAR molecule, e.g., a CD19 CAR molecule, a CD20 CAR molecule, or a CD22 CAR molecule e.g., a CAR molecule described herein,

optionally administered in combination with a B-cell inhibitor, are co-administered with a low, immune enhancing dose of an mTOR inhibitor. While not wishing to be bound by theory, it is believed that treatment with a low, immune enhancing, dose (e.g., a dose that is insufficient to completely suppress the immune system but sufficient to improve immune function) is accompanied by a decrease in PD-1 positive T cells or an increase in PD-1 negative cells. PD-1 positive T cells, but not PD-1 negative T cells, can be exhausted by engagement with cells which express a PD-1 ligand, e.g., PD-L1 or PD-L2.

In an embodiment this approach can be used to optimize the performance of CAR cells described herein in the subject. While not wishing to be bound by theory, it is believed that, in an embodiment, the performance of endogenous, non-modified immune effector cells, e.g., T cells, is improved. While not wishing to be bound by theory, it is believed that, in an embodiment, the performance of a CAR expressing cell is improved. In other embodiments, cells, e.g., T cells, which have, or will be engineered to express a CAR, can be treated ex vivo by contact with an amount of an mTOR inhibitor that increases the number of PD1 negative immune effector cells, e.g., T cells or increases the ratio of PD1 negative immune effector cells, e.g., T cells/ PD1 positive immune effector cells, e.g., T cells.

In an embodiment, administration of a low, immune enhancing, dose of an mTOR inhibitor, e.g., an allosteric inhibitor, e.g., RAD001, or a catalytic inhibitor, is initiated prior to administration of an CAR expressing cell described herein, e.g., T cells. In an embodiment, the CAR cells are administered after a sufficient time, or sufficient dosing, of an mTOR inhibitor, such that the level of PD1 negative immune effector cells, e.g., T cells, or the ratio of PD1 negative immune effector cells, e.g., T cells/ PD1 positive immune effector cells, e.g., T cells, has been, at least transiently, increased.

In an embodiment, the cell, e.g., T cell, to be engineered to express a CAR, is harvested after a sufficient time, or after sufficient dosing of the low, immune enhancing, dose of an mTOR inhibitor, such that the level of PD1 negative immune effector cells, e.g., T cells, or the ratio of PD1 negative immune effector cells, e.g., T cells/ PD1 positive immune effector cells, e.g., T cells, in the subject or harvested from the subject has been, at least transiently, increased.

Additional features or embodiments of the compositions or methods described herein include one or more of the following:

In embodiments, the B-cell inhibitor comprises an inhibitor of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1. In embodiments, the B-cell inhibitor comprises an effective number of one or more cells that express a CAR molecule that binds one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1.

In embodiments, the one or more cells that express a CAR molecule that binds CD19 are administered concurrently with, before, or after the one or more B-cell inhibitors.

In embodiments, the subject has or is identified as having a difference, e.g., a statistically significant difference, between a determined level compared to a reference level of one or more markers listed in **Table 29** in a biological sample.

In embodiments, the subject has or is identified as having a difference between a determined characteristic compared to a reference characteristic, in a characteristic of CD19, e.g., a mutation causing a frameshift or a premature stop codon or both, in a biological sample.

In embodiments, the subject has or is identified as having a difference, e.g., a statistically significant difference, between a determined level compared to a reference level of Treg cells in a biological sample.

In an embodiment, the method comprises administering to the subject a therapeutically effective dose of a chimeric antigen receptor (CAR) therapy, e.g., a CAR therapy as described herein, e.g., a therapy comprising a CD19 CAR-expressing cell and optionally one or more B-cell inhibitor, and if the subject is identified as having a difference, e.g., a statistically significant difference, between a determined level compared to a reference level, or a determined characteristic compared to a reference characteristic, in one or more of (i) a level or activity of one or more markers listed in **Table 29**; (ii) a characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) a level of T_{REG} cells in a biological sample. In an embodiment, the method comprises determining if the subject has a difference, e.g., a statistically significant difference, between a determined level compared to a reference level, or a determined characteristic compared to a reference characteristic, in one or more of (i) a level of one or more markers listed in **Table 29**; (ii) a characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) a level or activity of T_{REG} cells in a biological sample, and administering to the subject a therapeutically effective dose of a chimeric antigen receptor (CAR) therapy, e.g., a CAR therapy

as described herein, e.g., a therapy comprising a CD19 CAR-expressing cell and optionally one or more B-cell inhibitor. In an embodiment, the method comprises determining if the subject has a difference, e.g., a statistically significant difference, between a determined level compared to a reference level, or a determined characteristic compared to a reference characteristic, in one or more of (i) a level of one or more markers listed in **Table 29**; (ii) a characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) a level or activity of T_{REG} cells in a biological sample, and administering to the subject a therapeutically effective dose of a chimeric antigen receptor (CAR) therapy, e.g., a CAR therapy as described herein, e.g., a therapy comprising a CD19 CAR-expressing cell and optionally one or more B-cell inhibitor. In an embodiment, the method comprises administering to a subject a therapeutically effective dose of a chimeric antigen receptor (CAR) therapy, e.g., a CAR therapy as described herein, e.g., a therapy comprising a CD19 CAR-expressing cell, determining if the subject has a difference, e.g., a statistically significant difference, between a determined level compared to a reference level, or a determined characteristic compared to a reference characteristic, in one or more of (i) a level of one or more markers listed in **Table 29**; (ii) a characteristic of CD19, e.g., a mutation, e.g., a mutation causing a frameshift or a premature stop codon or both, or (iii) a level or activity of T_{REG} cells in a biological sample, and if the difference is present, administering to a subject a therapeutically effective dose of one or more B-cell inhibitor.

In embodiments, the subject has or is identified as having an increase, e.g., a statistically significant increase, between a determined level and to a reference level of Treg cells in a biological sample.

In embodiments, the subject has relapsed or is identified as having relapsed after treatment with the one or more cells that express a CAR molecule that binds CD19, e.g., a CD19 CAR.

In embodiments, the B-cell inhibitor comprises an effective number of one or more cells that express: a CAR molecule that binds CD10, e.g., a CD10 CAR as described herein; a CAR molecule that binds CD20, e.g., a CD20 CAR as described herein; a CAR molecule that binds CD22, e.g., a CD22 CAR as described herein; a CAR molecule that binds CD34, e.g., a CD34 CAR as described herein; a CAR molecule that binds CD123, e.g., a CD123 CAR as described herein; a CAR molecule that binds FLT-3, e.g., a FLT-3 CAR as described herein; or a CAR

molecule that binds ROR1, e.g., an ROR1 CAR as described herein.

In embodiments, the CD19 inhibitor comprises an antibody or antibody fragment which includes a CD19 binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said CD19 binding domain comprises one or more of (e.g., all three of) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD19 light chain binding domain amino acid sequence listed in **Tables 2 or 3**, and one or more of (e.g., all three of) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD19 heavy chain binding domain amino acid sequence listed in **Tables 2 or 3**.

In embodiments, a CD19 CAR comprises light chain variable region listed in **Tables 2 or 3** and any heavy chain variable region listed **Tables 2 or 3**.

In embodiments, the CD19 inhibitor comprises a CD19 binding domain which comprises a sequence selected from a group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and SEQ ID NO:12, or a sequence with 95-99% identity thereof. In embodiments, the CD19 CAR comprises a polypeptide of SEQ ID NO:58.

In embodiments, the B-cell inhibitor comprises a CD20 CAR which comprises an antibody or antibody fragment which includes a CD20 binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said CD20 binding domain comprises one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD20 light chain binding domain amino acid sequence listed in **Table 13**, and one or more of heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD20 heavy chain binding domain amino acid sequence listed in **Table 12A or 12B**.

In embodiments, the B-cell inhibitor comprises a CD22 CAR which comprises an antibody or antibody fragment which includes a CD22 binding domain, a transmembrane

domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said CD22 binding domain comprises one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD22 light chain binding domain amino acid sequence listed in **Table 8A, 8B, 10A and/or 10B**, and one or more of heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD22 heavy chain binding domain amino acid sequence listed in **Table 7A, 7B, 7C, 9A, and/or 9B**.

In embodiments, the CD22 CAR comprises any light chain variable region listed in **Table 10A or 10B**. In embodiments, the CD22 CAR comprises any heavy chain variable region listed in **Table 9A or 9B**. In embodiments, the CD22 CAR comprises any light chain variable region listed in **Table 10A or 10B** and any heavy chain variable region listed **Table 9A or 9B**.

In embodiments, the B-cell inhibitor comprises a CAR which comprises an antibody or antibody fragment which includes an antigen binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said antigen binding domain comprises one or more of (e.g., all of) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3), and one or more of (e.g., all of) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3).

In embodiments, the B-cell inhibitor comprises a CAR which comprises a scFv. In embodiments, the B-cell inhibitor comprises a CAR which comprises a transmembrane domain that comprises a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. In embodiments, the antigen binding domain is connected to the transmembrane domain by a hinge region. In embodiments, the hinge region comprises SEQ ID NO:14, or a sequence with 95-99% identity thereof. In embodiments, the costimulatory domain is a functional signaling domain obtained from a protein selected from the group consisting of OX40, CD2, CD27,

CD28, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), and 4-1BB (CD137). In embodiments, the costimulatory domain is a functional signaling domain obtained from a protein selected from the group consisting of MHC class I molecule, TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83. In embodiments, the costimulatory domain comprises a sequence of SEQ ID NO:16 or SEQ ID NO:51. In embodiments, the intracellular signaling domain comprises a functional signaling domain of 4-1BB and/or a functional signaling domain of CD3 zeta.

In embodiments, the intracellular signaling domain comprises the sequence of SEQ ID NO: 16 and/or the sequence of SEQ ID NO:17 or SEQ ID NO:43. In embodiments, the CAR further comprises a leader sequence. In embodiments, the leader sequence comprises SEQ ID NO: 13.

In embodiments, the cells that express the CAR molecule comprise T cells or NK cells.

In embodiments, the disease associated with CD19 expression is selected from a proliferative disease such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia, or is a non-cancer related indication associated with expression of CD19. In embodiments, the disease is one or more of a hematologic cancer, acute leukemia, B-cell acute lymphoid leukemia (BALL), T-cell acute lymphoid leukemia (TALL), small lymphocytic leukemia (SLL), acute lymphoid leukemia (ALL); chronic leukemia, chronic myelogenous leukemia (CML), or chronic lymphocytic

leukemia (CLL).

In embodiments, the method further comprises administering an agent that increases the efficacy of a cell expressing a CAR molecule. In embodiments, the method further comprises administering an agent that ameliorates one or more side effects associated with administration of a cell expressing a CAR molecule. In embodiments, the cells expressing a CAR molecule are administered in combination with an agent that treats the disease associated with CD19.

In embodiments, in accordance with a method described herein, e.g., a method of providing anti-tumor immunity to a mammal, or method of treating a mammal, a mammal is a non-responder, partial responder, or complete responder to a previously administered cancer therapy, e.g., a CD19 CAR therapy or a cancer therapy other than a CD19 CAR-expressing cell. In embodiments, the mammal is a non-relapser, partial relapse, or complete relapse to a previously administered cancer therapy, e.g., a CD19 CAR therapy or a cancer therapy other than a CD19 CAR-expressing cell. In embodiments, the mammal comprises a CD19-negative cancer cell or a CD19-positive cancer cell, optionally wherein the mammal further comprises a CD22-positive, CD123-positive, FLT-3-positive, ROR-1-positive, CD79b-positive, CD179b-positive, CD79a-positive, CD10-positive, CD34-positive, and/or CD20-positive cancer cell. In embodiments, the mammal has a relapsed ALL cancer. In embodiments, the mammal was previously administered a CD19 CAR-expressing cell and is refractory to CD19 CAR treatment.

In embodiments, the agent is an mTOR inhibitor and the subject is administered a low, immune enhancing, dose of an mTOR inhibitor, e.g., RAD001 or rapamycin. In embodiments, the mTOR inhibitor is a RAD001. In embodiments, the dose comprises an allosteric and a catalytic mTOR inhibitor. In embodiments, the mTOR inhibitor is administered for an amount of time sufficient to decrease the proportion of PD-1 positive T cells, increase the proportion of PD-1 negative T cells, or increase the ratio of PD-1 negative T cells/ PD-1 positive T cells, in the peripheral blood of the subject, or in a preparation of T cells isolated from the subject.

In embodiments, the immune effector cell, e.g., T cell, to be engineered to express a CAR, is harvested after a sufficient time, or after sufficient dosing of the low, immune enhancing, dose of an mTOR inhibitor, such that the level of PD1 negative immune effector cells, e.g., T cells, or the ratio of PD1 negative immune effector cells, e.g., T cells/ PD1 positive immune effector cells, e.g., T cells, in the subject or harvested from the subject has been, at least

transiently, increased. In embodiments, the dose of an mTOR inhibitor is associated with mTOR inhibition of at least 5 but no more than 90%, e.g., as measured by p70 S6 K inhibition. In embodiments, the dose of an mTOR inhibitor is associated with mTOR inhibition of at least 10% but no more than 40%, e.g., as measured by p70 S6 K inhibition.

In an embodiment, the method further comprises administering a checkpoint inhibitor. In embodiments, the subject receives a pre-treatment of with an agent, e.g., an mTOR inhibitor, and/or a checkpoint inhibitor, prior to the initiation of a CART therapy. In embodiments, the subject receives concurrent treatment with an agent, e.g., an mTOR inhibitor, and/or a checkpoint inhibitor. In embodiments, the subject receives treatment with an agent, e.g., an mTOR inhibitor, and/or a checkpoint inhibitor, post-CART therapy.

In embodiments, the determined level or determined characteristic is acquired before, at the same time, or during a course of CART therapy.

In embodiments, the method comprises assaying a gene signature that indicates whether the subject is likely to relapse, or has relapsed. In embodiments, the method comprises assaying a gene signature in a subject prior to treatment with a CAR-expressing cell, e.g., CART treatment (e.g., a CART19 treatment, e.g., CTL019 therapy) that predicts relapse to CAR treatment. In embodiments, the level of one or more markers is the level of at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 markers listed in **Table 29**. In embodiments, the level of the marker comprises an mRNA level or a level of a soluble protein.

In embodiments, the characteristic of CD19 is a mutation in exon 2, e.g., a mutation causing a frameshift or a premature stop codon or both. In embodiments, the level of T_{REG} cells is determined by staining a sample for a marker expressed by T_{REG} cells. In embodiments, the level of T_{REG} cells is the level of Treg cells in a relevant location in the subject's body, e.g., in a cancer microenvironment.

In embodiments, the method further comprises decreasing the T_{REG} signature in the subject prior to apheresis. In embodiments, the method further comprises decreasing the T_{REG} signature in the subject, e.g., by administering cyclophosphamide, an anti-GITR antibody, or both to the subject. In embodiments, the method comprises pre-treating a subject with cyclophosphamide, an anti-GITR antibody, or both, prior to collection of cells for CAR-expressing cell product manufacturing. In embodiments, the method further comprises

obtaining a sample from the subject, wherein the sample comprises a cellular fraction (e.g., which comprises blood), a tissue fraction, an apheresis sample, or a bone marrow sample.

In embodiments, the cell expresses an inhibitory molecule that comprises a first polypeptide that comprises at least a portion of an inhibitory molecule, associated with a second polypeptide that comprises a positive signal from an intracellular signaling domain. In embodiments, the inhibitory molecule comprise first polypeptide that comprises at least a portion of PD1 and a second polypeptide comprising a costimulatory domain and primary signaling domain.

In embodiments, the method comprises assaying a gene signature that indicates whether a subject treated with the cell is likely to relapse, or has relapsed. In embodiments, the method comprises assaying the gene signature in the cell prior to infusion into the subject. In embodiments, the method further comprises decreasing the T_{REG} signature of a population of cells comprising the transduced cell. In embodiments, decreasing the T_{REG} signature comprises performing CD25-depletion on the population of cells.

In embodiments, the subject is a mammal, e.g., a human.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein (e.g., sequence database reference numbers) are incorporated by reference in their entirety. For example, all GenBank, Unigene, and Entrez sequences referred to herein, e.g., in any Table herein, are incorporated by reference. Unless otherwise specified, the sequence accession numbers specified herein, including in any Table herein, refer to the database entries current as of April 8, 2015. When one gene or protein references a plurality of sequence accession numbers, all of the sequence variants are encompassed.

In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Headings, sub-headings or numbered or lettered elements, e.g., (a), (b), (i) etc, are presented merely for ease of reading. The use of headings or numbered or lettered elements in

this document does not require the steps or elements be performed in alphabetical order or that the steps or elements are necessarily discrete from one another.

Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A and 1B are schematics of representative CARs.

FIG. 2 contains images of immunohistochemical analysis of a Hodgkin lymphoma showing CD19 expressing cells present in the tumor. The left panel is at 1X magnification and the right panel is at 20X magnification.

FIG. 3 is a schematic diagram of the experimental set-up for a study to assess the therapeutic efficacy of CART19 treatment in patients with Hodgkin lymphoma.

FIGS. 4A, 4B, 4C, and 4D show flow cytometry analysis of PD1 and CAR19 expression on T cells. FIG. 4A and 4B are representative flow cytometry profiles demonstrating the distribution of PD-1 and CAR19 expression on CD4+ T cells from subjects that are complete responders (CR) or non-responders (NR) to CART therapy. FIG. 4C is a graph showing the percent of PD1 cells in the CD4+ T cell population from groups of subjects with different responses to CART therapy. FIG. 4D is a graph showing the percent of PD1 cells in the CD8+ T cell population from groups of subjects with different responses to CART therapy.

FIGS. 5A and 5B show the distribution of PD1 expression in CD4 and CAR19-expressing cells (FIG. 5A) or CD8 and CAR19-expressing cells (FIG. 5B) from groups of subjects with different responses to CART therapy.

FIG. 6 shows flow cytometry analysis of PD1, CAR 19, LAG3, and TIM3 expression on T cells from subjects that are complete responders (CR) or non-responders (NR) to CART therapy.

FIGS. 7A and 7B show the distribution of PD1 and LAG3 expression (FIG. 7A) or PD1 and TIM3 expression (FIG. 7B) from groups of subjects with different responses to CART therapy.

FIG. 8 shows the plasma cell IgA immunophenotyping from a myeloma patient who received CART19, demonstrating the response to CART19 therapy.

FIGS. 9A and 9B show IL-7 receptor (CD127) expression on cancer cell lines and CART cells. Expression of CD127 was measured by flow cytometry analysis in three cancer cell lines: RL (mantle cell lymphoma), JEKO (also known as Jeko-1, mantle cell lymphoma), and Nalm-6 (B-ALL) (Fig. 9A). CD127 expression was measured by flow cytometry analysis on CD3 positive (CART) cells that had been infused and circulating in NSG mice (Fig. 9B).

FIGS. 10A, 10B, and 10C show the anti-tumor response after CART19 treatment and subsequent IL-7 treatment. NSG mice engrafted with a luciferase-expressing mantle lymphoma cell line (RL-luc) at Day 0 were treated with varying dosages of CART19 cells at Day 6, and tumor burden was monitored. Mice were divided into 4 groups and received no CART19 cells, $0.5x10^6$ CART19 cells (CART19 0.5E6), $1x10^6$ CART19 cells (CART19 1E6), or $2x10^6$ CART19 cells (CART19 2E6). Tumor burden after CART treatment was measured by detection of bioluminescence (mean BLI) (Fig. 10A). Mice receiving $0.5x10^6$ CART19 cells (CART19 0.5E6) or $1x10^6$ CART19 cells (CART19 1E6) were randomized to receive recombinant human IL-7 (rhIL-7) or not. Tumor burden, represented here by mean bioluminescence (BLI), was monitored for the three mice (#3827, #3829, and #3815, receiving the indicated initial CART19 dose) from FIG. 10A that were treated with IL-7 starting at Day 85 (FIG. 10B). IL-7 was administered through IP injection 3 times weekly. Tumor burden, represented here by mean bioluminescence (BLI) before Day 85 (PRE) and after Day 115 (POST) was compared between mice that did not receive IL-7 (CTRL) and mice that received IL-7 treatment (IL-7) (Fig. 10C).

FIGS. 11A and 11B show the T cell dynamics after IL-7 treatment. The level of human T cells detected in the blood was monitored for each of the mice receiving IL-7 or control mice (Fig. 11A). The level of CART19 cells (CD3+ cells) detected in the blood was measured before (PRE) and 14 days after (Day 14) initiation of IL-7 treatment (Fig. 11B).

FIG. 12 depicts the structures of two exemplary RCAR configurations. The antigen binding members comprise an antigen binding domain, a transmembrane domain, and a switch domain. The intracellular binding members comprise a switch domain, a co-stimulatory signaling domain and a primary signaling domain. The two configurations demonstrate that the first and second switch domains described herein can be in different orientations with respect to

the antigen binding member and the intracellular binding member. Other RCAR configurations are further described herein.

FIG. 13 depicts two constructs for bispecific CARs with anti-C22 and anti-CD19 binding domains. "4G4S" represents the linker sequence GGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 1311).

FIG. 14 is a graph depicting the activity of bispecific CD19/CD22 CAR constructs in an NFAT assay.

FIGS. 15A, 15B, and 15C are graphs showing the extent of CAR T-cell activation (measured by relative luminescence) in the presence of various tumor target cell lines. FIG. 15A shows CAR T-cell activation in the presence of CD20 expressing target cell line, Daudi. FIG. 15B shows CAR T-cell activation in the presence of CD20 expressing target cell line, Raji. FIG. 15C shows CAR T-cell activation in the presence of a non CD20 expressing negative control, K562.

FIG. 16 is an exemplary schematic illustrating an overview of the gene signature analysis. Briefly, for each gene set, a 2-group statistical model was applied to determine whether the meta-gene was statistically different between the CRs, PRs, and NRs. CRs are more like resting T_{EFF} cells, whereas NR are more like activated T_{EFF} cells. Genes upregulated in activated versus resting T_{EFF} cells are also upregulated in NRs.

FIG. 17 depicts exemplary results (p=0.000215) illustrating that T_{REG} genes have high expression levels in samples from pediatric patients who were complete responders who became relapsers (R) compared to complete responders (CR) who did not relapse. The x-axis is samples by response group where CR=complete responder without relapse and R=relapser. The y-axis is normalized meta-gene expression scores.

FIGS. 18A, 18B, and 18C are graphs showing CAR T-cell activation in the presence of tumor target cell lines. In FIG. 18A, CAR-expressing JNL cells were mixed with the Daudi CD22 expressing target cell line at the indicated E:T ratios. In FIG. 18B, CAR-expressing JNL cells were mixed with the Raji CD22 expressing target cell line at the indicated E:T ratios. In FIG. 18C, CAR-expressing JNL cells were mixed with the negative control K562 cell line at the indicated E:T ratios.

FIG. 19 is a graph showing primary T-cells expression of chimeric antigen receptor on the cell surface. Protein-L-biotin/SA-PE (Fig. 19-1 and Fig. 19-2) and rhCD22-Fc/anti-Fc488 (Fig. 19-3 and 19-4) were used to determine CAR surface expression levels. Cells with no CAR were used as a negative control.

FIGS. 20A, 20B, 20C, 20D, 20E, and 20F are graphs showing a primary T-cell tumor target killing assay. Primary T-cells activated and transduced with CD22 CAR were mixed with target cell lines stably expressing luciferase at the ratios indicated and target cell killing was measured. The percent killing was normalized to hCD22-8 (28.8% transduction). CD22-experessing cell lines Raji (FIG. 20A), SEM (FIG. 20B), K562-hCD22 (FIG. 20C), Daudi (FIG. 20D), and Nalm6 (FIG. 20E) were used to test the function CD22 CAR clones in comparison with positive control CD22 CAR m971 (m971-HL), negative control CAR m971-LH, and untransduced T-cells as a negative control. K562 cell line does not express CD22 and was used as a negative control (FIG. 20F).

FIGS. 21A, 21B, 21C, 21D, 21E, and 21F are graphs showing induction of a significant proinflammatory cytokine response by CD22 CAR clones. Primary T-cell killing assays were used to determine the ability of CD22 CAR clone to produce the proinflammatory cytokines IFN-g, IL-2 and TNFa. Effector cells were co-cultured for 20 hours with each of the different target cell lines, normalized to 28.8% transduction. Supernatants were taken from different cultures with varying E:T ratios of 2.5:1 and 10:1 from Raji CD22 expressing target cells (FIG. 21A), Nalm6 CD22 expressing target cells (FIG. 21B), Daudi CD22 expressing target cells (FIG. 21C), SEM CD22 expressing target cells (FIG. 21D), K562-hCD22 CD22 expressing target cells (FIG. 21E), and K562 non-CD22 expressing cells (negative control) (FIG. 21F).

FIG. 22 is a graph depicting the expression of various B-cell antigens in relapsed ALL is a graph depicting the expression of various B-cell antigens in relapsed ALL as detected by flow cytometry. Samples from 16 r/r patients were screened by multiparametric flow cytometry for the following markers: CD19 (16 pts), CD22 (16 pts), CD123 (16 pts), FLT-3 (9 pts), ROR-1 (3 pts), CD79b (15 pts), CD179b (8 pts), CD79a (16 pts), CD10 (16 pts), CD34 (16 pts), and CD20 (16 pts). CD22 and CD123 were highly (>60%) and homogeneously expressed in the blasts of r/r ALL patients (bar indicates median % expression, respectively 99.50%, 98.80%, 95.70%, 72.00%, 47.00%,15.00%, 13.45%, 4.200%, 98.00%, 87.65%, and 7.00%). For each patient, the percentage of cells expressing the marker indicated is shown as a single data point.

FIG. 23 is a set of graphs showing is a set of graphs showing expression of CD22 and CD123 in 6 patients relapsing with CD19-negative leukemia, both before CART19 treatment (baseline) and after (CD19-neg relapse). In all analyses, the population of interest was gated based on forward vs. side scatter characteristics followed by singlet gating, and live cells were gated using Live Dead Aqua (Invitrogen). Time gating was included for quality control. The gating strategy included: time gating → SSC low → singlets → live → CD45dim → CD10+.

FIG. 24 is a set of graphs showing the expression of CD22 in in blasts from patient relapsing with CD19-neg disease after CART19 treatment (clinical trials UPCC04409/CHP959, patient UPN indicated in the squared box). The top row shows the CD19 and CD22 expression in blasts before CART19 treatment while the bottom row shows the disease phenotype at relapse. CD22 expression was maintained also at relapse when CD19 expression was lost.

FIG. 25 is a set of graphs showing the expression of CD123 in is a set of graphs showing expression of CD123 in blasts from patient relapsing with CD19-neg disease after CART19 treatment (clinical trials UPCC04409/CHP959, patient UPN indicated in the squared box). The top row shows the CD19 and CD123 expression in blasts before CART19 treatment while the bottom row shows the disease phenotype at relapse. CD123 expression was maintained at relapse in most of the patients while CD19 expression was lost.

FIG. 26 is a graph showing the median expression of CD19, CD22 and CD123 before and after CART19 treatment in patients relapsing with a CD19-negative disease. CD19 expression was lost at relapse (94.25% vs. 0%, p=0.0009), while CD22 (99.20% vs. 97.30%, p=ns) and CD123 (63.00% vs. 48.75%, p=ns) were still expressed. For each patient, the percentage of cells expressing the marker indicated is shown as a single data point.

FIGS. 27A and 27B are a series of graphs showing CD22 expression in samples from 16 r/r patients and 4 patients relapsing with CD19-negative disease after treatment with CART19 therapy. Samples were screened by multiparametric flow cytometry for the B cell marker, CD22. CD22 was highly (>60%) and homogeneously expressed in the blasts of 11/15 r/r ALL patients (FIG. 27A). CD22 was positive in 4/4 patients relapsing with CD19-negative leukemia, both before CART19 treatment (baseline) and after (CD19-neg relapse) (2 pts shown) (FIG. 27B). Gating strategy: SSC low→ singlets → live → CD45dim

FIGS. 28A, 28B, and 28C are a series of graphs showing the effect of CD22 CART on CD19 and CD22 expression. Schema of the two CAR22 constructs that were generated using different chain orientations (H to L and L to H) is shown (FIG. 28A). The anti-CD22 scFv (m971) was codon optimized and cloned in the murine CAR19 vector containing CD8 hinge, 41-BB costimulatory and CD3 zeta signaling domains (FIG. 28A). The expression of CD19, CD22 and isotype control on NALM6 ALL cell line is shown as mean fluorescence intensity (MFI) (FIG. 28B) and antibody-binding capacity (ABC) (FIG. 28C). In NALM-6 the expression of CD19 was higher than CD22. However, in most primary ALL samples the CD19 and CD22 expressions were similar (see Fig. 27A).

FIGS. 29A, 29B, and 29C are a series of graphs showing normal donor T cell expansions for generating CART22 and CART19 (together with UTD cells). Population doublings (PD) versus days in culture: at the end of the expansion (day 11) CART22 and control T cells reached around 4.5 PD, with no significant difference in comparison to CART19 or UTD cells (FIG. 29A). T cell volume (fl) versus days in culture: at day 6 there was peak volume (around 450 fl) while in the following days the volume decreased down to 300 fl when the cells are harvested and frozen. No significant different was observed versus CART19 or UTD cells (FIG. 29B). CAR expression on CD4-positive and CD8-positive T-cells at day 11 of expansion is shown in FIG. 29C. Gating for CAR expression is based on UTD. Gating strategy: FSS vs SSC lymphocytes→ singlets → live → CD3+.

FIG. 30 is a series of graphs showing a CD107a degranulation assay with intra-cytoplasmic cytokine production. CART19, CART22 HtoL and LtoH were co-cultured with different targets (alone, PMA/IONOMYCIN, MOLM-14 and NALM-6). CART19 and CART22 HtoL show high levels of CD107a degranulation, IL-2, IFNg and TNFa production when co-cultured with the ALL cell line (NALM-6) but not when co-cultured with negative controls. UTD and CART22 LtoH did not show degranulation nor cytokine productions. Gating strategy: FSS vs SSC lymphocytes→ singlets → live → CD3+.

FIG. 31 is a graph showing a luciferase-based killing assay. CART22 and CART19 HtoL but not UTD cells were able to lyse NALM-6 cells when co-cultured os for 24 hours. A direct correlation between cytotoxic activity and E:T ratios was observed, with better anti-leukemia effect at 2:1 E:T ratio (78% and 75% killing for CART19 and CART22).

FIGS. 32A and 32B are a series of graphs showing a CFSE-based proliferation assay. Co-culture for 5 days of CART22 and CART19 with the ALL cell line NALM-6 led to significant T cell proliferation (94% and 92.9% respectively). Controls are also shown (TCM= media alone, P-I= PMA/Ionomycin, MOLM-14) (FIG. 32A). In histograms showing the dynamics of CFSE dilution in CART19 and CART22, most of T cells underwent multiple proliferative cycles (FIG. 32B). Gating strategy: FSS vs SSC lymphocytes→ singlets → live → CD3+.

FIG. 33 is a series of graphs showing cytokine production. CART22, CART19 and UTD were incubated for 24 hours with different irradiated targets (alone, PMA/Ionomycin, MOLM-14 and NALM-6). When co-cultured with the ALL cell line NALM-6 only CART22 and CART19 HtoL were able to release multiple cytokines (here shown IFNg, IL-2, GM-CSF, TNFa and MIP1b). Results are shown as mean intensity fluorescence (MFI).

FIGS. 34A and 34B are a series of graphs showing T-cell degranulation with primary ALL blasts. CART22, CART19 and UTD cells were co-incubated for 4 hours with blasts derived from an ALL patient (CHP-959-101) at baseline and after CART19 treatment when the patient relapsed with a CD19-neg disease. Both CART19 and CART22 were able to degranulate at baseline (when blasts are CD19+ and CD22+) but at relapse only CART22 was degranulating (when the disease is CD19-neg) (FIG. 34A). Dot-plots showing CD107a degranulation in CD8-pos and CD8-neg CART19 and CART22 effector after incubation with CHP101 sample at relapse demonstrate only CART22 showed degranulation in both CD8 and CD4 T cells (FIG. 34B). Gating strategy: FSS vs SSC lymphocytes→ singlets → live → CD3+.

FIGS. 35A, 35B, 35C, and 35D are a series of graphs showing in vivo CART22 efficacy against NALM-6. A. Schema of the experiment: 1 million NALM-6 luciferase+ cells/mouse were injected i.v. in NSG mice. After 6 days tumor engraftment was assessed by bioluminescence. Mice were then randomized to receive untransduced T cells or different doses of CART22 (from 1.25 to 5 million total cells/mouse, with 75% CAR expression). Mice were then monitored for tumor burden, PB T cell expansion, and survival (FIG. 35A). Tumor burden by bioluminescence (BLI) detected a dose-related anti leukemia response. Mice receiving $5e^6$ CART22 cells showed better tumor control (FIG. 35B). CART22 treated mice showed a statistically significant better overall survival (OS) in comparison to mice treated with UTD cells. For OS there was a significant correlation between higher dose of CART22 and better OS

(FIG. 35C). T-cell in vivo expansion was monitored weekly by retro-orbital bleedings. One week after T cell infusion mice receiving the higher dose of CART22 showed better CART expansion (median of 12 T cells/μl) (FIG. 35D).

FIGS. 36A and 36B are a series of graphs showing an in vivo comparison between CART22 and CART19 against NALM-6. Schema of the experiment: 1 million NALM-6 luciferase+ cells/mouse were injected i.v. in NSG mice. After 6 days tumor engraftment was assessed by bioluminescence. Mice were then randomized to receive untransduced T cells, CART19 or CART22 (5 million total cells, with 75% CAR expression). Mice were then monitored for tumor burden, PB T cell expansion, and survival (FIG. 36A). Tumor burden by bioluminescence (BLI) demonstrated anti leukemia response in both CART22 and CART19 treated mice, while UTD mice rapidly progressed (FIG. 36B). CART19 treated mice showed better overall survival (OS) in comparison to CART22, possibly due to the different target expression in NALM-6 (CD19>>CD22) (FIG. 36C).

FIGS. 37A and 37B are a series of graphs showing an in vivo comparison between CART22 and CART19 in a model of primary ALL. The blasts of a primary ALL patient (JH331) were passaged in vivo and transduced with luciferase to follow tumor burden. Schema of the experiment: 1 million JH331 luciferase+ cells/mouse were injected i.v. in NSG mice. After 14 days tumor engraftment was assessed by bioluminescence. Mice were then randomized to receive untransduced T cells, CART19 or CART22 (5 million total cells, with 75% CAR expression). Mice were then monitored for tumor burden, PB T cell expansion, and survival (FIG. 37A). Tumor burden by bioluminescence (BLI) detected anti leukemia response in both CART22 and CART19 treated mice, while UTD mice rapidly progressed (FIG. 37B).

FIGs. 38A, 38B, and 38C are a series of images showing tissue microarrays for CD22 expression on 28 human normal tissues by immunohistochemistry staining. Lymphoid organs resulted positive for CD22 expression (tonsil, lymph node, spleen and thymus) (FIG. 38A). Non-lymphoid organs showed no expression of CD22 (FIG. 38B). CD22-positive resident B-cells were observed in multiple tissues (FIG. 38C). *= non-specific staining.

FIG. 39 is a graph showing CD22 RNA-expression data from GeneAtlas U133A. CD22 expression was observed at high level in B-cells, tonsil and lymph node. B-lymphoblast and leukemia/lymphoma cell lines were also highly positive.

FIG. 40 is a series of graphs showing a 51-Chromium-release assay for CART22 toxicity. Both CART22 and CART19 but not UTD cells triggered the lysis of the ALL cell line NALM-6. No cytotoxic effect of CART22 was observed in any normal tissue (CD34+, human neuronal progenitors or neurons and keratinocytes) or control (K562 cell line).

FIG. 41 shows a graphical representation of CAR expression in JNL cells transduced with anti-CD123 CAR constructs as evaluated by FACS and reported as the percent of cells showing signal above the level of signal in untransduced (CAR negative) cells using Protein L as a detection reagent.

FIGS. 42A, 42B, and 42C show graphical representations of CD123 CAR activity in JNL cells. Anti-CD123 CAR constructs were evaluated for activity using a Jurkat cell line containing the luciferase reporter driven by the NFAT promoter (termed JNL cells). CAR activity is measured as activation of this NFAT-driven reporter.

FIGS. 43A and 43B show CD123 expressing and activity. Figure 43A shows a graphical representation of CD123 CAR expression in primary T-cells. Percentage of cells transduced (expressing the anti-CD123 CAR on the cell surface) and their relative fluorescence intensity of expression were determined by flow cytometric analysis on a BD LSRFortessa or BD-FACSCanto using Protein L as a detection reagent. Gating histogram plots of relative fluorescent intensity from that FACS for signal above unstained cells shows the percentage of transduced T cells. Transduction resulted in a range of CAR positive cells from 12-42%. Figure 43B shows a graphical representation of CD123-CART-mediated cell killing. T cell killing was directed towards CD123-expressing MOLM13 acute myelogenous leukemia cells stably expressing luciferase. Untransduced T cells were used to determine non-specific background killing levels. The cytolytic activities of CART-CD123 were measured over a range of effector:target cell ratios of 4:1 and 2-fold downward dilutions of T cells where effectors were defined as T cells expressing the anti-CD123 chimeric receptor. Assays were initiated by mixing an appropriate number of T cells with a constant number of targets cells. After 20 hours luciferase signal was measured using the Bright-Glo™ Luciferase Assay on the EnVision instrument.

FIGS. 44A and 44B show transduction efficiency of T cells with CD123-CARs. Figure 44A shows transduction efficiency of T cells with 1172 and 1176. Figure 44B shows transduction efficiency of T cells with CD123 CARs 2-4.

FIG. 45 shows flow cytometry of CD123 CARs 2-4 and 1172 and 1176 to determine the CD4:CD8 ratio.

FIG.46 shows degranulation of CD123 CARs 2-4 and 1172 and 1176 upon exposure to CD123+ tumor cells.

FIG.47 shows a graphical representation of a luciferase assay to assess cytotoxicity of CART cells (NVS 2-4, 1172 and 1176 clones) towards tumor target cells (MOLM14).

FIG.48 shows a comparison of tumor burden in NSG mice injected with luciferase expressing MOLM14 cells at D6 (before CART injection) and at day 13 (6 days post injection with NVS 2-4, 1172 or 1176 clones) or at day 20.

FIGS..49A, 49B, 49C, 49D, 49E, and 49F show CD123 is highly expressed in CD19-neg B-cell acute lymphoblastic leukemia relapses occurring after CART19 treatment. FIG. 49A shows expression of CD123 compared to CD19 in 42 relapsing/refractory ALL samples. FIG. 49B shows CD123 and CD19 co-expression in B-ALL blasts. Gated on blasts (SSC low, singlet, live, CD45dim). FIG. 49C shows the gating strategy for the leukemia stem cell (LSC). CD123 is highly expressed in this subset. FIG. 49D shows CD123 and CD19 co-expression and results from FISH analysis. FIGS. 49E and 49F show the comparison of CD19 and CD123 expression at baseline or after relapse.

FIGS. 50A, 50B, 50C, 50D, 50E, and 50F shows results from various in vitro assays using T cells expressing a CD19 CAR (CAR19) or a CD123 CAR (CAR123). FIG. 50A shows CD19 and CD123 expression; FIG. 50B shows a CD107a degranulation assay; FIG. 50C shows the capability for targeted cell killing; FIGS. 50D and 50E shows proliferation capacity; FIG. 50F shows cytokine production for the indicated cytokines.

FIGS. 51A, 51B, and 51C show that CART cells expressing CD19 CAR (CAR19) or CD123 CAR (CAR123) had an anti-tumor effect in an in vivo mouse model. FIG. 51A shows the tumor burden represented by bioluminescent imaging; FIG. 51B shows the overall survival curve of mice receiving CART therapy; and FIG. 51C shows the expansion of CART123 cells in the peripheral blood.

FIGS. 52A, 52B, 52C, 52D, 52E, and 52F show that CART123 is active in an in vivo mouse model of antigen-loss relapse. FIG. 52A shows the experimental schema; FIG. 52B shows disease progression as represented by bioluminescent imaging in baseline and relapse

disease with respect to CD19 expression (top graph) and in response to treatment with CART19 therapy (bottom graph); FIG. 52C shows bioluminescent images of mice administered untransduced T cells or CART19 cells; FIG. 52D shows the experimental schema for treating with CART19 or CART123; FIG. 52E shows the disease progression; and FIG. 52F shows the overall survival of the treated mice.

FIGS. 53A, 53B, and 53C show ALL-CART interactions in skull bone marrow of xenograft mice. FIG. 53A shows the experimental schema; FIG. 53B shows representative multiphoton XY plane images of CART19 cells and CART123 cells interacting with ALL tumor engineered to express either CD19 and CD123 or CD123 alone (motile cells are indicated in dashed circles, non-motile cells are indicated with the arrows); and FIG. 53C is a graphic representation of the microscopy images.

FIGS. 54A, 54B, and 54C show the prevention of CD19-neg relapses using CART19 and CART123. FIG. 54A shows the experimental schema; FIG. 54B shows the disease progression (tumor burden as represented by BLI) of mice treated with untransduced T cells (top graph), CART19 (middle graph), or the combination of CART19 and CART123 (bottom graph); and FIG. 54C shows the overall survival from this experiment.

FIGS. 55A and 55B, show T cells expressing both CAR19 and CAR123 (FIG. 55A) and the results from a degranulation assay (FIG. 55B).

FIGS. 56A and 56B show characterization of ALL blasts. FIG. 56A shows expression of various markers CD19, CD123, CD10, CD34, and CD20; and FIG. 56B shows the gating strategy for sorting CD19-CD123+ cells.

FIGS. 57A, 57B, 57C, and 57D show anti-leukemia activity of CART123. FIG. 57A shows the expression of CD19 and CD123 on the NALM6 cells; FIG. 57B shows the tumor burden (as represented by BLI) in response to CART19 or CART123 therapy; FIG. 57C shows the overall survival of mice administered CART19 or CART123; and FIG. 57D shows the overall survival of mice administered varying doses of CART123.

FIGS. 58A and 58B show the characterization of the in vivo model of antigen-loss relapse. FIG. 58A shows the expression of CD123 in CD19 negative relapse disease; and FIG. 58B shows the degranulation assay of CART19 or CART123 cells when cultured with baseline or relapse cells in vitro.

FIG. 59 shows that the proliferation of CAR-expressing, transduced T cells is enhanced by low doses of RAD001 in a cell culture system. CARTs were co-cultured with NALM6 (Nalm-6) cells in the presence of different concentrations of RAD001 (nM). The number of CAR-positive CD3-positive T cells (black) and total T cells (white) was assessed after 4 days of co-culture.

FIG. 60 depicts tumor growth measurements of NALM6-luc cells with daily RAD001 dosing at 0.3, 1, 3, and 10 mg/kg (mpk) or vehicle dosing. Circles denote the vehicle; squares denote the 10 mg/kg dose of RAD001; triangles denote the 3 mg/kg dose of RAD001, inverted triangles denote the 1 mg/kg dose of RAD001; and diamonds denote the 0.3 mg/kg dose of RAD001.

FIGS. 61A and 61B show pharmacokinetic curves showing the amount of RAD001 in the blood of NSG mice with NALM6 tumors. FIG. 61A shows day 0 PK following the first dose of RAD001. FIG. 61B shows Day 14 PK following the final RAD001 dose. Diamonds denote the 10 mg/kg dose of RAD001; squares denote the 1 mg/kg dose of RAD001; triangles denote the 3 mg/kg dose of RAD001; and x's denote the 10 mg/kg dose of RAD001.

FIGS. 62A and 62B show in vivo proliferation of humanized CD19 CART cells with and without RAD001 dosing. Low doses of RAD001 (0.003 mg/kg) daily lead to an enhancement in CAR T cell proliferation, above the normal level of huCAR19 proliferation. Figures 62A shows CD4+ CAR T cells; FIG. 62B shows CD8+ CAR T cells. Circles denote PBS; squares denote huCTL019; triangles denote huCTL019 with 3 mg/kg RAD001; inverted triangles denote huCTL019 with 0.3 mg/kg RAD001; diamonds denote huCTL019 with 0.03 mg/kg RAD001; and circles denote huCTL019 with 0.003 mg/kg RAD001.

FIG. 63 shows multiplex FIHC AQUA analysis showing significant difference between CD3+/PD-1+ cell populations in primary and secondary human DLBCL patient samples.

FIG. 64 shows AQUA analysis showing various levels of CD19 (lower panel) and PD-L1 (upper panel) in primary and secondary sites of DLBCL samples. A total of 40 human DLBCL patient samples, 25 primary and 15 secondary sites, were subjected to multiplex FIHC and followed by AQUA analysis to identify expression levels of CD19 and PD-L1 proteins.

FIG. 65 shows a schematic of two populations of CAR-expressing cells. In the population on the left (pooled), each cell expresses one type of CAR. In the population on the right (bicistronic CAR), each cell expresses two types of CAR.

FIG. 66 shows diagrams of bicistronic CARs. The upper CAR has a CD19 CAR and a CD22 CAR, separated by a P2A protease cleavage site. The lower CAR has a CD19 CAR and a CD123 CAR, separated by a P2A protease cleavage site.

FIG. 67 shows co-expression of CD19 and CD22 CARs from a bicistronic vector.

FIG. 68, top panel, shows co-expression of CD19 and CD123 CARs from a bicistronic vector. FIG. 68, bottom panel, shows the anti-leukemic effect of these cells.

FIG. 69 shows the tumor burden in mice bearing CD19-negative B-ALL xenografts after treatment with a UTD control, CART19, or CART22.

FIG. 70 shows the expression of PD-L1, PD1, LAG3, and TIM3 (from left to right in each set of four bars) in lymph node and bone marrow samples from five CR patients, one unclassified patient, and six PD patients.

FIG. 71 is a graph showing the activation (in RLU) of several CD22 CAR constructs in the presence and absence of a m971 competitor.

FIG. 72 is a graph showing the activation (in RLU) of additional CD22 CAR constructs.

FIG. 73 shows three bar graphs indicating CD22 CAR activity in an IFN-gamma assay.

FIG. 74 shows binding activity of CD22-64 and CD22-65 CARs.

FIG. 75 is a diagram mapping the epitopes bound by various CD22 scFvs.

DETAILED DESCRIPTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains.

The term “a” and “an” refers to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, “an element” means one element or more than one element.

The term “about” when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or in some instances ±10%, or in some instances ±5%, or in some instances ±1%, or in some instances ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

The term "apheresis" as used herein refers to the art-recognized extracorporeal process by which the blood of a donor or patient is removed from the donor or patient and passed through an apparatus that separates out selected particular constituent(s) and returns the remainder to the circulation of the donor or patient, e.g., by retransfusion. Thus, "an apheresis sample" refers to a sample obtained using apheresis.

The term "bioequivalent" refers to an amount of an agent other than the reference compound (e.g., RAD001), required to produce an effect equivalent to the effect produced by the reference dose or reference amount of the reference compound (e.g., RAD001). In an embodiment the effect is the level of mTOR inhibition, e.g., as measured by P70 S6 kinase inhibition, e.g., as evaluated in an *in vivo* or *in vitro* assay, e.g., as measured by an assay described herein, e.g., the Boulay assay, or measurement of phosphorylated S6 levels by western blot. In an embodiment, the effect is alteration of the ratio of PD-1 positive/PD-1 negative T cells, as measured by cell sorting. In an embodiment a bioequivalent amount or dose of an mTOR inhibitor is the amount or dose that achieves the same level of P70 S6 kinase inhibition as does the reference dose or reference amount of a reference compound. In an embodiment, a bioequivalent amount or dose of an mTOR inhibitor is the amount or dose that achieves the same level of alteration in the ratio of PD-1 positive/PD-1 negative T cells as does the reference dose or reference amount of a reference compound.

The term "inhibition" or "inhibitor" includes a reduction in a certain parameter, e.g., an activity, of a given molecule, e.g., CD20, CD10, CD19, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. For example, inhibition of an activity, e.g., an activity of CD20, CD10, CD19, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a, of at least 5%, 10%, 20%, 30%, 40%, or more is included by this term. Thus, inhibition need not be 100%. Activities for the inhibitors can be determined as described herein or by assays known in the art. A "B-cell inhibitor" is a molecule, e.g., a small molecule, antibody, CAR or cell comprising a CAR, which causes the reduction in a certain parameter, e.g., an activity, e.g., growth or proliferation, of a B-cell, or which causes a reduction in a certain parameter, e.g., an activity, of a molecule associated with a B cell. Non-limiting examples of molecules associated with a B cell include proteins expressed on the surface of B cells, e.g., CD20, CD10, CD19, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

The term "Chimeric Antigen Receptor" or alternatively a "CAR" refers to a set of

polypeptides, typically two in the simplest embodiments, which when in an immune effector cell, provides the cell with specificity for a target cell, typically a cancer cell, and with intracellular signal generation. In some embodiments, a CAR comprises at least an extracellular antigen binding domain, a transmembrane domain and a cytoplasmic signaling domain (also referred to herein as "an intracellular signaling domain") comprising a functional signaling domain derived from a stimulatory molecule and/or costimulatory molecule as defined below. In some embodiments, the set of polypeptides are in the same polypeptide chain, e.g., comprise a chimeric fusion protein. In some embodiments, the set of polypeptides are not contiguous with each other, e.g., are in different polypeptide chains. In some embodiments, the set of polypeptides include a dimerization switch that, upon the presence of a dimerization molecule, can couple the polypeptides to one another, e.g., can couple an antigen binding domain to an intracellular signaling domain. In one aspect, the stimulatory molecule of the CAR is the zeta chain associated with the T cell receptor complex (e.g., CD3 zeta). In one aspect, the cytoplasmic signaling domain comprises a primary signaling domain (e.g., a primary signaling domain of CD3-zeta). In one aspect, the cytoplasmic signaling domain further comprises one or more functional signaling domains derived from at least one costimulatory molecule as defined below. In one aspect, the costimulatory molecule is chosen from the costimulatory molecules described herein, e.g., 4-1BB (i.e., CD137), CD27, and/or CD28. In one aspect, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain derived from a stimulatory molecule. In one aspect, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising a functional signaling domain derived from a costimulatory molecule and a functional signaling domain derived from a stimulatory molecule. In one aspect, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising two functional signaling domains derived from one or more costimulatory molecule(s) and a functional signaling domain derived from a stimulatory molecule. In one aspect, the CAR comprises a chimeric fusion protein comprising an extracellular antigen binding domain, a transmembrane domain and an intracellular signaling domain comprising at least two functional signaling domains derived from one or more costimulatory molecule(s) and a functional

signaling domain derived from a stimulatory molecule. In one aspect the CAR comprises an optional leader sequence at the amino-terminus (N-ter) of the CAR fusion protein. In one aspect, the CAR further comprises a leader sequence at the N-terminus of the extracellular antigen binding domain, wherein the leader sequence is optionally cleaved from the antigen binding domain (e.g., a scFv) during cellular processing and localization of the CAR to the cellular membrane.

The phrase "disease associated with expression of CD20" as used herein includes but is not limited to, a disease associated with expression of CD20 (e.g., wild-type or mutant CD20) or condition associated with cells which express, or at any time expressed, CD20 (e.g., wild-type or mutant CD20) including, e.g., a proliferative disease such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia; or a noncancer related indication associated with cells which express CD20 (e.g., wild-type or mutant CD20). For the avoidance of doubt, a disease associated with expression of CD20 may include a condition associated with cells which do not presently express CD20, e.g., because CD20 expression has been downregulated, e.g., due to treatment with a molecule targeting CD20, e.g., a CD20 CAR, but which at one time expressed CD20. In one aspect, a cancer associated with expression of CD20 is a hematological cancer. In one aspect, a hematological cancer includes but is not limited to AML, myelodysplastic syndrome, ALL, hairy cell leukemia, Prolymphocytic leukemia, Chronic myeloid leukemia, Hodgkin lymphoma, Blastic plasmacytoid dendritic cell neoplasm, and the like. Further disease associated with expression of CD20 expression include, but are not limited to, e.g., atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative diseases associated with expression of CD20. Non-cancer related indications associated with expression of CD20 may also be included. In some embodiments, the CD20-expressing cells express, or at any time expressed, CD20 mRNA. In an embodiment, the CD20-expressing cells produce a CD20 protein (e.g., wild-type or mutant), and the CD20 protein may be present at normal levels or reduced levels. In an embodiment, the CD20-expressing cells produced detectable levels of a CD20 protein at one point, and subsequently produced substantially no detectable CD20 protein.

The phrase "disease associated with expression of CD22" as used herein includes but is not limited to, a disease associated with expression of CD22 (e.g., wild-type or mutant CD22) or condition associated with cells which express, or at any time expressed, CD22 (e.g., wild-type or

mutant CD22) including, e.g., a proliferative disease such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia; or a noncancer related indication associated with cells which express CD22 (e.g., wild-type or mutant CD22). For the avoidance of doubt, a disease associated with expression of CD22 may include a condition associated with cells which do not presently express CD22, e.g., because CD22 expression has been downregulated, e.g., due to treatment with a molecule targeting CD22, e.g., a CD22 CAR, but which at one time expressed CD22. In one aspect, a cancer associated with expression of CD22 is a hematological cancer. In one aspect, a hematological cancer includes but is not limited to AML, myelodysplastic syndrome, ALL, hairy cell leukemia, Prolymphocytic leukemia, Chronic myeloid leukemia, Hodgkin lymphoma, Blastic plasmacytoid dendritic cell neoplasm, and the like. Further disease associated with expression of CD22 expression include, but are not limited to, e.g., atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative diseases associated with expression of CD22. Non-cancer related indications associated with expression of CD22 may also be included. In some embodiments, the CD22-expressing cells express, or at any time expressed, CD22 mRNA. In an embodiment, the CD22-expressing cells produce a CD22 protein (e.g., wild-type or mutant), and the CD22 protein may be present at normal levels or reduced levels. In an embodiment, the CD22-expressing cells produced detectable levels of a CD22 protein at one point, and subsequently produced substantially no detectable CD22 protein.

As used herein, unless otherwise specified, the terms "prevent," "preventing" and "prevention" refer to an action that occurs before the subject begins to suffer from the condition, or relapse of the condition. Prevention need not result in a complete prevention of the condition; partial prevention or reduction of the condition or a symptom of the condition, or reduction of the risk of developing the condition, is encompassed by this term.

Administered "in combination", as used herein, means that two (or more) different treatments are delivered to the subject during the course of the subject's affliction with the disorder, e.g., the two or more treatments are delivered after the subject has been diagnosed with the disorder and before the disorder has been cured or eliminated or treatment has ceased for other reasons. In some embodiments, the delivery of one treatment is still occurring when the delivery of the second begins, so that there is overlap in terms of administration. This is sometimes referred to herein as "simultaneous" or "concurrent delivery". In other embodiments,

the delivery of one treatment ends before the delivery of the other treatment begins. In some embodiments of either case, the treatment is more effective because of combined administration. For example, the second treatment is more effective, e.g., an equivalent effect is seen with less of the second treatment, or the second treatment reduces symptoms to a greater extent, than would be seen if the second treatment were administered in the absence of the first treatment, or the analogous situation is seen with the first treatment. In some embodiments, delivery is such that the reduction in a symptom, or other parameter related to the disorder is greater than what would be observed with one treatment delivered in the absence of the other. The effect of the two treatments can be partially additive, wholly additive, or greater than additive. The delivery can be such that an effect of the first treatment delivered is still detectable when the second is delivered. In one embodiment, the CAR-expressing cell is administered at a dose and/or dosing schedule described herein, and the B-cell inhibitor, or agent that enhances the activity of the CD19 CAR-expressing cell is administered at a dose and/or dosing schedule described herein.

"Derived from" as that term is used herein, indicates a relationship between a first and a second molecule. It generally refers to structural similarity between the first molecule and a second molecule and does not connote or include a process or source limitation on a first molecule that is derived from a second molecule. For example, in the case of an intracellular signaling domain that is derived from a CD3zeta molecule, the intracellular signaling domain retains sufficient CD3zeta structure such that is has the required function, namely, the ability to generate a signal under the appropriate conditions. It does not connote or include a limitation to a particular process of producing the intracellular signaling domain, e.g., it does not mean that, to provide the intracellular signaling domain, one must start with a CD3zeta sequence and delete unwanted sequence, or impose mutations, to arrive at the intracellular signaling domain.

The term "signaling domain" refers to the functional portion of a protein which acts by transmitting information within the cell to regulate cellular activity via defined signaling pathways by generating second messengers or functioning as effectors by responding to such messengers.

As used herein, the term "CD19" refers to the Cluster of Differentiation 19 protein, which is an antigenic determinant detectable on leukemia precursor cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequence of human CD19 can be found as

UniProt/Swiss-Prot Accession No. P15391 and the nucleotide sequence encoding of the human CD19 can be found at Accession No. NM_001178098. As used herein, "CD19" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type CD19. CD19 is expressed on most B lineage cancers, including, e.g., acute lymphoblastic leukemia, chronic lymphocyte leukemia and non-Hodgkin lymphoma. Other cells with express CD19 are provided below in the definition of "disease associated with expression of CD19." It is also an early marker of B cell progenitors. See, e.g., Nicholson et al. Mol. Immun. 34 (16-17): 1157-1165 (1997). In one aspect the antigen-binding portion of the CART recognizes and binds an antigen within the extracellular domain of the CD19 protein. In one aspect, the CD19 protein is expressed on a cancer cell.

The term "antibody," as used herein, refers to a protein, or polypeptide sequence derived from an immunoglobulin molecule which specifically binds with an antigen. Antibodies can be polyclonal or monoclonal, multiple or single chain, or intact immunoglobulins, and may be derived from natural sources or from recombinant sources. Antibodies can be tetramers of immunoglobulin molecules.

The term "antibody fragment" refers to at least one portion of an antibody, that retains the ability to specifically interact with (e.g., by binding, steric hindrance, stabilizing/destabilizing, spatial distribution) an epitope of an antigen. Examples of antibody fragments include, but are not limited to, Fab, Fab', $F(ab')_2$, Fv fragments, scFv antibody fragments, disulfide-linked Fvs (sdFv), a Fd fragment consisting of the VH and CH1 domains, linear antibodies, single domain antibodies such as sdAb (either VL or VH), camelid VHH domains, multi-specific antibodies formed from antibody fragments such as a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region, and an isolated CDR or other epitope binding fragments of an antibody. An antigen binding fragment can also be incorporated into single domain antibodies, maxibodies, minibodies, nanobodies, intrabodies, diabodies, triabodies, tetrabodies, v-NAR and bis-scFv (see, e.g., Hollinger and Hudson, Nature Biotechnology 23:1126-1136, 2005). Antigen binding fragments can also be grafted into scaffolds based on polypeptides such as a fibronectin type III (Fn3)(see U.S. Patent No.: 6,703,199, which describes fibronectin polypeptide minibodies).

The term "scFv" refers to a fusion protein comprising at least one antibody fragment comprising a variable region of a light chain and at least one antibody fragment comprising a

variable region of a heavy chain, wherein the light and heavy chain variable regions are contiguously linked, e.g., via a synthetic linker, e.g., a short flexible polypeptide linker, and capable of being expressed as a single chain polypeptide, and wherein the scFv retains the specificity of the intact antibody from which it is derived. Unless specified, as used herein an scFv may have the VL and VH variable regions in either order, e.g., with respect to the N-terminal and C-terminal ends of the polypeptide, the scFv may comprise VL-linker-VH or may comprise VH-linker-VL.

The term "complementarity determining region" or "CDR," as used herein, refers to the sequences of amino acids within antibody variable regions which confer antigen specificity and binding affinity. For example, in general, there are three CDRs in each heavy chain variable region (e.g., HCDR1, HCDR2, and HCDR3) and three CDRs in each light chain variable region (LCDR1, LCDR2, and LCDR3). The precise amino acid sequence boundaries of a given CDR can be determined using any of a number of well-known schemes, including those described by Kabat et al. (1991), "Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD ("Kabat" numbering scheme), Al-Lazikani et al., (1997) JMB 273,927-948 ("Chothia" numbering scheme), or a combination thereof. Under the Kabat numbering scheme, in some embodiments, the CDR amino acid residues in the heavy chain variable domain (VH) are numbered 31-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3); and the CDR amino acid residues in the light chain variable domain (VL) are numbered 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3). Under the Chothia numbering scheme, in some embodiments, the CDR amino acids in the VH are numbered 26-32 (HCDR1), 52-56 (HCDR2), and 95-102 (HCDR3); and the CDR amino acid residues in the VL are numbered 26-32 (LCDR1), 50-52 (LCDR2), and 91-96 (LCDR3). In a combined Kabat and Chothia numbering scheme, in some embodiments, the CDRs correspond to the amino acid residues that are part of a Kabat CDR, a Chothia CDR, or both. For instance, in some embodiments, the CDRs correspond to amino acid residues 26-35 (HCDR1), 50-65 (HCDR2), and 95-102 (HCDR3) in a VH, e.g., a mammalian VH, e.g., a human VH; and amino acid residues 24-34 (LCDR1), 50-56 (LCDR2), and 89-97 (LCDR3) in a VL, e.g., a mammalian VL, e.g., a human VL.

As used herein, the term "binding domain" or "antibody molecule" refers to a protein, e.g., an immunoglobulin chain or fragment thereof, comprising at least one immunoglobulin

variable domain sequence. The term "binding domain" or "antibody molecule" encompasses antibodies and antibody fragments. In an embodiment, an antibody molecule is a multispecific antibody molecule, e.g., it comprises a plurality of immunoglobulin variable domain sequences, wherein a first immunoglobulin variable domain sequence of the plurality has binding specificity for a first epitope and a second immunoglobulin variable domain sequence of the plurality has binding specificity for a second epitope. In an embodiment, a multispecific antibody molecule is a bispecific antibody molecule. A bispecific antibody has specificity for no more than two antigens. A bispecific antibody molecule is characterized by a first immunoglobulin variable domain sequence which has binding specificity for a first epitope and a second immunoglobulin variable domain sequence that has binding specificity for a second epitope.

The portion of the CAR of the invention comprising an antibody or antibody fragment thereof may exist in a variety of forms where the antigen binding domain is expressed as part of a contiguous polypeptide chain including, for example, a single domain antibody fragment (sdAb), a single chain antibody (scFv), a humanized antibody, or bispecific antibody (Harlow et al., 1999, In: Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, In: Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426). In one aspect, the antigen binding domain of a CAR composition of the invention comprises an antibody fragment. In a further aspect, the CAR comprises an antibody fragment that comprises a scFv.

The term "antibody heavy chain," refers to the larger of the two types of polypeptide chains present in antibody molecules in their naturally occurring conformations, and which normally determines the class to which the antibody belongs.

The term "antibody light chain," refers to the smaller of the two types of polypeptide chains present in antibody molecules in their naturally occurring conformations. Kappa (κ) and lambda (λ) light chains refer to the two major antibody light chain isotypes.

The term "recombinant antibody" refers to an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage or yeast expression system. The term should also be construed to mean an antibody which has

been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody protein, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using recombinant DNA or amino acid sequence technology which is available and well known in the art.

The term "antigen" or "Ag" refers to a molecule that provokes an immune response. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells, or both. The skilled artisan will understand that any macromolecule, including virtually all proteins or peptides, can serve as an antigen. Furthermore, antigens can be derived from recombinant or genomic DNA. A skilled artisan will understand that any DNA, which comprises a nucleotide sequences or a partial nucleotide sequence encoding a protein that elicits an immune response therefore encodes an "antigen" as that term is used herein. Furthermore, one skilled in the art will understand that an antigen need not be encoded solely by a full length nucleotide sequence of a gene. It is readily apparent that the present invention includes, but is not limited to, the use of partial nucleotide sequences of more than one gene and that these nucleotide sequences are arranged in various combinations to encode polypeptides that elicit the desired immune response. Moreover, a skilled artisan will understand that an antigen need not be encoded by a "gene" at all. It is readily apparent that an antigen can be generated synthesized or can be derived from a biological sample, or might be macromolecule besides a polypeptide. Such a biological sample can include, but is not limited to a tissue sample, a tumor sample, a cell or a fluid with other biological components.

The terms "compete" or "cross-compete" are used interchangeably herein to refer to the ability of an antibody molecule to interfere with binding of an antibody molecule, e.g., an anti-CD20 or CD22 antibody molecule provided herein, to a target, e.g., human CD20 or CD22. The interference with binding can be direct or indirect (e.g., through an allosteric modulation of the antibody molecule or the target). The extent to which an antibody molecule is able to interfere with the binding of another antibody molecule to the target, and therefore whether it can be said to compete, can be determined using a competition binding assay, e.g., as described herein. In some embodiments, a competition binding assay is a quantitative competition assay. In some embodiments, a first antibody molecule is said to compete for binding to the target with a second antibody molecule when the binding of the first antibody molecule to the target is reduced by 10% or more, e.g., 20% or more, 30% or more, 40% or more, 50% or more, 55% or more, 60%

or more, 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 98% or more, 99% or more in a competition binding assay (e.g., a competition assay described herein).

As used herein, the term "epitope" refers to the moieties of an antigen (e.g., human CD20 or CD22) that specifically interact with an antibody molecule. Such moieties, referred to herein as epitopic determinants, typically comprise, or are part of, elements such as amino acid side chains or sugar side chains. An epitopic determinate can be defined, e.g., by methods known in the art or disclosed herein, e.g., by crystallography or by hydrogen-deuterium exchange. At least one or some of the moieties on the antibody molecule, that specifically interact with an epitopic determinant, are typically located in a CDR(s). Typically an epitope has a specific three dimensional structural characteristics. Typically an epitope has specific charge characteristics. Some epitopes are linear epitopes while others are conformational epitopes.

The term "anti-cancer effect" refers to a biological effect which can be manifested by various means, including but not limited to, e.g., a decrease in tumor volume, a decrease in the number of cancer cells, a decrease in the number of metastases, an increase in life expectancy, decrease in cancer cell proliferation, decrease in cancer cell survival, or amelioration of various physiological symptoms associated with the cancerous condition. An "anti-cancer effect" can also be manifested by the ability of the peptides, polynucleotides, cells and antibodies described herein in prevention of the occurrence of cancer in the first place. The term "anti-tumor effect" refers to a biological effect which can be manifested by various means, including but not limited to, e.g., a decrease in tumor volume, a decrease in the number of tumor cells, a decrease in tumor cell proliferation, or a decrease in tumor cell survival.

The term "autologous" refers to any material derived from the same individual to whom it is later to be re-introduced into the individual.

The term "allogeneic" refers to any material derived from a different animal of the same species as the individual to whom the material is introduced. Two or more individuals are said to be allogeneic to one another when the genes at one or more loci are not identical. In some aspects, allogeneic material from individuals of the same species may be sufficiently unlike genetically to interact antigenically

The term "xenogeneic" refers to a graft derived from an animal of a different species.

The term "cancer" refers to a disease characterized by the uncontrolled growth of aberrant cells. Cancer cells can spread locally or through the bloodstream and lymphatic system to other parts of the body. Examples of various cancers are described herein and include but are not limited to, breast cancer, prostate cancer, ovarian cancer, cervical cancer, skin cancer, pancreatic cancer, colorectal cancer, renal cancer, liver cancer, brain cancer, lymphoma, leukemia, lung cancer and the like. The terms "tumor" and "cancer" are used interchangeably herein, e.g., both terms encompass solid and liquid, e.g., diffuse or circulating, tumors. As used herein, the term "cancer" or "tumor" includes premalignant, as well as malignant cancers and tumors.

The terms "cancer associated antigen" or "tumor antigen" or "proliferative disorder antigen" or "antigen associated with a proliferative disorder" interchangeably refers to a molecule (typically protein, carbohydrate or lipid) that is preferentially expressed on the surface of a cancer cell, either entirely or as a fragment (e.g., MHC/peptide), in comparison to a normal cell, and which is useful for the preferential targeting of a pharmacological agent to the cancer cell. In some embodiments, a tumor antigen is a marker expressed by both normal cells and cancer cells, e.g., a lineage marker, e.g., CD19 on B cells. In certain aspects, the tumor antigens of the present invention are derived from, cancers including but not limited to primary or metastatic melanoma, thymoma, lymphoma, sarcoma, lung cancer, liver cancer, non-Hodgkin lymphoma, Hodgkin lymphoma, leukemias, uterine cancer, cervical cancer, bladder cancer, kidney cancer and adenocarcinomas such as breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, and the like. In some embodiments, the tumor antigen is an antigen that is common to a specific proliferative disorder. In some embodiments, a cancer-associated antigen is a cell surface molecule that is overexpressed in a cancer cell in comparison to a normal cell, for instance, 1-fold over expression, 2-fold overexpression, 3-fold overexpression or more in comparison to a normal cell. In some embodiments, a cancer-associated antigen is a cell surface molecule that is inappropriately synthesized in the cancer cell, for instance, a molecule that contains deletions, additions or mutations in comparison to the molecule expressed on a normal cell. In some embodiments, a cancer-associated antigen will be expressed exclusively on the cell surface of a cancer cell, entirely or as a fragment (e.g., MHC/peptide), and not synthesized or expressed on the surface of a normal cell. In some embodiments, the CARs of the present invention includes CARs comprising an antigen binding domain (e.g., antibody or antibody fragment) that binds to a MHC presented peptide. Normally, peptides derived from endogenous

proteins fill the pockets of Major histocompatibility complex (MHC) class I molecules, and are recognized by T cell receptors (TCRs) on CD8 + T lymphocytes. The MHC class I complexes are constitutively expressed by all nucleated cells. In cancer, virus-specific and/or tumor-specific peptide/MHC complexes represent a unique class of cell surface targets for immunotherapy. TCR-like antibodies targeting peptides derived from viral or tumor antigens in the context of human leukocyte antigen (HLA)-A1 or HLA-A2 have been described (see, e.g., Sastry et al., J Virol. 2011 85(5):1935-1942; Sergeeva et al., Bood, 2011 117(16):4262-4272; Verma et al., J Immunol 2010 184(4):2156-2165; Willemsen et al., Gene Ther 2001 8(21) :1601-1608 ; Dao et al., Sci Transl Med 2013 5(176) :176ra33 ; Tassev et al., Cancer Gene Ther 2012 19(2):84-100). For example, TCR-like antibody can be identified from screening a library, such as a human scFv phage displayed library.

The phrase "disease associated with expression of CD19" includes, but is not limited to, a disease associated with expression of CD19 (e.g., wild-type or mutant CD19) or condition associated with cells which express, or at any time expressed, CD19 (e.g., wild-type or mutant CD19) including, e.g., proliferative diseases such as a cancer or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia; or a noncancer related indication associated with cells which express CD19. For the avoidance of doubt, a disease associated with expression of CD19 may include a condition associated with cells which do not presently express CD19, e.g., because CD19 expression has been downregulated, e.g., due to treatment with a molecule targeting CD19, e.g., a CD19 CAR, but which at one time expressed CD19. In one aspect, a cancer associated with expression of CD19 is a hematological cancer. In one aspect, the hematological cancer is a leukemia or a lymphoma. In one aspect, a cancer associated with expression of CD19 includes cancers and malignancies including, but not limited to, e.g., one or more acute leukemias including but not limited to, e.g., B-cell acute Lymphoid Leukemia (BALL), T-cell acute Lymphoid Leukemia (TALL), acute lymphoid leukemia (ALL); one or more chronic leukemias including but not limited to, e.g., chronic myelogenous leukemia (CML), Chronic Lymphoid Leukemia (CLL). Additional cancers or hematologic conditions associated with expression of CD19 comprise, but are not limited to, e.g., B cell prolymphocytic leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma, Follicular lymphoma, Hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions,

MALT lymphoma, mantle cell lymphoma (MCL), Marginal zone lymphoma, multiple myeloma, myelodysplasia and myelodysplastic syndrome, non-Hodgkin lymphoma, Hodgkin lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, and "preleukemia" which are a diverse collection of hematological conditions united by ineffective production (or dysplasia) of myeloid blood cells, and the like. Further diseases associated with expression of CD19 expression include, but not limited to, e.g., atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative diseases associated with expression of CD19. Non-cancer related indications associated with expression of CD19 include, but are not limited to, e.g., autoimmune disease, (e.g., lupus), inflammatory disorders (allergy and asthma) and transplantation. In some embodiments, the CD19-expressing cells express, or at any time expressed, CD19 mRNA. In an embodiment, the CD19-expressing cells produce a CD19 protein (e.g., wild-type or mutant), and the CD19 protein may be present at normal levels or reduced levels. In an embodiment, the CD19-expressing cells produced detectable levels of a CD19 protein at one point, and subsequently produced substantially no detectable CD19 protein.

The term "conservative sequence modifications" refers to amino acid modifications that do not significantly affect or alter the binding characteristics of the antibody or antibody fragment containing the amino acid sequence. Such conservative modifications include amino acid substitutions, additions and deletions. Modifications can be introduced into an antibody or antibody fragment of the invention by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, one or more amino acid residues within a CAR of the invention can be replaced with other amino acid residues from the same side chain family and the altered CAR can be tested using the functional assays described herein.

The term "stimulation," refers to a primary response induced by binding of a stimulatory molecule (e.g., a TCR/CD3 complex or CAR) with its cognate ligand (or tumor antigen in the case of a CAR) thereby mediating a signal transduction event, such as, but not limited to, signal transduction via the TCR/CD3 complex or signal transduction via the appropriate NK receptor or signaling domains of the CAR. Stimulation can mediate altered expression of certain molecules.

The term "stimulatory molecule," refers to a molecule expressed by an immune cell, e.g., T cell, NK cell, or B cell) that provides the cytoplasmic signaling sequence(s) that regulate activation of the immune cell in a stimulatory way for at least some aspect of the immune cell signaling pathway. In one aspect, the signal is a primary signal that is initiated by, for instance, binding of a TCR/CD3 complex with an MHC molecule loaded with peptide, and which leads to mediation of a T cell response, including, but not limited to, proliferation, activation, differentiation, and the like. A primary cytoplasmic signaling sequence (also referred to as a "primary signaling domain") that acts in a stimulatory manner may contain a signaling motif which is known as immunoreceptor tyrosine-based activation motif or ITAM. Examples of an ITAM containing cytoplasmic signaling sequence that is of particular use in the invention includes, but is not limited to, those derived from CD3 zeta, common FcR gamma (FCER1G), Fc gamma RIIa, FcR beta (Fc Epsilon R1b), CD3 gamma, CD3 delta , CD3 epsilon, CD79a, CD79b, DAP10, and DAP12. In a specific CAR of the invention, the intracellular signaling domain in any one or more CARS of the invention comprises an intracellular signaling sequence, e.g., a primary signaling sequence of CD3-zeta. In a specific CAR of the invention, the primary signaling sequence of CD3-zeta is the sequence provided as SEQ ID NO:17, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like. In a specific CAR of the invention, the primary signaling sequence of CD3-zeta is the sequence as provided in SEQ ID NO:43, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like.

The term "antigen presenting cell" or "APC" refers to an immune system cell such as an accessory cell (e.g., a B-cell, a dendritic cell, and the like) that displays a foreign antigen complexed with major histocompatibility complexes (MHC's) on its surface. T-cells may recognize these complexes using their T-cell receptors (TCRs). APCs process antigens and present them to T-cells.

"Immune effector cell," as that term is used herein, refers to a cell that is involved in an immune response, *e.g.*, in the promotion of an immune effector response. Examples of immune effector cells include T cells, e.g., alpha/beta T cells and gamma/delta T cells, B cells, natural killer (NK) cells, natural killer T (NK-T) cells, mast cells, and myeloid-derived phagocytes.

"Immune effector function or immune effector response," as that term is used herein, refers to function or response, e.g., of an immune effector cell, that enhances or promotes an immune attack of a target cell. E.g., an immune effector function or response refers a property of a T or NK cell that promotes killing or the inhibition of growth or proliferation, of a target cell. In the case of a T cell, primary stimulation and co-stimulation are examples of immune effector function or response.

The term "effector function" refers to a specialized function of a cell. Effector function of a T cell, for example, may be cytolytic activity or helper activity including the secretion of cytokines.

An "intracellular signaling domain," as the term is used herein, refers to an intracellular portion of a molecule. The intracellular signaling domain can generate a signal that promotes an immune effector function of the CAR containing cell, e.g., a CART cell. Examples of immune effector function, e.g., in a CART cell, include cytolytic activity and helper activity, including the secretion of cytokines. In embodiments, the intracellular signal domain is the portion of the protein which transduces the effector function signal and directs the cell to perform a specialized function. While the entire intracellular signaling domain can be employed, in many cases it is not necessary to use the entire chain. To the extent that a truncated portion of the intracellular signaling domain is used, such truncated portion may be used in place of the intact chain as long as it transduces the effector function signal. The term intracellular signaling domain is thus meant to include any truncated portion of the intracellular signaling domain sufficient to transduce the effector function signal.

In an embodiment, the intracellular signaling domain can comprise a primary intracellular signaling domain. Exemplary primary intracellular signaling domains include those derived from the molecules responsible for primary stimulation, or antigen dependent simulation. In an embodiment, the intracellular signaling domain can comprise a costimulatory intracellular

domain. Exemplary costimulatory intracellular signaling domains include those derived from molecules responsible for costimulatory signals, or antigen independent stimulation. For example, in the case of a CART, a primary intracellular signaling domain can comprise a cytoplasmic sequence of a T cell receptor, and a costimulatory intracellular signaling domain can comprise cytoplasmic sequence from co-receptor or costimulatory molecule.

A primary intracellular signaling domain can comprise a signaling motif which is known as an immunoreceptor tyrosine-based activation motif or ITAM. Examples of ITAM containing primary cytoplasmic signaling sequences include, but are not limited to, those derived from CD3 zeta, FcR gamma, common FcR gamma (FCER1G), Fc gamma RIIa, FcR beta (Fc Epsilon R1b), CD3 gamma, CD3 delta, CD3 epsilon, CD22, CD79a, CD79b, CD278 ("ICOS"), FcεRI, CD66d, CD32, DAP10 and DAP12.

The term "zeta" or alternatively "zeta chain", "CD3-zeta" or "TCR-zeta" is defined as the protein provided as GenBank Acc. No. BAG36664.1, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like, and a "zeta stimulatory domain" or alternatively a "CD3-zeta stimulatory domain" or a "TCR-zeta stimulatory domain" is defined as the amino acid residues from the cytoplasmic domain of the zeta chain, or functional derivatives thereof, that are sufficient to functionally transmit an initial signal necessary for T cell activation. In one aspect the cytoplasmic domain of zeta comprises residues 52 through 164 of GenBank Acc. No. BAG36664.1 or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like, that are functional orthologs thereof. In one aspect, the "zeta stimulatory domain" or a "CD3-zeta stimulatory domain" is the sequence provided as SEQ ID NO:17. In one aspect, the "zeta stimulatory domain" or a "CD3-zeta stimulatory domain" is the sequence provided as SEQ ID NO:43.

The term "costimulatory molecule" refers to the cognate binding partner on a T cell that specifically binds with a costimulatory ligand, thereby mediating a costimulatory response by the T cell, such as, but not limited to, proliferation. Costimulatory molecules are cell surface molecules other than antigen receptors or their ligands that contribute to an efficient immune response. Costimulatory molecules include, but are not limited to an MHC class I molecule, TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signalling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1

(CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83.

A costimulatory intracellular signaling domain refers to the intracellular portion of a costimulatory molecule. The intracellular signaling domain can comprise the entire intracellular portion, or the entire native intracellular signaling domain, of the molecule from which it is derived, or a functional fragment or derivative thereof.

The term "4-1BB" refers to a member of the TNFR superfamily with an amino acid sequence provided as GenBank Acc. No. AAA62478.2, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like; and a "4-1BB costimulatory domain" is defined as amino acid residues 214-255 of GenBank Acc. No. AAA62478.2, or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like. In one aspect, the "4-1BB costimulatory domain" is the sequence provided as SEQ ID NO:16 or the equivalent residues from a non-human species, e.g., mouse, rodent, monkey, ape and the like.

The term "encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (e.g., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene, cDNA, or RNA, encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the

protein or other product of that gene or cDNA.

Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. The phrase nucleotide sequence that encodes a protein or a RNA may also include introns to the extent that the nucleotide sequence encoding the protein may in some version contain an intron(s).

The term "effective amount" or "therapeutically effective amount" are used interchangeably herein, and refer to an amount of a compound, formulation, material, or composition, as described herein effective to achieve a particular biological result.

The term "endogenous" refers to any material from or produced inside an organism, cell, tissue or system.

The term "exogenous" refers to any material introduced from or produced outside an organism, cell, tissue or system.

The term "expression" refers to the transcription and/or translation of a particular nucleotide sequence driven by a promoter.

The term "transfer vector" refers to a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "transfer vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to further include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, a polylysine compound, liposome, and the like. Examples of viral transfer vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, lentiviral vectors, and the like.

The term "expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system.

Expression vectors include all those known in the art, including cosmids, plasmids (e.g., naked or contained in liposomes) and viruses (e.g., lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

The term "lentivirus" refers to a genus of the Retroviridae family. Lentiviruses are unique among the retroviruses in being able to infect non-dividing cells; they can deliver a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. HIV, SIV, and FIV are all examples of lentiviruses.

The term "lentiviral vector" refers to a vector derived from at least a portion of a lentivirus genome, including especially a self-inactivating lentiviral vector as provided in Milone et al., Mol. Ther. 17(8): 1453–1464 (2009). Other examples of lentivirus vectors that may be used in the clinic, include but are not limited to, e.g., the LENTIVECTOR® gene delivery technology from Oxford BioMedica, the LENTIMAX™ vector system from Lentigen and the like. Nonclinical types of lentiviral vectors are also available and would be known to one skilled in the art.

The term "homologous" or "identity" refers to the subunit sequence identity between two polymeric molecules, e.g., between two nucleic acid molecules, such as, two DNA molecules or two RNA molecules, or between two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit; e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous or identical at that position. The homology between two sequences is a direct function of the number of matching or homologous positions; e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two sequences are homologous, the two sequences are 50% homologous; if 90% of the positions (e.g., 9 of 10), are matched or homologous, the two sequences are 90% homologous.

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies and antibody fragments thereof are human immunoglobulins (recipient antibody or antibody fragment) in which residues from a complementary-determining region (CDR) of the recipient are replaced

by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, a humanized antibody/antibody fragment can comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications can further refine and optimize antibody or antibody fragment performance. In general, the humanized antibody or antibody fragment thereof will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or a significant portion of the FR regions are those of a human immunoglobulin sequence. The humanized antibody or antibody fragment can also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., Nature, 321: 522-525, 1986; Reichmann et al., Nature, 332: 323-329, 1988; Presta, Curr. Op. Struct. Biol., 2: 593-596, 1992.

"Fully human" refers to an immunoglobulin, such as an antibody or antibody fragment, where the whole molecule is of human origin or consists of an amino acid sequence identical to a human form of the antibody or immunoglobulin.

The term "isolated" means altered or removed from the natural state. For example, a nucleic acid or a peptide naturally present in a living animal is not "isolated," but the same nucleic acid or peptide partially or completely separated from the coexisting materials of its natural state is "isolated." An isolated nucleic acid or protein can exist in substantially purified form, or can exist in a non-native environment such as, for example, a host cell.

In the context of the present invention, the following abbreviations for the commonly occurring nucleic acid bases are used. "A" refers to adenosine, "C" refers to cytosine, "G" refers to guanosine, "T" refers to thymidine, and "U" refers to uridine.

The term "operably linked" or "transcriptional control" refers to functional linkage between a regulatory sequence and a heterologous nucleic acid sequence resulting in expression of the latter. For example, a first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence

if the promoter affects the transcription or expression of the coding sequence. Operably linked DNA sequences can be contiguous with each other and, e.g., where necessary to join two protein coding regions, are in the same reading frame.

The term "parenteral" administration of an immunogenic composition includes, e.g., subcutaneous (s.c.), intravenous (i.v.), intramuscular (i.m.), or intrasternal injection, intratumoral, or infusion techniques.

The term "nucleic acid" or "polynucleotide" refers to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof in either single- or double-stranded form. The term "nucleic acid" includes a gene, cDNA, or an mRNA. In one embodiment, the nucleic acid molecule is synthetic (e.g., chemically synthesized) or recombinant. Unless specifically limited, the term encompasses nucleic acids containing analogues or derivatives of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, SNPs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al., Nucleic Acid Res. 19:5081 (1991); Ohtsuka et al., J. Biol. Chem. 260:2605-2608 (1985); and Rossolini et al., Mol. Cell. Probes 8:91-98 (1994)).

The terms "peptide," "polypeptide," and "protein" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. A protein or peptide must contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that can comprise a protein's or peptide's sequence. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Polypeptides" include, for example, biologically active fragments, substantially homologous polypeptides, oligopeptides, homodimers, heterodimers, variants of polypeptides, modified polypeptides, derivatives, analogs, fusion proteins, among others. A polypeptide includes a natural peptide, a

recombinant peptide, or a combination thereof.

The term "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a polynucleotide sequence.

The term "promoter/regulatory sequence" refers to a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a tissue specific manner.

The term "constitutive" promoter refers to a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell under most or all physiological conditions of the cell.

The term "inducible" promoter refers to a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a cell substantially only when an inducer which corresponds to the promoter is present in the cell.

The term "tissue-specific" promoter refers to a nucleotide sequence which, when operably linked with a polynucleotide encodes or specified by a gene, causes the gene product to be produced in a cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

The term "flexible polypeptide linker" or "linker" as used in the context of a scFv refers to a peptide linker that consists of amino acids such as glycine and/or serine residues used alone or in combination, to link variable heavy and variable light chain regions together. In one embodiment, the flexible polypeptide linker is a Gly/Ser linker and comprises the amino acid sequence $(Gly\text{-}Gly\text{-}Gly\text{-}Ser)_n$, where n is a positive integer equal to or greater than 1. For example, n=1, n=2, n=3. n=4, n=5, n=6, n=7, n=8, n=9 and n=10 (SEQ ID NO:105). In one embodiment, the flexible polypeptide linkers include, but are not limited to, $(Gly_4\ Ser)_4$ (SEQ ID NO:106) or $(Gly_4\ Ser)_3$ (SEQ ID NO:107). In another embodiment, the linkers include multiple repeats of (Gly_2Ser), (GlySer) or (Gly_3Ser) (SEQ ID NO:108). Also included within the scope

of the invention are linkers described in WO2012/138475, incorporated herein by reference.

As used herein, a 5' cap (also termed an RNA cap, an RNA 7-methylguanosine cap or an RNA m^7G cap) is a modified guanine nucleotide that has been added to the "front" or 5' end of a eukaryotic messenger RNA shortly after the start of transcription. The 5' cap consists of a terminal group which is linked to the first transcribed nucleotide. Its presence is important for recognition by the ribosome and protection from RNases. Cap addition is coupled to transcription, and occurs co-transcriptionally, such that each influences the other. Shortly after the start of transcription, the 5' end of the mRNA being synthesized is bound by a cap-synthesizing complex associated with RNA polymerase. This enzymatic complex catalyzes the chemical reactions that are required for mRNA capping. Synthesis proceeds as a multi-step biochemical reaction. The capping moiety can be modified to modulate functionality of mRNA such as its stability or efficiency of translation.

As used herein, "in vitro transcribed RNA" refers to RNA, e.g., mRNA, that has been synthesized in vitro. Generally, the in vitro transcribed RNA is generated from an in vitro transcription vector. The in vitro transcription vector comprises a template that is used to generate the in vitro transcribed RNA.

As used herein, a "poly(A)" is a series of adenosines attached by polyadenylation to the mRNA. In some embodiments of a construct for transient expression, the polyA is between 50 and 5000 (SEQ ID NO: 28), e.g., greater than 64, e.g., greater than 100, e.g., than 300 or 400. Poly(A) sequences can be modified chemically or enzymatically to modulate mRNA functionality such as localization, stability or efficiency of translation.

As used herein, "polyadenylation" refers to the covalent linkage of a polyadenylyl moiety, or its modified variant, to a messenger RNA molecule. In eukaryotic organisms, most messenger RNA (mRNA) molecules are polyadenylated at the 3' end. The 3' poly(A) tail is a long sequence of adenine nucleotides (often several hundred) added to the pre-mRNA through the action of an enzyme, polyadenylate polymerase. In higher eukaryotes, the poly(A) tail is added onto transcripts that contain a specific sequence, the polyadenylation signal. The poly(A) tail and the protein bound to it aid in protecting mRNA from degradation by exonucleases. Polyadenylation is also important for transcription termination, export of the mRNA from the nucleus, and translation. Polyadenylation occurs in the nucleus immediately after transcription of DNA into

RNA, but additionally can also occur later in the cytoplasm. After transcription has been terminated, the mRNA chain is cleaved through the action of an endonuclease complex associated with RNA polymerase. The cleavage site is usually characterized by the presence of the base sequence AAUAAA near the cleavage site. After the mRNA has been cleaved, adenosine residues are added to the free 3' end at the cleavage site.

As used herein, "transient" refers to expression of a non-integrated transgene for a period of hours, days or weeks, wherein the period of time of expression is less than the period of time for expression of the gene if integrated into the genome or contained within a stable plasmid replicon in the host cell.

The term "signal transduction pathway" refers to the biochemical relationship between a variety of signal transduction molecules that play a role in the transmission of a signal from one portion of a cell to another portion of a cell. The phrase "cell surface receptor" includes molecules and complexes of molecules capable of receiving a signal and transmitting signal across the membrane of a cell.

The term "subject" is intended to include living organisms in which an immune response can be elicited (e.g., mammals, human).

The term, a "substantially purified" cell refers to a cell that is essentially free of other cell types. A substantially purified cell also refers to a cell which has been separated from other cell types with which it is normally associated in its naturally occurring state. In some instances, a population of substantially purified cells refers to a homogenous population of cells. In other instances, this term refers simply to cell that have been separated from the cells with which they are naturally associated in their natural state. In some aspects, the cells are cultured in vitro. In other aspects, the cells are not cultured in vitro.

The term "therapeutic" as used herein means a treatment. A therapeutic effect is obtained by reduction, suppression, remission, or eradication of a disease state.

The term "prophylaxis" as used herein means the prevention of or protective treatment for a disease or disease state.

In the context of the present invention, "tumor antigen" or "hyperproliferative disorder antigen" or "antigen associated with a hyperproliferative disorder" refers to antigens that are

common to specific hyperproliferative disorders. In certain aspects, the hyperproliferative disorder antigens of the present invention are derived from, cancers including but not limited to primary or metastatic melanoma, thymoma, lymphoma, sarcoma, lung cancer, liver cancer, non-Hodgkin lymphoma, Hodgkin lymphoma, leukemias, uterine cancer, cervical cancer, bladder cancer, kidney cancer and adenocarcinomas such as breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, and the like.

The term "transfected" or "transformed" or "transduced" refers to a process by which exogenous nucleic acid is transferred or introduced into the host cell. A "transfected" or "transformed" or "transduced" cell is one which has been transfected, transformed or transduced with exogenous nucleic acid. The cell includes the primary subject cell and its progeny.

A subject "responds" to treatment if a parameter of a cancer (e.g., a hematological cancer, e.g., cancer cell growth, proliferation and/or survival) in the subject is retarded or reduced by a detectable amount, e.g., about 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more as determined by any appropriate measure, e.g., by mass, cell count or volume. In one example, a subject responds to treatment if the subject experiences a life expectancy extended by about 5%, 10%, 20%, 30%, 40%, 50% or more beyond the life expectancy predicted if no treatment is administered. In another example, a subject responds to treatment, if the subject has an increased disease-free survival, overall survival or increased time to progression. Several methods can be used to determine if a patient responds to a treatment including, for example, criteria provided by NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®). For example, in the context of B-ALL, a complete response or complete responder, may involve one or more of: < 5% BM blast, >1000 neutrophil/ANC (/μL). >100,000 platelets (/μL) with no circulating blasts or extramedullary disease (no lymphadenopathy, splenomegaly, skin/gum infiltration/testicular mass/CNS involvement), Trilineage hematopoiesis, and no recurrence for 4 weeks. A partial responder may involve one or more of >50% reduction in BM blast, >1000 neutrophil/ANC (/μL). >100,000 platelets (/μL). A non-responder can show disease progression, e.g., > 25% in BM blasts.

"Refractory" as used herein refers to a disease, e.g., cancer, that does not respond to a treatment. In embodiments, a refractory cancer can be resistant to a treatment before or at the beginning of the treatment. In other embodiments, the refractory cancer can become resistant during a treatment. A refractory cancer is also called a resistant cancer.

The term "relapse" as used herein refers to reappearance of a cancer after an initial period of responsiveness (e.g., complete response or partial response). The initial period of responsiveness may involve the level of cancer cells falling below a certain threshold, e.g., below 20%, 1%, 10%, 5%, 4%, 3%, 2%, or 1%. The reappearance may involve the level of cancer cells rising above a certain threshold, e.g., above 20%, 1%, 10%, 5%, 4%, 3%, 2%, or 1%. For example, e.g., in the context of B-ALL, the reappearance may involve, e.g., a reappearance of blasts in the blood, bone marrow (> 5%), or any extramedullary site, after a complete response. A complete response, in this context, may involve < 5% BM blast. More generally, in an embodiment, a response (e.g., complete response or partial response) can involve the absence of detectable MRD (minimal residual disease). In an embodiment, the initial period of responsiveness lasts at least 1, 2, 3, 4, 5, or 6 days; at least 1, 2, 3, or 4 weeks; at least 1, 2, 3, 4, 6, 8, 10, or 12 months; or at least 1, 2, 3, 4, or 5 years.

In some embodiments, a therapy that includes a CD19 inhibitor, e.g., a CD19 CAR therapy, may relapse or be refractory to treatment. The relapse or resistance can be caused by CD19 loss (e.g., an antigen loss mutation) or other CD19 alteration that reduces the level of CD19 (e.g., caused by clonal selection of CD19-negative clones). A cancer that harbors such CD19 loss or alteration is referred to herein as a "CD19-negative cancer" or a "CD19-negative relapsed cancer"). It shall be understood that a CD19-negative cancer need not have 100% loss of CD19, but a sufficient reduction to reduce the effectiveness of a CD19 therapy such that the cancer relapses or becomes refractory. In some embodiments, a CD19-negative cancer results from a CD19 CAR therapy.

The term "specifically binds," refers to an antibody, or a ligand, which recognizes and binds with a binding partner (e.g., a stimulatory tumor antigen) protein present in a sample, but which antibody or ligand does not substantially recognize or bind other molecules in the sample.

As used herein, the term "pharmaceutically acceptable salt" refers to those salts which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of subjects without undue toxicity, irritation, allergic response and the like, and are commensurate with a reasonable benefit/risk ratio. Pharmaceutically acceptable salts are well known in the art. For example, Berge *et al.* describes pharmaceutically acceptable salts in detail in *J. Pharmaceutical Sciences* (1977) 66:1–19.

"Regulatable chimeric antigen receptor (RCAR),"as that term is used herein, refers to a

set of polypeptides, typically two in the simplest embodiments, which when in a RCARX cell, provides the RCARX cell with specificity for a target cell, typically a cancer cell, and with regulatable intracellular signal generation or proliferation, which can optimize an immune effector property of the RCARX cell. An RCARX cell relies at least in part, on an antigen binding domain to provide specificity to a target cell that comprises the antigen bound by the antigen binding domain. In an embodiment, an RCAR includes a dimerization switch that, upon the presence of a dimerization molecule, can couple an intracellular signaling domain to the antigen binding domain.

"Membrane anchor" or "membrane tethering domain", as that term is used herein, refers to a polypeptide or moiety, e.g., a myristoyl group, sufficient to anchor an extracellular or intracellular domain to the plasma membrane.

"Switch domain," as that term is used herein, e.g., when referring to an RCAR, refers to an entity, typically a polypeptide-based entity, that, in the presence of a dimerization molecule, associates with another switch domain. The association results in a functional coupling of a first entity linked to, e.g., fused to, a first switch domain, and a second entity linked to, e.g., fused to, a second switch domain. A first and second switch domain are collectively referred to as a dimerization switch. In embodiments, the first and second switch domains are the same as one another, e.g., they are polypeptides having the same primary amino acid sequence, and are referred to collectively as a homodimerization switch. In embodiments, the first and second switch domains are different from one another, e.g., they are polypeptides having different primary amino acid sequences, and are referred to collectively as a heterodimerization switch. In embodiments, the switch is intracellular. In embodiments, the switch is extracellular. In embodiments, the switch domain is a polypeptide-based entity, e.g., FKBP or FRB-based, and the dimerization molecule is small molecule, e.g., a rapalogue. In embodiments, the switch domain is a polypeptide-based entity, e.g., an scFv that binds a myc peptide, and the dimerization molecule is a polypeptide, a fragment thereof, or a multimer of a polypeptide, e.g., a myc ligand or multimers of a myc ligand that bind to one or more myc scFvs. In embodiments, the switch domain is a polypeptide-based entity, e.g., myc receptor, and the dimerization molecule is an antibody or fragments thereof, e.g., myc antibody.

"Dimerization molecule," as that term is used herein, e.g., when referring to an RCAR, refers to a molecule that promotes the association of a first switch domain with a second switch

domain. In embodiments, the dimerization molecule does not naturally occur in the subject, or does not occur in concentrations that would result in significant dimerization. In embodiments, the dimerization molecule is a small molecule, e.g., rapamycin or a rapalogue, e.g., RAD001.

The term "low, immune enhancing, dose" when used in conjunction with an mTOR inhibitor, e.g., an allosteric mTOR inhibitor, e.g., RAD001 or rapamycin, or a catalytic mTOR inhibitor, refers to a dose of mTOR inhibitor that partially, but not fully, inhibits mTOR activity, e.g., as measured by the inhibition of P70 S6 kinase activity. Methods for evaluating mTOR activity, e.g., by inhibition of P70 S6 kinase, are discussed herein. The dose is insufficient to result in complete immune suppression but is sufficient to enhance the immune response. In an embodiment, the low, immune enhancing, dose of mTOR inhibitor results in a decrease in the number of PD-1 positive T cells and/or an increase in the number of PD-1 negative T cells, or an increase in the ratio of PD-1 negative T cells/PD-1 positive T cells. In an embodiment, the low, immune enhancing, dose of mTOR inhibitor results in an increase in the number of naive T cells. In an embodiment, the low, immune enhancing, dose of mTOR inhibitor results in one or more of the following:

an increase in the expression of one or more of the following markers: $CD62L^{high}$, $CD127^{high}$, $CD27^{+}$, and BCL2, e.g., on memory T cells, e.g., memory T cell precursors;

a decrease in the expression of KLRG1, e.g., on memory T cells, e.g., memory T cell precursors; and

an increase in the number of memory T cell precursors, e.g., cells with any one or combination of the following characteristics: increased $CD62L^{high}$, increased $CD127^{high}$, increased $CD27^{+}$, decreased KLRG1, and increased BCL2;

wherein any of the changes described above occurs, e.g., at least transiently, e.g., as compared to a non-treated subject.

Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically

disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. As another example, a range such as 95-99% identity, includes something with 95%, 96%, 97%, 98% or 99% identity, and includes subranges such as 96-99%, 96-98%, 96-97%, 97-99%, 97-98% and 98-99% identity. This applies regardless of the breadth of the range.

Description

CD19 Inhibitors and Binding Domains

Provided herein are compositions of matter and methods of use for the treatment of a disease such as cancer using CD19 chimeric antigen receptors (CAR). The methods include, inter alia, administering a CD19 CAR described herein in combination with another agent such as B-cell inhibitor. The methods also include, e.g., administering a CD19 CAR described herein to treat a lymphoma such as Hodgkin lymphoma.

In one aspect, the invention provides a number of chimeric antigen receptors (CAR) comprising an antibody or antibody fragment engineered for specific binding to a CD19 protein. In one aspect, the invention provides a cell (e.g., T cell) engineered to express a CAR, wherein the CAR T cell ("CART") exhibits an anticancer property. In one aspect a cell is transformed with the CAR and the CAR is expressed on the cell surface. In some embodiments, the cell (e.g., T cell) is transduced with a viral vector encoding a CAR. In some embodiments, the viral vector is a retroviral vector. In some embodiments, the viral vector is a lentiviral vector. In some such embodiments, the cell may stably express the CAR. In another embodiment, the cell (e.g., T cell) is transfected with a nucleic acid, e.g., mRNA, cDNA, DNA, encoding a CAR. In some such embodiments, the cell may transiently express the CAR.

In one aspect, the anti-CD19 protein binding portion of the CAR is a scFv antibody fragment. In one aspect such antibody fragments are functional in that they retain the equivalent binding affinity, e.g., they bind the same antigen with comparable affinity, as the IgG antibody from which it is derived. In one aspect such antibody fragments are functional in that they provide a biological response that can include, but is not limited to, activation of an immune response, inhibition of signal-transduction origination from its target antigen, inhibition of kinase activity, and the like, as will be understood by a skilled artisan. In one aspect, the anti-

CD19 antigen binding domain of the CAR is a scFv antibody fragment that is humanized compared to the murine sequence of the scFv from which it is derived. In one aspect, the parental murine scFv sequence is the CAR19 construct provided in PCT publication WO2012/079000 and provided herein as SEQ ID NO:59. In one embodiment, the anti-CD19 binding domain is a scFv described in WO2012/079000 and provided in SEQ ID NO:59, or a sequence at least 95%, e.g., 95-99%, identical thereto. In an embodiment, the anti-CD19 binding domain is part of a CAR construct provided in PCT publication WO2012/079000 and provided herein as SEQ ID NO:58, or a sequence at least 95%, e.g., 95%-99%, identical thereto. In an embodiment, the anti-CD19 binding domain comprises at least one (e.g., 2, 3, 4, 5, or 6) CDRs selected from **Table 4** and/or **Table 5**.

In some aspects, the antibodies of the invention are incorporated into a chimeric antigen receptor (CAR). In one aspect, the CAR comprises the polypeptide sequence provided as SEQ ID NO: 12 in PCT publication WO2012/079000, and provided herein as SEQ ID NO: 58, wherein the scFv domain is substituted by one or more sequences selected from SEQ ID NOS: 1-12. In one aspect, the scFv domains of SEQ ID NOS:1-12 are humanized variants of the scFv domain of SEQ ID NO:59, which is an scFv fragment of murine origin that specifically binds to human CD19. Humanization of this mouse scFv may be desired for the clinical setting, where the mouse-specific residues may induce a human-anti-mouse antigen (HAMA) response in patients who receive CART19 treatment, e.g., treatment with T cells transduced with the CAR19 construct.

In one aspect, the anti-CD19 binding domain, e.g., humanized scFv, portion of a CAR of the invention is encoded by a transgene whose sequence has been codon optimized for expression in a mammalian cell. In one aspect, entire CAR construct of the invention is encoded by a transgene whose entire sequence has been codon optimized for expression in a mammalian cell. Codon optimization refers to the discovery that the frequency of occurrence of synonymous codons (i.e., codons that code for the same amino acid) in coding DNA is biased in different species. Such codon degeneracy allows an identical polypeptide to be encoded by a variety of nucleotide sequences. A variety of codon optimization methods is known in the art, and include, e.g., methods disclosed in at least US Patent Numbers 5,786,464 and 6,114,148.

In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:1. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID

NO:2. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:3. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:4. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:5. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:6. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:7. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:8. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:9. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:10. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:11. In one aspect, the humanized CAR19 comprises the scFv portion provided in SEQ ID NO:12.

In one aspect, the CARs of the invention combine an antigen binding domain of a specific antibody with an intracellular signaling molecule. For example, in some aspects, the intracellular signaling molecule includes, but is not limited to, CD3-zeta chain, 4-1BB and CD28 signaling modules and combinations thereof. In one aspect, the CD19 CAR comprises a CAR selected from the sequence provided in one or more of SEQ ID NOS: 31 - 42. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:31. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:32. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:33. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:34. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:35. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:36. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:37. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:38. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:39. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:40. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:41. In one aspect, the CD19 CAR comprises the sequence provided in SEQ ID NO:42.

Thus, in one aspect, the antigen binding domain comprises a humanized antibody or an antibody fragment. In one embodiment, the humanized anti-CD19 binding domain comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary

determining region 3 (LC CDR3) of a murine or humanized anti-CD19 binding domain described herein, and/or one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a murine or humanized anti-CD19 binding domain described herein, e.g., a humanized anti-CD19 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. In one embodiment, the humanized anti-CD19 binding domain comprises one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a murine or humanized anti-CD19 binding domain described herein, e.g., the humanized anti-CD19 binding domain has two variable heavy chain regions, each comprising a HC CDR1, a HC CDR2 and a HC CDR3 described herein. In one embodiment, the humanized anti-CD19 binding domain comprises a humanized light chain variable region described herein (e.g., in **Table 2**) and/or a humanized heavy chain variable region described herein (e.g., in **Table 2**). In one embodiment, the humanized anti-CD19 binding domain comprises a humanized heavy chain variable region described herein (e.g., in **Table 2**), e.g., at least two humanized heavy chain variable regions described herein (e.g., in **Table 2**). In one embodiment, the anti-CD19 binding domain is a scFv comprising a light chain and a heavy chain of an amino acid sequence of **Table 2**. In an embodiment, the anti-CD19 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 2**, or a sequence with 95-99% identity with an amino acid sequence of **Table 2**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 2**, or a sequence with 95-99% identity to an amino acid sequence of **Table 2**. In one embodiment, the humanized anti-CD19 binding domain comprises a sequence selected from a group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12, or a sequence with 95-99% identity thereof. In one embodiment, the nucleic

acid sequence encoding the humanized anti-CD19 binding domain comprises a sequence selected from a group consisting of SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:70, SEQ ID NO:71 and SEQ ID NO:72, or a sequence with 95-99% identity thereof. In one embodiment, the humanized anti-CD19 binding domain is a scFv, and a light chain variable region comprising an amino acid sequence described herein, e.g., in **Table 2**, is attached to a heavy chain variable region comprising an amino acid sequence described herein, e.g., in **Table 2**, via a linker, e.g., a linker described herein. In one embodiment, the humanized anti-CD19 binding domain includes a $(Gly_4\text{-}Ser)n$ linker, wherein n is 1, 2, 3, 4, 5, or 6, e.g., 3 or 4 (SEQ ID NO:53). The light chain variable region and heavy chain variable region of a scFv can be, e.g., in any of the following orientations: light chain variable region-linker-heavy chain variable region or heavy chain variable region-linker-light chain variable region.

In one aspect, the antigen binding domain portion comprises one or more sequence selected from SEQ ID NOS:1-12. In one aspect the humanized CAR is selected from one or more sequence selected from SEQ ID NOS: 31-42. In some aspects, a non-human antibody is humanized, where specific sequences or regions of the antibody are modified to increase similarity to an antibody naturally produced in a human or fragment thereof.

In one embodiment, the CAR molecule comprises an anti-CD19 binding domain comprising one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of an anti-CD19 binding domain described herein, and one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of an anti-CD19 binding domain described herein, e.g., an anti-CD19 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. In one embodiment, the anti-CD19 binding domain comprises one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of an anti-CD19 binding domain described herein, e.g., the anti-CD19 binding domain has two variable heavy chain regions, each comprising a HC CDR1, a HC CDR2 and a HC CDR3 described herein.

In one aspect, the anti-CD19 binding domain is characterized by particular functional features or properties of an antibody or antibody fragment. For example, in one aspect, the portion of a CAR composition of the invention that comprises an antigen binding domain specifically binds human CD19. In one aspect, the invention relates to an antigen binding domain comprising an antibody or antibody fragment, wherein the antibody binding domain specifically binds to a CD19 protein or fragment thereof, wherein the antibody or antibody fragment comprises a variable light chain and/or a variable heavy chain that includes an amino acid sequence of SEQ ID NO: 1-12 or SEQ ID NO:59. In one aspect, the antigen binding domain comprises an amino acid sequence of an scFv selected from SEQ ID NOs: 1-12 or SEQ ID NO:59. In certain aspects, the scFv is contiguous with and in the same reading frame as a leader sequence. In one aspect the leader sequence is the polypeptide sequence provided as SEQ ID NO:13.

In one aspect, the portion of the CAR comprising the antigen binding domain comprises an antigen binding domain that targets CD19. In one aspect, the antigen binding domain targets human CD19. In one aspect, the antigen binding domain of the CAR has the same or a similar binding specificity as, or includes, the FMC63 scFv fragment described in Nicholson et al. Mol. Immun. 34 (16-17): 1157-1165 (1997). In one aspect, the portion of the CAR comprising the antigen binding domain comprises an antigen binding domain that targets a B-cell antigen, e.g., a human B-cell antigen. A CD19 antibody molecule can be, e.g., an antibody molecule (e.g., a humanized anti-CD19 antibody molecule) described in WO2014/153270, which is incorporated herein by reference in its entirety. WO2014/153270 also describes methods of assaying the binding and efficacy of various CART constructs.

In one embodiment, the anti-CD19 binding domain comprises a murine light chain variable region described herein (e.g., in **Table 3**) and/or a murine heavy chain variable region described herein (e.g., in **Table 3**). In one embodiment, the anti-CD19 binding domain is a scFv comprising a murine light chain and a murine heavy chain of an amino acid sequence of **Table 3**. In an embodiment, the anti-CD19 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 3**, or a sequence with 95-99% identity with an amino acid sequence of **Table 3**; and/or a heavy chain

variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 3**, or a sequence with 95-99% identity to an amino acid sequence of **Table 3**. In one embodiment, the anti-CD19 binding domain comprises a sequence of SEQ ID NO:59, or a sequence with 95-99% identity thereof. In one embodiment, the anti-CD19 binding domain is a scFv, and a light chain variable region comprising an amino acid sequence described herein, e.g., in **Table 3**, is attached to a heavy chain variable region comprising an amino acid sequence described herein, e.g., in **Table 3**, via a linker, e.g., a linker described herein. In one embodiment, the antigen binding domain includes a $(Gly_4\text{-}Ser)n$ linker, wherein n is 1, 2, 3, 4, 5, or 6, e.g., 3 or 4 (SEQ ID NO: 53). The light chain variable region and heavy chain variable region of a scFv can be, e.g., in any of the following orientations: light chain variable region-linker-heavy chain variable region or heavy chain variable region-linker-light chain variable region.

Furthermore, the present invention provides (among other things) CD19 CAR compositions, optionally in combination with a B-cell inhibitor, and their use in medicaments or methods for treating, among other diseases, cancer or any malignancy or autoimmune diseases involving cells or tissues which express CD19.

In one aspect, the CAR of the invention can be used to eradicate CD19-expressing normal cells, thereby applicable for use as a cellular conditioning therapy prior to cell transplantation. In one aspect, the CD19-expressing normal cell is a CD19-expressing normal stem cell and the cell transplantation is a stem cell transplantation.

In one aspect, the invention provides a cell (e.g., T cell) engineered to express a chimeric antigen receptor (CAR), wherein the CAR-expressing cell, e.g., CAR T cell ("CART") exhibits an anticancer property. A suitable antigen is CD19. In one aspect, the antigen binding domain of the CAR comprises a partially humanized anti-CD19 antibody fragment. In one aspect, the antigen binding domain of the CAR comprises a partially humanized anti-CD19 antibody fragment comprising an scFv. Accordingly, the invention provides (among other things) a CD19-CAR that comprises a humanized anti-CD19 binding domain and is engineered into an immune effector cell, e.g., a T cell or an NK cell, and methods of their use for adoptive therapy.

In one aspect, the CAR, e.g., CD19-CAR comprises at least one intracellular domain

selected from the group of a CD137 (4-1BB) signaling domain, a CD28 signaling domain, a CD3zeta signal domain, and any combination thereof. In one aspect, the CAR, e.g., CD19-CAR comprises at least one intracellular signaling domain is from one or more co-stimulatory molecule(s) other than a CD137 (4-1BB) or CD28.

The present invention encompasses, but is not limited to, a recombinant DNA construct comprising sequences encoding a CAR, wherein the CAR comprises an antibody or antibody fragment that binds specifically to CD19, CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1, e.g., human CD19, CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1, wherein the sequence of the antibody fragment is contiguous with and in the same reading frame as a nucleic acid sequence encoding an intracellular signaling domain. The intracellular signaling domain can comprise a costimulatory signaling domain and/or a primary signaling domain, e.g., a zeta chain. The costimulatory signaling domain refers to a portion of the CAR comprising at least a portion of the intracellular domain of a costimulatory molecule. In one embodiment, the antigen binding domain is a murine antibody or antibody fragment described herein. In one embodiment, the antigen binding domain is a humanized antibody or antibody fragment.

In specific aspects, a CAR construct of the invention comprises a scFv domain selected from the group consisting of SEQ ID NOS:1-12 or an scFV domain of SEQ ID NO:59, wherein the scFv may be preceded by an optional leader sequence such as provided in SEQ ID NO: 13, and followed by an optional hinge sequence such as provided in SEQ ID NO:14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49, a transmembrane region such as provided in SEQ ID NO:15, an intracellular signalling domain that includes SEQ ID NO:16 or SEQ ID NO:51 and a CD3 zeta sequence that includes SEQ ID NO:17 or SEQ ID NO:43, wherein the domains are contiguous with and in the same reading frame to form a single fusion protein. Also included in the invention (among other things) is a nucleotide sequence that encodes the polypeptide of each of the scFv fragments selected from the group consisting of SEQ IS NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ IS NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:59. Also included in the invention (among other things) is a nucleotide sequence that encodes the polypeptide of each of the scFv fragments selected from the group consisting of SEQ IS NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ IS NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:59,

and each of the domains of SEQ ID NOS: 13-17, plus an encoded CD19 CAR fusion protein of the invention. In one aspect an exemplary CD19 CAR constructs comprise an optional leader sequence, an extracellular antigen binding domain, a hinge, a transmembrane domain, and an intracellular stimulatory domain. In one aspect an exemplary CD19 CAR construct comprises an optional leader sequence, an extracellular antigen binding domain, a hinge, a transmembrane domain, an intracellular costimulatory domain and an intracellular stimulatory domain. In some embodiments, specific CD19 CAR constructs containing humanized scFv domains of the invention are provided as SEQ ID NOS: 31-42, or a murine scFv domain as provided as SEQ ID NO:59.

Full-length CAR sequences are also provided herein as SEQ ID NOS: 31-42 and 58, as shown in **Table 2** and **Table 3**.

An exemplary leader sequence is provided as SEQ ID NO: 13. An exemplary hinge/spacer sequence is provided as SEQ ID NO: 14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49. An exemplary transmembrane domain sequence is provided as SEQ ID NO:15. An exemplary sequence of the intracellular signaling domain of the 4-1BB protein is provided as SEQ ID NO: 16. An exemplary sequence of the intracellular signaling domain of CD27 is provided as SEQ ID NO:51. An exemplary CD3zeta domain sequence is provided as SEQ ID NO: 17 or SEQ ID NO:43. These sequences may be used, e.g., in combination with an scFv that recognizes one or more of CD19, CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1.

Exemplary sequences of various scFv fragments and other CAR components are provided herein. It is noted that these CAR components (e.g., of SEQ ID NO: 121, or a sequence of **Table 2, 3, 6, 11A, 11B, 16, or 25**) without a leader sequence (e.g., without the amino acid sequence of SEQ ID NO: 13 or a nucleotide sequence of SEQ ID NO: 54), are also provided herein.

In embodiments, the CAR sequences described herein contain a Q/K residue change in the signal domain of the co-stimulatory domain derived from CD3zeta chain.

In one aspect, the present invention encompasses a recombinant nucleic acid construct comprising a nucleic acid molecule encoding a CAR, wherein the nucleic acid molecule comprises the nucleic acid sequence encoding an anti-CD19 binding domain, e.g., described herein, that is contiguous with and in the same reading frame as a nucleic acid sequence

encoding an intracellular signaling domain. In one aspect, the anti-CD19 binding domain is selected from one or more of SEQ ID NOS:1-12 and 58. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of the sequence provided in one or more of SEQ ID NOS:61-72 and 97. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:61. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:62. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:63. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:64. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:65. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:66. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:67. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:68. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:69. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:70. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:71. In one aspect, the anti-CD19 binding domain is encoded by a nucleotide residues 64 to 813 of SEQ ID NO:72.

Provided herein are CD19 inhibitors and combination therapies. In some embodiments, the CD19 inhibitor (e.g., a cell therapy or an antibody) is administered in combination with a B cell inhibitor, e.g., one or more inhibitors of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, or ROR1. A CD19 inhibitor includes but is not limited to a CD19 CAR-expressing cell, e.g., a CD19 CART cell, or an anti-CD19 antibody (e.g., an anti-CD19 mono- or bispecific antibody) or a fragment or conjugate thereof. In an embodiment, the CD19 inhibitor is administered in combination with a B-cell inhibitor, e.g., a CAR-expressing cell described herein.

Numerous CD19 CAR-expressing cells are described in this disclosure. For instance, in some embodiments, a CD19 inhibitor includes an anti-CD19 CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-CD19 CAR construct described in **Table 2** or encoded by a CD19 binding CAR comprising a scFv, CDRs, or VH and VL chains described in **Tables 2, 4, or 5**. For example, an anti-CD19 CAR-expressing cell, e.g., CART, is a generated by engineering a CD19-CAR (that comprises a CD19 binding domain) into a cell (e.g., a T cell or NK cell), e.g.,

for administration in combination with a CAR-expressing cell described herein. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In one embodiment, an antigen binding domain comprises one, two three (e.g., all three) heavy chain CDRs, HC CDR1, HC CDR2 and HC CDR3, from an antibody listed herein, e.g., in **Table 2, 4, or 5** and/or one, two, three (e.g., all three) light chain CDRs, LC CDR1, LC CDR2 and LC CDR3, from an antibody listed herein, e.g., in **Table 2, 4, or 5**. In one embodiment, the antigen binding domain comprises a heavy chain variable region and/or a variable light chain region of an antibody listed or described above.

In an embodiment, the CD19 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 2**, or a sequence with 95-99% identity with an amino acid sequence of **Table 2**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 2**, or a sequence with 95-99% identity to an amino acid sequence of **Table 2**. In embodiments, the CD19 binding domain comprises one or more CDRs (e.g., one each of a HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, and LC CDR3) of **Table 4** or **Table 5**, or CDRs having one, two, three, four, five, or six modifications (e.g., substitutions) of one or more of the CDRs.

Exemplary anti-CD19 antibodies or fragments or conjugates thereof include but are not limited to blinatumomab, SAR3419 (Sanofi), MEDI-551 (MedImmune LLC), Combotox, DT2219ARL (Masonic Cancer Center), MOR-208 (also called XmAb-5574; MorphoSys), XmAb-5871 (Xencor), MDX-1342 (Bristol-Myers Squibb), SGN-CD19A (Seattle Genetics), and AFM11 (Affimed Therapeutics). See, e.g., Hammer. MAbs. 4.5(2012): 571–77.

Blinatomomab is a bispecific antibody comprised of two scFvs—one that binds to CD19 and one that binds to CD3. Blinatomomab directs T cells to attack cancer cells. See, e.g., Hammer et al.; Clinical Trial Identifier No. NCT00274742 and NCT01209286. MEDI-551 is a humanized anti-CD19 antibody with a Fc engineered to have enhanced antibody-dependent cell-mediated cytotoxicity (ADCC). See, e.g., Hammer et al.; and Clinical Trial Identifier No. NCT01957579. Combotox is a mixture of immunotoxins that bind to CD19 and CD22. The

immunotoxins are made up of scFv antibody fragments fused to a deglycosylated ricin A chain. See, e.g., Hammer et al.; and Herrera et al. J. Pediatr. Hematol. Oncol. 31.12(2009):936-41; Schindler et al. Br. J. Haematol. 154.4(2011):471-6. DT2219ARL is a bispecific immunotoxin targeting CD19 and CD22, comprising two scFvs and a truncated diphtheria toxin. See, e.g., Hammer et al.; and Clinical Trial Identifier No. NCT00889408. SGN-CD19A is an antibody-drug conjugate (ADC) comprised of an anti-CD19 humanized monoclonal antibody linked to a synthetic cytotoxic cell-killing agent, monomethyl auristatin F (MMAF). See, e.g., Hammer et al.; and Clinical Trial Identifier Nos. NCT01786096 and NCT01786135. SAR3419 is an anti-CD19 antibody-drug conjugate (ADC) comprising an anti-CD19 humanized monoclonal antibody conjugated to a maytansine derivative via a cleavable linker. See, e.g., Younes et al. J. Clin. Oncol. 30.2(2012): 2776-82; Hammer et al.; Clinical Trial Identifier No. NCT00549185; and Blanc et al. Clin Cancer Res. 2011;17:6448-58. XmAb-5871 is an Fc-engineered, humanized anti-CD19 antibody. See, e.g., Hammer et al. MDX-1342 is a human Fc-engineered anti-CD19 antibody with enhanced ADCC. See, e.g., Hammer et al. In embodiments, the antibody molecule is a bispecific anti-CD19 and anti-CD3 molecule. For instance, AFM11 is a bispecific antibody that targets CD19 and CD3. See, e.g., Hammer et al.; and Clinical Trial Identifier No. NCT02106091. In some embodiments, an anti-CD19 antibody described herein is conjugated or otherwise bound to a therapeutic agent, e.g., a chemotherapeutic agent, peptide vaccine (such as that described in Izumoto et al. 2008 J Neurosurg 108:963-971), immunosuppressive agent, or immunoablative agent, e.g., cyclosporin, azathioprine, methotrexate, mycophenolate, FK506, CAMPATH, anti-CD3 antibody, cytoxin, fludarabine, rapamycin, mycophenolic acid, steroid, FR901228, or cytokine.

Exemplary anti-CD19 antibody molecules (including antibodies or fragments or conjugates thereof) can include a scFv, CDRs, or VH and VL chains described in **Tables 2, 4, or 5**. In an embodiment, the CD19-binding antibody molecule comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 2**, or a sequence with 95-99% identity with an amino acid sequence of **Table 2**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid

sequence of a heavy chain variable region provided in **Table 2**, or a sequence with 95-99% identity to an amino acid sequence of **Table 2**. In embodiments, the CD19-binding antibody molecule comprises one or more CDRs (e.g., one each of a HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, and LC CDR3) of **Table 4** or **Table 5**, or CDRs having one, two, three, four, five, or six modifications (e.g., substitutions) of one or more of the CDRs. The antibody molecule may be, e.g., an isolated antibody molecule.

In one embodiment, an antigen binding domain against CD19 is an antigen binding portion, e.g., CDRs, of an antigen binding domain described in a Table herein. In one embodiment, a CD19 antigen binding domain can be from any CD19 CAR, e.g., LG-740; US Pat. No. 8,399,645; US Pat. No. 7,446,190; Xu et al., Leuk Lymphoma. 2013 54(2):255-260(2012); Cruz et al., Blood 122(17):2965-2973 (2013); Brentjens et al., Blood, 118(18):4817-4828 (2011); Kochenderfer et al., Blood 116(20):4099-102 (2010); Kochenderfer et al., Blood 122 (25):4129-39(2013); and 16th Annu Meet Am Soc Gen Cell Ther (ASGCT) (May 15-18, Salt Lake City) 2013, Abst 10, each of which is herein incorporated by reference in its entirety.

In one embodiment, the CAR T cell that specifically binds to CD19 has the USAN designation TISAGENLECLEUCEL-T. CTL019 is made by a gene modification of T cells is mediated by stable insertion via transduction with a self-inactivating, replication deficient Lentiviral (LV) vector containing the CTL019 transgene under the control of the EF-1 alpha promoter. CTL019 can be a mixture of transgene positive and negative T cells that are delivered to the subject on the basis of percent transgene positive T cells.

In one aspect the nucleic acid sequence of a CAR construct of the invention is selected from one or more of SEQ ID NOS:85-96. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:85. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:86. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:87. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:88. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:89. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:90. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:91. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:92. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:93. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:94. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:95. In one aspect the nucleic acid

sequence of a CAR construct is SEQ ID NO:96. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:97. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:98. In one aspect the nucleic acid sequence of a CAR construct is SEQ ID NO:99.

CD20 Inhibitors and Binding Domains

As used herein, the term "CD20" refers to an antigenic determinant known to be detectable on B cells. Human CD20 is also called membrane-spanning 4-domains, subfamily A, member 1 (MS4A1). The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequence of human CD20 can be found at Accession Nos. NP_690605.1 and NP_068769.2, and the nucleotide sequence encoding transcript variants 1 and 3 of the human CD20 can be found at Accession No. NM_152866.2 and NM_021950.3, respectively. As used herein, "CD20" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type CD20. In one aspect the antigen-binding portion of the CAR recognizes and binds an antigen within the extracellular domain of the CD20 protein. In one aspect, the CD20 protein is expressed on a cancer cell.

In some aspects, the present disclosure provides a CD20 inhibitor or binding domain, e.g., a CD20 inhibitor or binding domain as described herein. The disclosure also provides a nucleic acid encoding the CD20 binding domain, or a CAR comprising the CD20 binding domain. A CD20 inhibitor includes but is not limited to a CD20 CAR-expressing cell, e.g., a CD20 CART cell or an anti-CD20 antibody (e.g., an anti-CD20 mono- or bispecific antibody) or a fragment thereof. The composition may also comprise a second agent, e.g., an anti-CD19 CAR-expressing cell or a CD19 binding domain. The agents may be, e.g., encoded by a single nucleic acid or different nucleic acids.

In some aspects, a CD20 inhibitor or binding domain is administered as a monotherapy. In some aspects, the CD20 inhibitor or binding domain is administered in combination with a second agent such as an anti-CD19 CAR-expressing cell. In an embodiment, the CD20 inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

CD20 CAR-expressing cells, e.g., CARTs

In an embodiment, the CD20 antibody molecule comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 15A or 15B**, or a sequence with 95-99% identity with an amino acid sequence of **Table 15A or 15B**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 14A or 14B**, or a sequence with 95-99% identity to an amino acid sequence of **Table 14A or 14B**. In one embodiment, the CD20 antibody molecule comprises one or more (e.g., two or all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a CD20 binding domain described herein, and/or one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a CD20 binding domain described herein, e.g., a CD20 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. These CDRs may be, e.g., those of **Table 12A, 12B,** and/or **Table 13**, or a sequence substantially identical thereto. In an embodiment, the CD20 antibody molecule comprises one or more CDRs (e.g., a HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, or LC CDR3) comprising an amino acid sequence having one, two, three, four, five, or six modifications (e.g., substitutions) of an amino acid sequence of **Table 12A, 12B,** and/or **Table 13.** The antibody molecule may be, e.g., an isolated antibody molecule.

In some embodiments, a CD20 inhibitor includes an anti-CD20 CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-CD20 CAR construct described in **Table 11A or 11B,** or a sequence substantially identical thereto, or encoded by a CD20 binding CAR comprising a scFv, CDRs, or VH and VL chains described in **Tables 11A-15B,** or a sequence substantially identical thereto. For example, an anti-CD20 CAR-expressing cell, e.g., CART, is a generated by engineering a CD20-CAR (that comprises a CD20 binding domain) into a cell (e.g., a T cell or NK cell), e.g., for administration in combination with a CAR-expressing cell described

herein. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In an embodiment, the CD20 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 15A or 15B**, or a sequence with 95-99% identity with an amino acid sequence of **Table 15A or 15B**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 14A or 14B**, or a sequence with 95-99% identity to an amino acid sequence of **Table 14A or 14B**.

In one embodiment, the CD20 binding domain comprises one or more (e.g., two or all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a CD20 binding domain described herein, and/or one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a CD20 binding domain described herein, e.g., a CD20 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. These CDRs may be, e.g., those of **Table 12A, 12B,** and/or **Table 13**, or a sequence substantially identical thereto. In an embodiment, the CD20 binding domain (e.g., an scFv) comprises one or more CDRs (e.g., a HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, or LC CDR3) comprising an amino acid sequence having one, two, three, four, five, or six modifications (e.g., substitutions) of an amino acid sequence of **Table 12A, 12B,** and/or **Table 13.**

In some embodiments, the CAR comprises an antibody or antibody fragment which includes a CD20 binding domain, a transmembrane domain, and an intracellular signaling domain. The CD20 binding domain may comprise one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD20 light chain binding domain amino acid sequence listed in **Table 13, 15A, or 15B**, and one or more of heavy

chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD20 heavy chain binding domain amino acid sequence listed in **Table 12A, 12B, 14A, or 14B**.

In an embodiment, the CD20 binding domain comprises six CDRs (e.g., one each of a HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, and LC CDR3) of any one of CAR20-1, CAR20-2, CAR20-3, CAR20-4, CAR20-5, CAR20-6, CAR20-7, CAR20-8, CAR20-9, CAR20-10, CAR20-11, CAR20-12, CAR20-13, CAR20-14, CAR20-15, or CAR20-16, or a sequence substantially identical thereto. In an embodiment, the CD20 binding domain comprises three CDRs (e.g., one each of a HC CDR1, HC CDR2, and HC CDR3, or one each of a LC CDR1, LC CDR2, and LC CDR3) of any one of CAR20-1, CAR20-2, CAR20-3, CAR20-4, CAR20-5, CAR20-6, CAR20-7, CAR20-8, CAR20-9, CAR20-10, CAR20-11, CAR20-12, CAR20-13, CAR20-14, CAR20-15, or CAR20-16, or a sequence substantially identical thereto.

In one embodiment, the CD20 binding domain comprises a light chain variable region described herein (e.g., in **Table 15A or 15B**) and/or a heavy chain variable region described herein (e.g., in **Table 14A or 14B**), or a sequence substantially identical thereto. In an embodiment, the CD20 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 15A or 15B**, or a sequence with 95-99% identity with an amino acid sequence of **Table 15A or 15B**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 14A or 14B**, or a sequence with 95-99% identity to an amino acid sequence of **Table 14A or 14B**.

Further embodiments include a nucleotide sequence that encodes a polypeptide described in this section. For example, further embodiments include a nucleotide sequence that encodes a polypeptide of **any of Tables 11A-15B.** For instance, the nucleotide sequence can comprise a CAR construct or scFv of **Table 11A or 11B**. The nucleotide may encode a VH of **Table 14A or 14B**, a VL of **Table 15A or 15B**, or both. The nucleotide may encode one or more of (e.g., two or three of) a VH CDR1, VH CDR2, or VH CDR3 of **Table 12A or 12B** and/or the

nucleotide may encode one or more of (e.g., two or three of) a VL CDR1, VL CDR2, or VL CDR3 of **Table 13**. The nucleotide sequence can also include one or more of, e.g., all of the domains of SEQ ID NOS: 13, 14, 15, 16, 17, and 51.

In another aspect, provided herein is a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD20 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD20 CAR. In one embodiment, the population of CAR-expressing cells includes, e.g., a first cell expressing a CAR (e.g., a CD19 CAR, a ROR1 CAR, a CD20 CAR, or a CD22 CAR) that includes a primary intracellular signaling domain, and a second cell expressing a CAR (e.g., a CD19 CAR, a ROR1 CAR, a CD20 CAR, or a CD22 CAR)) that includes a secondary signaling domain.

The CD20 CAR may also comprise one or more of a a transmembrane domain, e.g., a transmembrane domain as described herein, an intracellular signaling domain, e.g., intracellular signaling domain as described herein, a costimulatory domain, e.g., a costimulatory domain as described herein, a leader sequence, e.g. a leader sequence as described herein, or a hinge, e.g., a hinge as described herein.

Exemplary anti-CD20 antibodies include but are not limited to rituximab, ofatumumab, ocrelizumab, veltuzumab, obinutuzumab, TRU-015 (Trubion Pharmaceuticals), ocaratuzumab, and Pro131921 (Genentech). See, e.g., Lim et al. Haematologica. 95.1(2010):135-43.

In some embodiments, the anti-CD20 antibody comprises rituximab. Rituximab is a chimeric mouse/human monoclonal antibody IgG1 kappa that binds to CD20 and causes cytolysis of a CD20 expressing cell, e.g., as described in www.accessdata.fda.gov/drugsatfda_docs/label/2010/103705s5311lbl.pdf. In some embodiments, rituximab can be used to treat B-cell malignancies, such as non-Hodgkin lymphoma (NHL) (e.g., follicular NHL, diffuse large B-cell lymphoma) and chronic lymphocytic leukemia (CLL). In other embodiments, rituximab can be used to treat autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, chronic inflammatory demyelinating polyneuropathy, autoimmune anemia, autoimmune hemolytic anemia, pure red cell aplasia, idiopathic thrombocytopenic purpura (ITP), Evans syndrome, vasculitis, bullous skin disorders, type 1 diabetes mellitus, Sjogren's

syndrome, anti-NMDA receptor encephalitis and Devic's disease, Graves' ophthalmopathy, and autoimmune pancreatitis. In some embodiments, rituximab can be used to treat transplant rejection.

In some embodiments, rituximab is administered intravenously, e.g., as an intravenous infusion. For example, each infusion provides about 500-2000 mg (e.g., about 500-550, 550-600, 600-650, 650-700, 700-750, 750-800, 800-850, 850-900, 900-950, 950-1000, 1000-1100, 1100-1200, 1200-1300, 1300-1400, 1400-1500, 1500-1600, 1600-1700, 1700-1800, 1800-1900, or 1900-2000 mg) of rituximab.

In some embodiments, rituximab is administered at a dose of 150 mg/m^2 to 750 mg/m^2, e.g., about 150-175 mg/m^2, 175-200 mg/m^2, 200-225 mg/m^2, 225-250 mg/m^2, 250-300 mg/m^2, 300-325 mg/m^2, 325-350 mg/m^2, 350-375 mg/m^2, 375-400 mg/m^2, 400-425 mg/m^2, 425-450 mg/m^2, 450-475 mg/m^2, 475-500 mg/m^2, 500-525 mg/m^2, 525-550 mg/m^2, 550-575 mg/m^2, 575-600 mg/m^2, 600-625 mg/m^2, 625-650 mg/m^2, 650-675 mg/m^2, or 675-700 mg/m^2, where m^2 indicates the body surface area of the subject.

In some embodiments, rituximab is administered at a dosing interval of at least 4 days, e.g., 4, 7, 14, 21, 28, 35 days, or more. For example, rituximab is administered at a dosing interval of at least 0.5 weeks, e.g., 0.5, 1, 2, 3, 4, 5, 6, 7, 8 weeks, or more.

In some embodiments, rituximab is administered at a dose and dosing interval described herein for a period of time, e.g., at least 2 weeks, e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 weeks, or greater. For example, rituximab is administered at a dose and dosing interval described herein for a total of at least 4 doses per treatment cycle (e.g., at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, or more doses per treatment cycle).

In some aspects, the anti-CD20 antibody comprises ofatumumab. Ofatumumab is an anti-CD20 IgG1κ human monoclonal antibody with a molecular weight of approximately 149 kDa. For example, ofatumumab is generated using transgenic mouse and hybridoma technology and is expressed and purified from a recombinant murine cell line (NS0). See, e.g., www.accessdata.fda.gov/drugsatfda_docs/label/2009/125326lbl.pdf; and Clinical Trial Identifier number NCT01363128, NCT01515176, NCT01626352, and NCT01397591.

Ofatumumab can be used to treat diseases such as CLL, non-Hodgkin lymphoma (NHL) (e.g., follicular NHL and DLBCL), B-Cell Prolymphocytic Leukemia, Acute Lymphoblastic

Leukemia (ALL), mantle cell lymphoma, rheumatoid arthritis, and multiple sclerosis.

In some embodiments, ofatumumab is administered as an intravenous infusion. For example, each infusion provides about 150-3000 mg (e.g., about 150-200, 200-250, 250-300, 300-350, 350-400, 400-450, 450-500, 500-550, 550-600, 600-650, 650-700, 700-750, 750-800, 800-850, 850-900, 900-950, 950-1000, 1000-1200, 1200-1400, 1400-1600, 1600-1800, 1800-2000, 2000-2200, 2200-2400, 2400-2600, 2600-2800, or 2800-3000 mg) of ofatumumab.

In some embodiments, ofatumumab is administered at a dosing interval of at least 4 days, e.g., 4, 7, 14, 21, 28, 35 days, or more. For example, ofatumumab is administered at a dosing interval of at least 1 week, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 26, 28, 20, 22, 24, 26, 28, 30 weeks, or more.

In some embodiments, ofatumumab is administered at a dose and dosing interval described herein for a period of time, e.g., at least 1 week, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 40, 50, 60 weeks or greater, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months or greater, or 1, 2, 3, 4, 5 years or greater. For example, ofatumumab is administered at a dose and dosing interval described herein for a total of at least 2 doses per treatment cycle (e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, or more doses per treatment cycle).

In some aspects, the anti-CD20 antibody comprises ocrelizumab. Ocrelizumab is a humanized anti-CD20 monoclonal antibody, e.g., as described in Clinical Trials Identifier Nos. NCT00077870, NCT01412333, NCT00779220, NCT00673920, NCT01194570, and Kappos et al. Lancet. 19.378(2011):1779-87. For example, ocrelizumab can be used to treat diseases such as rheumatoid arthritis, multiple sclerosis, and lupus.

In some embodiments, ocrelizumab is administered as an intravenous infusion. For example, each infusion provides about 50-2000 mg (e.g., about 50-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400-450, 450-500, 500-550, 550-600, 600-650, 650-700, 700-750, 750-800, 800-850, 850-900, 900-950, 950-1000, 1000-1100, 1100-1200, 1200-1300, 1300-1400, 1400-1500, 1500-1600, 1600-1700, 1700-1800, 1800-1900, or 1900-2000 mg) of ocrelizumab.

In some embodiments, ocrelizumab is administered at a dosing interval of at least 7 days, e.g., 7, 14, 21, 28, 35 days, or more. For example, ocrelizumab is administered at a dosing

interval of at least 1 week, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 26, 28, 20, 22, 24, 26, 28, 30 weeks, or more.

In some embodiments, ocrelizumab is administered at a dose and dosing interval described herein for a period of time, e.g., at least 2 weeks, e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 40, 50, 60 weeks or greater, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months or greater, or 1, 2, 3, 4, 5 years or greater. For example, ocrelizumab is administered at a dose and dosing interval described herein for a total of at least 4 doses per treatment cycle (e.g., at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, or more doses per treatment cycle).

In some aspects, the anti-CD20 antibody comprises veltuzumab. Veltuzumab is a humanized monoclonal antibody against CD20. See, e.g., Clinical Trial Identifier No. NCT00547066, NCT00546793, NCT01101581, and Goldenberg et al. Leuk Lymphoma. 51(5)(2010):747-55. For example, veltuzumab can be used to treat NHL (e.g., DLBCL, follicular lymphoma), CLL, and autoimmune diseases such as Immune Thrombocytopenic Purpura (ITP).

In some embodiments, veltuzumab is administered subcutaneously or intravenously, e.g., as an intravenous infusion. In some embodiments, veltuzumab is administered at a dose of 50-800 mg/m^2, e.g., about 50-60, 60-70, 70-80, 80-90, 90-100, 100-110, 110-120, 120-130, 130-140, 140-150, 150-160, 160-170, 170-180, 180-190, 190-200, 200-225, 225-250, 250-275, 275-300, 300-325, 325-350, 350-375, 375-400, 400-425, 425-450, 450-475, 475-500, 500-525, 525-550, 550-575, 575-600, 600-625, 625-650, 650-675, 675-700, 700-725, 725-750, 750-775, or 775-800 mg/m^2. In some embodiments, a dose of 50-400 mg, e.g., 50, 75, 100, 125, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, or 400 mg of veltuzumab is administered.

In some embodiments, veltuzumab is administered at a dosing interval of at least 7 days, e.g., 7, 14, 21, 28, 35 days, or more. For example, veltuzumab is administered at a dosing interval of at least 1 week, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 26, 28, 20, 22, 24, 26, 28, 30 weeks, or more.

In some embodiments, veltuzumab is administered at a dose and dosing interval described herein for a period of time, e.g., at least 2 weeks, e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 40, 50, 60 weeks or greater, or 1, 2, 3, 4,

5, 6, 7, 8, 9, 10, 11, 12 months or greater, or 1, 2, 3, 4, 5 years or greater. For example, veltuzumab is administered at a dose and dosing interval described herein for a total of at least 4 doses per treatment cycle (e.g., at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, or more doses per treatment cycle).

In some aspects, the anti-CD20 antibody comprises GA101. GA101 (also called obinutuzumab or RO5072759) is a humanized and glyco-engineered anti-CD20 monoclonal antibody. For example, GA101 can be used to treat diseases such as B-cell lymphoid malignancies, e.g., CLL, non-Hodgkin lymphoma (NHL) and diffuse large B-cell lymphoma (DLBCL). See, e.g., Robak. Curr. Opin. Investig. Drugs. 10.6(2009):588-96; Clinical Trial Identifier Numbers: NCT01995669, NCT01889797, NCT02229422, and NCT01414205; and www.accessdata.fda.gov/drugsatfda_docs/label/2013/125486s000lbl.pdf.

In some embodiments, GA101 is administered intravenously, e.g., as an intravenous infusion. For example, each infusion provides about 100-3000 mg (e.g., about 100-150, 150-200, 200-250, 250-500, 300-350, 350-400, 400-450, 450-500, 500-550, 550-600, 600-650, 650-700, 700-750, 750-800, 800-850, 850-900, 900-950, 950-1000, 1000-1200, 1200-1400, 1400-1600, 1600-1800, 1800-2000, 2000-2200, 2200-2400, 2400-2600, 2600-2800, or 2800-3000 mg) of GA101.

In some embodiments, GA101 is administered at a dosing interval of at least 7 days, e.g., 7, 14, 21, 28, 35 days, or more. For example, GA101 is administered at a dosing interval of at least 1 week, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 26, 28, 20, 22, 24, 26, 28, 30 weeks, or more. For example, GA101 is administered at a dosing interval of at least 1 month, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more months. In some embodiments, GA101 is administered at a dosing interval of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days.

In some embodiments, GA101 is administered at a dose and dosing interval described herein for a period of time, e.g., at least 1 week, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 40, 50, 60 weeks or greater, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months or greater, or 1, 2, 3, 4, 5 years or greater. For example, GA101 is administered at a dose and dosing interval described herein for a total of at least 2 doses per treatment cycle (e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, or more doses per treatment cycle).

In some aspects, the anti-CD20 antibody comprises AME-133v. AME-133v (also called LY2469298 or ocaratuzumab) is a humanized IgG1 monoclonal antibody against CD20 with increased affinity for the FcγRIIIa receptor and an enhanced antibody dependent cellular cytotoxicity (ADCC) activity compared with rituximab. See, e.g., Robak et al. BioDrugs 25.1(2011):13-25. In some embodiments, AME-133v can be used to treat cancers such as NHL, e.g., follicular lymphoma. See, e.g., Forero-Torres et al. Clin Cancer Res. 18.5(2012):1395-403.

In some aspects, the anti-CD20 antibody comprises PRO131921. PRO131921 is a humanized anti-CD20 monoclonal antibody engineered to have better binding to FcγRIIIa and enhanced ADCC compared with rituximab. See, e.g., Robak et al. BioDrugs 25.1(2011):13-25; and Casulo et al. Clin Immunol. 154.1(2014):37-46. In some embodiments, PRO131921 can be used to treat NHL. See, e.g., Clinical Trial Identifier No. NCT00452127. In some embodiments, PRO131921 is administered intravenously, e.g., as an intravenous infusion. In some embodiments, PRO131921 is administered at a dose of 15 mg/m^2 to 1000 mg/m^2, e.g., about 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, 95-100, 100-125, 125-150, 150-175, 175-200, 200-226, 225-250, 250-300, 300-325, 325-350, 350-375, 375-400, 400-425, 425-450, 450-475, 475-500, 500-525, 525-550, 550-575, 575-600, 600-625, 625-650, 650-675, 675-700, 700-725, 725-750, 750-775, 775-800, 800-825, 825-850, 850-875, 875-900, 900-925, 925-950, 950-975, or 975-1000 mg/m^2, where m^2 indicates the body surface area of the subject.

In some aspects, the anti-CD20 antibody comprises TRU-015. TRU-015 is an anti-CD20 fusion protein derived from domains of an antibody against CD20. TRU-015 is smaller than monoclonal antibodies, but retains Fc-mediated effector functions. See, e.g., Robak et al. BioDrugs 25.1(2011):13-25. TRU-015 contains an anti-CD20 single-chain variable fragment (scFv) linked to human IgG1 hinge, CH2, and CH3 domains but lacks CH1 and CL domains. In some embodiments, TRU-015 can be used to treat B-cell lymphomas and rheumatoid arthritis. In some cases, TRU-015 is administered intravenously, e.g., as an intravenous infusion. In some embodiments, TRU-015 is administered at a dose of 0.01-30 mg/kg, e.g., 0.01-0.015, 0.015-0.05, 0.05-0.15, 0.15-0.5, 0.5-1, 1-1.5, 1.5-2.5, 2.5-5, 5-10, 10-15, 15-20, 20-25, or 25-30 mg/kg body weight. In some embodiments, TRU-015 is administered at a dosing interval of at least 1 day, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 days apart. See, e.g., Burge et al. Clin Ther. 30.10(2008):1806-16.

In some embodiments, an anti-CD20 antibody described herein is conjugated or otherwise bound to a therapeutic agent, e.g., a chemotherapeutic agent (e.g., cytoxan, fludarabine, histone deacetylase inhibitor, demethylating agent, peptide vaccine, anti-tumor antibiotic, tyrosine kinase inhibitor, alkylating agent, anti-microtubule or anti-mitotic agent, CD20 antibody, or CD20 antibody drug conjugate described herein), anti-allergic agent, anti-nausea agent (or anti-emetic), pain reliever, or cytoprotective agent described herein.

CD22 Inhibitors and Binding Domains

As used herein, the term "CD22," refers to an antigenic determinant known to be detectable on leukemia precursor cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequences of isoforms 1-5 human CD22 can be found at Accession Nos. NP 001762.2, NP 001172028.1, NP 001172029.1, NP 001172030.1, and NP 001265346.1, respectively, and the nucleotide sequence encoding variants 1-5 of the human CD22 can be found at Accession No. NM 001771.3, NM 001185099.1, NM 001185100.1, NM 001185101.1, and NM 001278417.1, respectively. As used herein, "CD22" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type CD22. In one aspect the antigen-binding portion of the CAR recognizes and binds an antigen within the extracellular domain of the CD22 protein. In one aspect, the CD22 protein is expressed on a cancer cell.

In some aspects, the present disclosure provides a CD22 inhibitor or binding domain, e.g., a CD22 inhibitor or binding domain as described herein. The disclosure also provides a nucleic acid encoding the CD22 binding domain, or a CAR comprising the CD22 binding domain. A CD22 inhibitor includes but is not limited to a CD22 CAR-expressing cell, e.g., a CD22 CART cell or an anti-CD22 antibody (e.g., an anti-CD22 mono- or bispecific antibody) or a fragment thereof. The composition may also comprise a second agent, e.g., an anti-CD19 CAR-expressing cell or a CD19 binding domain. The agents may be, e.g., encoded by a single nucleic acid or different nucleic acids.

In some aspects, a CD22 inhibitor or binding domain is administered as a monotherapy. In some aspects, the CD22 inhibitor or binding domain is administered in combination with a

second agent such as an anti-CD19 CAR-expressing cell. In an embodiment, the CD22 inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

CD22 CAR-expressing cells, e.g., CARTs

In one embodiment, the CD22 inhibitor is a CD22 CAR-expressing cell, e.g., a CD22-CAR that comprises a CD22 binding domain and is engineered into a cell (e.g., T cell or NK cell) for administration in combination with CD19 CAR-expressing cell, e.g., CART, and methods of their use for adoptive therapy.

In another aspect, the present invention provides a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD22 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD22 CAR. In one embodiment, the population of CAR-expressing cells includes, e.g., a first cell expressing a CAR (e.g., a CD19 CAR or CD22 CAR) that includes a primary intracellular signaling domain, and a second cell expressing a CAR (e.g., a CD19 CAR or CD22 CAR) that includes a secondary signaling domain.

In one aspect, the CD22-CAR comprises an optional leader sequence (e.g., an optional leader sequence described herein), an extracellular antigen binding domain, a hinge (e.g., hinge described herein), a transmembrane domain (e.g., transmembrane domain described herein), and an intracellular stimulatory domain (e.g., intracellular stimulatory domain described herein). In one aspect an exemplary CD22 CAR construct comprises an optional leader sequence (e.g., a leader sequence described herein), an extracellular antigen binding domain, a hinge, a transmembrane domain, an intracellular costimulatory domain (e.g., an intracellular costimulatory domain described herein) and an intracellular stimulatory domain.

In one aspect, the CAR22 binding domain comprises the scFv portion of an amino acid sequence (or encoded by a nucleotide sequence) provided in any of SEQ ID NOs: 200-428. In one aspect, the CAR22 binding domain comprises the scFv portion provided in any of SEQ ID NOs: 203, 209, 215, 221, 227, 232, 238, 244, 250, 256, 262, 268, 274, 280, 286, 292, 298, 304, 310, 316, 322, 328, 334, 340, 346, 352, 358, 364, 370, 376, 382, 388, 394, 400, 406, 412, 418, or 423.

In specific aspects, a CAR construct of the invention comprises a scFv domain selected from the group consisting of SEQ ID NOS: 203, 209, 215, 221, 227, 232, 238, 244, 250, 256, 262, 268, 274, 280, 286, 292, 298, 304, 310, 316, 322, 328, 334, 340, 346, 352, 358, 364, 370, 376, 382, 388, 394, 400, 406, 412, 418, or 423, wherein the scFv may be preceded by an optional leader sequence such as provided in SEQ ID NO: 13, and followed by an optional hinge sequence such as provided in SEQ ID NO:14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49, a transmembrane region such as provided in SEQ ID NO:15, an intracellular signalling domain that includes SEQ ID NO:16 or SEQ ID NO:51 and a CD3 zeta sequence that includes SEQ ID NO:17 or SEQ ID NO:43, e.g., wherein the domains are contiguous with and in the same reading frame to form a single fusion protein. In some embodiments, the scFv domain is a human scFv domain selected from the group consisting of SEQ ID NOS: 203, 209, 215, 221, 227, 232, 238, 244, 250, 256, 262, 268, 274, 280, 286, 292, 298, 304, 310, 316, 322, 328, 334, 340, 346, 352, 358, 364, 370, 376, 382, 388, 394, 400, 406, 412, 418, or 423.

Also included in the invention is a nucleotide sequence that encodes the polypeptide of each of the scFv fragments selected from the group consisting of SEQ ID NO: 203, 209, 215, 221, 227, 232, 238, 244, 250, 256, 262, 268, 274, 280, 286, 292, 298, 304, 310, 316, 322, 328, 334, 340, 346, 352, 358, 364, 370, 376, 382, 388, 394, 400, 406, 412, 418, or 423. Also included in the invention is a nucleotide sequence that encodes the polypeptide of each of the scFv fragments selected from the group consisting of SEQ ID NO: 203, 209, 215, 221, 227, 232, 238, 244, 250, 256, 262, 268, 274, 280, 286, 292, 298, 304, 310, 316, 322, 328, 334, 340, 346, 352, 358, 364, 370, 376, 382, 388, 394, 400, 406, 412, 418, or 423, and each of the domains of SEQ ID NOS: 13-17, plus the encoded CD22 CAR of the invention.

In some embodiments, full-length CD22 CAR sequences are also provided herein as SEQ ID NOS: 207, 213, 219, 225, 230, 236, 242, 248, 254, 260, 266, 272, 278, 284, 290, 296, 302, 308, 314, 320, 326, 332, 338, 344, 350, 356, 362, 368, 374, 380, 386, 392, 398, 404, 410, 416, 422, or 427, as shown in **Table 6A or 6B**.

In one aspect, the present invention encompasses a recombinant nucleic acid construct comprising a nucleic acid molecule encoding a CAR, wherein the nucleic acid molecule comprises the nucleic acid sequence encoding a CD22 binding domain, e.g., described herein, e.g., that is contiguous with and in the same reading frame as a nucleic acid sequence encoding an intracellular signaling domain. In one aspect, a CD22 binding domain is selected from one or

more of SEQ ID NOS: 203, 209, 215, 221, 227, 232, 238, 244, 250, 256, 262, 268, 274, 280, 286, 292, 298, 304, 310, 316, 322, 328, 334, 340, 346, 352, 358, 364, 370, 376, 382, 388, 394, 400, 406, 412, 418, or 423. In one aspect, the present invention encompasses a recombinant nucleic acid construct comprising a nucleic acid molecule encoding a CAR, wherein the nucleic acid molecule comprises a nucleic acid sequence encoding a CD22 binding domain, e.g., wherein the sequence is contiguous with and in the same reading frame as the nucleic acid sequence encoding an intracellular signaling domain. An exemplary intracellular signaling domain that can be used in the CAR includes, but is not limited to, one or more intracellular signaling domains of, e.g., CD3-zeta, CD28, 4-1BB, and the like. In some instances, the CAR can comprise any combination of CD3-zeta, CD28, 4-1BB, and the like.

In one aspect, the nucleic acid sequence of a CAR construct of the invention comprises the CAR construct of one or more of SEQ ID NOS: 200, 208, 214, 220, 226, 231, 237, 243, 249, 255, 261, 267, 273, 279, 285, 291, 297, 303, 309, 315, 321, 327, 333, 339, 345, 351, 357, 363, 369, 375, 381, 387, 393, 399, 405, 411, 417, 422, or 428. In one aspect, the nucleic acid sequence of a CAR construct of the invention comprises an scFv-encoding sequence of one or more of SEQ ID NOs: 204, 210, 216, 222, 116, 233, 239, 245, 251, 257, 263, 269, 275, 281, 287, 293, 299, 305, 311, 317, 323, 329, 335, 341, 347, 353, 359, 365, 371, 377, 383, 389, 395, 401, 407, 413, 117, or 424.

In some instances, it is beneficial for the antigen binding domain to be derived from the same species in which the CAR will ultimately be used in. For example, for use in humans, it may be beneficial for the antigen binding domain of the CAR to comprise human or humanized residues for the antigen binding domain of an antibody or antibody fragment. Thus, in one aspect, the antigen binding domain comprises a human antibody or an antibody fragment. In one embodiment, the human CD22 binding domain comprises one or more (e.g., all three) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of a human CD22 binding domain described herein, and/or one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a human CD22 binding domain described herein, e.g., a human CD22 binding domain comprising one or more, e.g., all three, LC CDRs and one or more, e.g., all three, HC CDRs. In one

embodiment, the human CD22 binding domain comprises one or more (e.g., all three) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of a human CD22 binding domain described herein, e.g., the human CD22 binding domain has two variable heavy chain regions, each comprising a HC CDR1, a HC CDR2 and a HC CDR3 described herein. In one embodiment, the human CD22 binding domain comprises a human light chain variable region described herein (e.g., in **Table 6A, 6B, 10A or 10B**) and/or a human heavy chain variable region described herein (e.g., in **Table 6A, 6B, 9A or 9B**). In one embodiment, the human CD22 binding domain comprises a human heavy chain variable region described herein (e.g., in **Table 6A, 6B, 9A or 9B**), e.g., at least two human heavy chain variable regions described herein (e.g., in **Table 6A, 8B, 9A or 9B**). In one embodiment, the CD22 binding domain is a scFv comprising a light chain and a heavy chain of an amino acid sequence of **Table 6A, 6B, 9A, 9B, 10A, or 10B**. In an embodiment, the CD22 binding domain (e.g., an scFv) comprises: a light chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a light chain variable region provided in **Table 6A, 6B, 10A, or 10B**, or a sequence with 95-99% identity with an amino acid sequence of **Table 6A, 6B, 10A, or 10B**; and/or a heavy chain variable region comprising an amino acid sequence having at least one, two or three modifications (e.g., substitutions) but not more than 30, 20 or 10 modifications (e.g., substitutions) of an amino acid sequence of a heavy chain variable region provided in **Table 6A, 6B, 9A, or 9B**, or a sequence with 95-99% identity to an amino acid sequence of **Table 6A, 6B, 9A or 9B**. In one embodiment, the human CD22 binding domain comprises a sequence selected from a group consisting of SEQ ID NO: 203, 209, 215, 221, 227, 232, 238, 244, 250, 256, 262, 268, 274, 280, 286, 292, 298, 304, 310, 316, 322, 328, 334, 340, 346, 352, 358, 364, 370, 376, 382, 388, 394, 400, 406, 412, 418, or 423, or a sequence with 95-99% identity thereof. In one embodiment, the human CD22 binding domain is a scFv, and a light chain variable region comprising an amino acid sequence described herein, e.g., in **Table 6A, 6B, 10A, or 10B**, is attached to a heavy chain variable region comprising an amino acid sequence described herein, e.g., in **Table 6A, 6B, 9A, or 9B**, via a linker, e.g., a linker described herein. In one embodiment, the human CD22 binding domain includes a $(Gly_4$-Ser)n linker, wherein n is 1, 2, 3, 4, 5, or 6, e.g., 3 or 4 (SEQ ID NO:53). The light chain

variable region and heavy chain variable region of a scFv can be, e.g., in any of the following orientations: light chain variable region-linker-heavy chain variable region or heavy chain variable region-linker-light chain variable region.

In one aspect, the CD22 binding domain is characterized by particular functional features or properties of an antibody or antibody fragment. For example, in one aspect, the portion of a CAR composition of the invention that comprises an antigen binding domain specifically binds human CD22 or a fragment thereof. In one aspect, the invention relates to an antigen binding domain comprising an antibody or antibody fragment, wherein the antibody binding domain specifically binds to a CD22 protein or fragment thereof, wherein the antibody or antibody fragment comprises a variable heavy chain that includes an amino acid sequence of any of SEQ ID NO:s 700, 701, 702, 703, 704, 705, 706, 707, 708, 709, 710, 711, 712, 713, 714, 715, 716, 717, 718, 719, 720, 721, 722, 723, 724, 725, 726, 727, 728, 729, 730, 731, 732, 733, 734, 735, 736, 737, or 738, and/or a variable light chain that includes an amino acid sequence of any of SEQ ID NOs 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 749, 750, 751, 752, 753, 754, 755, 756, 757, 758, 759, 760, 761, 762, 763, 764, 765, 766, 767, 768, 769, 770, 771, 772, 773, 774, 775, 776, or 777. In certain aspects, the scFv is contiguous with and in the same reading frame as a leader sequence. In one aspect the leader sequence is the polypeptide sequence provided as SEQ ID NO:13.

In embodiments, the CAR comprises an antibody or antibody fragment which includes a CD22 binding domain, a transmembrane domain, and an intracellular signaling domain. In embodiments, the CD22 binding domain comprises one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD22 light chain binding domain amino acid sequence listed in **Table 8A, 8B, 10A or 10B**, and one or more of heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD22 heavy chain binding domain amino acid sequence listed in **Table 7A, 7B, 7C, 9A, or 9B**.

In one aspect, the CD22 binding domain is a fragment, e.g., a single chain variable fragment (scFv). In one aspect, the CD22 binding domain is a Fv, a Fab, a (Fab')2, or a bi-functional (e.g. bi-specific) hybrid antibody (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105

(1987)). In one aspect, the antibodies and fragments thereof of the invention binds a CD22 protein or a fragment thereof with wild-type or enhanced affinity.

In some instances, a human scFv can be derived from a display library.

In one embodiment, the CD22 binding domain, e.g., scFv comprises at least one mutation such that the mutated scFv confers improved stability to the CART22 construct. In another embodiment, the CD22 binding domain, e.g., scFv comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mutations arising, e.g., from the humanization process such that the mutated scFv confers improved stability to the CART22 construct.

In one aspect, the present invention contemplates modifications of the starting antibody or fragment (e.g., scFv) amino acid sequence that generate functionally equivalent molecules. For example, the VH or VL of a CD22 binding domain, e.g., scFv, comprised in the CAR can be modified to retain at least about 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%,81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity of the starting VH or VL framework region of the CD22 binding domain, e.g., scFv. The present invention contemplates modifications of the entire CAR construct, e.g., modifications in one or more amino acid sequences of the various domains of the CAR construct in order to generate functionally equivalent molecules. The CAR construct can be modified to retain at least about 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity of the starting CAR construct.

In an embodiment, the CD22 binding domain comprises six CDRs (e.g., one each of a HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, and LC CDR3) of any one of CAR22-1, CAR22-2, CAR22-3, CAR22-4, CAR22-5, CAR22-6, CAR22-7, CAR22-8, CAR22-9, CAR22-10, CAR22-11, CAR22-12, CAR22-13, CAR22-14, CAR22-15, or CAR22-16, CAR22-17, CAR22-18, CAR22-19, CAR22-20, CAR22-21, CAR22-22, CAR22-23, CAR22-24, CAR22-25, CAR22-26, CAR22-27, CAR22-28, CAR22-29, CAR22-30, CAR22-31, CAR22-32, CAR22-33, CAR22-34, CAR22-35, CAR22-36, CAR22-37, or CAR22-38 (e.g., as described in **Table 7A, 7B, 7C, 8A and/or 8B**), or a sequence substantially identical thereto. In an embodiment, the CD22 binding domain comprises three CDRs (e.g., one each of a HC CDR1, HC CDR2, and HC CDR3, or one each of a LC CDR1, LC CDR2, and LC CDR3) of

any one of CAR22-1, CAR22-2, CAR22-3, CAR22-4, CAR22-5, CAR22-6, CAR22-7, CAR22-8, CAR22-9, CAR22-10, CAR22-11, CAR22-12, CAR22-13, CAR22-14, CAR22-15, or CAR22-16, CAR22-17, CAR22-18, CAR22-19, CAR22-20, CAR22-21, CAR22-22, CAR22-23, CAR22-24, CAR22-25, CAR22-26, CAR22-27, CAR22-28, CAR22-29, CAR22-30, CAR22-31, CAR22-32, CAR22-33, CAR22-34, CAR22-35, CAR22-36, CAR22-37, or CAR22-38 (e.g., as described in **Table 7A, 7B, 7C, 8A and/or 8B**), or a sequence substantially identical thereto.

Further embodiments include a nucleotide sequence that encodes a polypeptide described in this section. For example, further embodiments include a nucleotide sequence that encodes a polypeptide of **any of Tables 6A-10B.** For instance, the nucleotide sequence can comprise a CAR construct or scFv of **Table 6A or 6B**. The nucleotide may encode a VH of **Table 9A or 9B**, a VL or **Table 10A or 10B**, or both. The nucleotide may encode one or more of (e.g., two or three of) a VH CDR1, VH CDR2, or VH CDR3 of **Table 7A, 7B, or 7C** and/or the nucleotide may encode one or more of (e.g., two or three of) a VL CDR1, VL CDR2, or VL CDR3 of **Table 8A or 8B**. The nucleotide sequence can also include one or more of, e.g., all of the domains of SEQ ID NOS: 13, 14, 15, 16, 17, and 51.

The CD22 CAR may also comprise one or more of a a transmembrane domain, e.g., a transmembrane domain as described herein, an intracellular signaling domain, e.g., intracellular signaling domain as described herein, a costimulatory domain, e.g., a costimulatory domain as described herein, a leader sequence, e.g. a leader sequence as described herein, or a hinge, e.g., a hinge as described herein.

In one embodiment, the CD22 inhibitor is a CD22 inhibitor described herein. The CD22 inhibitor can be, e.g., an anti-CD22 antibody (e.g., an anti-CD22 mono- or bispecific antibody), a small molecule, or a CD22 CART. In some embodiments the anti-CD22 antibody is conjugated or otherwise bound to a therapeutic agent. Exemplary therapeutic agents include, e.g., microtubule disrupting agents (*e.g.*, monomethyl auristatin E) and toxins (*e.g.*, diphtheria toxin or Pseudomonas exotoxin-A, ricin). In an embodiment, the CD22 inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

In one embodiment, the anti-CD22 antibody is selected from an anti-CD19/CD22 bispecific ligand-directed toxin (e.g., two scFv ligands, recognizing human CD19 and CD22, linked to the first 389 amino acids of diphtheria toxin (DT), DT 390, e.g., DT2219ARL); anti-CD22 monoclonal antibody-MMAE conjugate (e.g., DCDT2980S); scFv of an anti-CD22 antibody RFB4 fused to a fragment of Pseudomonas exotoxin-A (e.g., BL22); deglycosylated ricin A chain-conjugated anti-CD19/anti-CD22 (e.g., Combotox); humanized anti-CD22 monoclonal antibody (e.g., epratuzumab); or the Fv portion of an anti-CD22 antibody covalently fused to a 38 KDa fragment of Pseudomonas exotoxin-A (e.g., moxetumomab pasudotox).

In one embodiment, the anti-CD22 antibody is an anti-CD19/CD22 bispecific ligand-directed toxin (e.g., DT2219ARL) and the anti-CD19/CD22 bispecific ligand-directed toxin is administered at a dose of about 1 µg/kg, 2 µg/kg, 3 µg/kg, 4 µg/kg, 5 µg/kg, 6 µg/kg, 7 µg/kg, 8 µg/kg, 9 µg/kg, 10 µg/kg, 11 µg/kg, 12 µg/kg, 13 µg/kg, 14 µg/kg,15 µg/kg, 20 µg/kg, 25 µg/kg, 30 µg/kg, 40 µg/kg, 60 µg/kg, 80 µg/kg, 100 µg/kg, 120 µg/kg, 140 µg/kg, 160 µg/kg, 180 µg/kg, 200 µg/kg, 220 µg/kg, 250 µg/kg, 300 µg/kg, 350 µg/kg, 400 µg/kg, 450 µg/kg, 500 µg/kg, 600 µg/kg, 700 µg/kg, 800 µg/kg, 900 µg/kg, 1 mg.kg (e.g., 30 µg/kg, 40 µg/kg, 60 µg/kg, or 80 µg/kg) for a period of time, e.g., every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more days. In some embodiments, the anti-CD19/CD22 bispecific ligand-directed toxin is administered via intravenous infusion.

In one embodiment, the anti-CD22 antibody is BL22 and BL22 is administered at a dose of about 1 µg/kg, 2 µg/kg, 3 µg/kg, 4 µg/kg, 5 µg/kg, 6 µg/kg, 7 µg/kg, 8 µg/kg, 9 µg/kg, 10 µg/kg, 11 µg/kg, 12 µg/kg, 13 µg/kg, 14 µg/kg,15 µg/kg, 20 µg/kg, 25 µg/kg, 30 µg/kg, 40 µg/kg, 60 µg/kg, 80 µg/kg, 100 µg/kg, 120 µg/kg, 140 µg/kg, 160 µg/kg, 180 µg/kg, 200 µg/kg, 220 µg/kg, 250 µg/kg, 300 µg/kg, 350 µg/kg, 400 µg/kg, 450 µg/kg, 500 µg/kg, 600 µg/kg, 700 µg/kg, 800 µg/kg, 900 µg/kg, 1 mg.kg (e.g., 3 µg/kg, 30 µg/kg, 40 µg/kg, or 50 µg/kg) for a period of time, e.g., every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more days. In some embodiments, BL22 is administered daily, every other day, every third, day, or every fourth day for a period of time, e.g., for a 4 day cycle, a 6 day cycle, an 8 day cycle, a 10 day cycle, a 12 day cycle, or a 14 day cycle. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more cycles of BL22 are administered. In some embodiments, BL22 is administered via intravenous infusion.

In one embodiment, the anti-CD22 antibody is a deglycosylated ricin A chain-conjugated anti-CD19/anti-CD22 (e.g., Combotox) and the deglycosylated ricin A chain-conjugated anti-

CD19/anti-CD22 is administered at a dose of about 500 µg/m^2, 600 µg/m^2, 700 µg/m^2, 800 µg/m^2, 900 µg/m^2, 1 mg/m^2, 2 mg/m^2, 3 mg/m^2, 4 mg/m^2, 5 mg/m^2, 6 mg/m^2, or 7 mg/m^2 for a period of time, e.g., every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 or more days. In some embodiments, the deglycosylated ricin A chain-conjugated anti-CD19/anti-CD22 is administered daily, every other day, every third, day, or every fourth day for a period of time, e.g., for a 4 day cycle, a 6 day cycle, an 8 day cycle, a 10 day cycle, a 12 day cycle, or a 14 day cycle (e.g., every other day for 6 days). In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more cycles of the deglycosylated ricin A chain-conjugated anti-CD19/anti-CD22 are administered. In some embodiments, the deglycosylated ricin A chain-conjugated anti-CD19/anti-CD22 is administered via intravenous infusion.

In one embodiment, the anti-CD22 antibody is a humanized anti-CD22 monoclonal antibody (e.g., epratuzumab) and the humanized anti-CD22 monoclonal antibody is administered at a dose of about 10 mg/m^2/week, 20 mg/m^2/week, 50 mg/m^2/week, 100 mg/m^2/week, 120 mg/m^2/week, 140 mg/m^2/week, 160 mg/m^2/week, 180 mg/m^2/week, 200 mg/m^2/week, 220 mg/m^2/week, 250 mg/m^2/week, 260 mg/m^2/week, 270 mg/m^2/week, 280 mg/m^2/week, 290 mg/m^2/week, 300 mg/m^2/week, 305 mg/m^2/week, 310 mg/m^2/week, 320 mg/m^2/week, 325 mg/m^2/week, 330 mg/m^2/week, 335 mg/m^2/week, 340 mg/m^2/week, 345 mg/m^2/week, 350 mg/m^2/week, 355 mg/m^2/week, 360 mg/m^2/week, 365 mg/m^2/week, 370 mg/m^2/week, 375 mg/m^2/week, 380 mg/m^2/week, 385 mg/m^2/week, 390 mg/m^2/week, 400 mg/m^2/week, 410 mg/m^2/week, 420 mg/m^2/week, 430 mg/m^2/week, 440 mg/m^2/week, 450 mg/m^2/week, 460 mg/m^2/week, 470 mg/m^2/week, 480 mg/m^2/week, 490 mg/m^2/week, 500 mg/m^2/week, 600 mg/m^2/week, 700 mg/m^2/week, 800 mg/m^2/week, 900 mg/m^2/week, 1 g/m^2/week, or 2 g/m^2/week (e.g., 360 mg/m^2/week or 480 mg/m^2/week) for a period of time, e.g., every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more weeks. In some embodiments a first dose is lower than subsequent doses (e.g. a first dose of 360 mg/m^2/week followed by subsequent doses of 370 mg/m^2/week). In some embodiments, the humanized anti-CD22 monoclonal antibody is administered via intravenous infusion.

In one embodiment, the anti-CD22 antibody is moxetumomab pasudotox and moxetumomab pasudotox is administered at a dose of about 1 µg/kg, 2 µg/kg, 3 µg/kg, 4 µg/kg, 5 µg/kg, 6 µg/kg, 7 µg/kg, 8 µg/kg, 9 µg/kg, 10 µg/kg, 11 µg/kg, 12 µg/kg, 13 µg/kg, 14 µg/kg,15 µg/kg, 20 µg/kg, 25 µg/kg, 30 µg/kg, 40 µg/kg, 60 µg/kg, 80 µg/kg, 100 µg/kg, 120

μg/kg, 140 μg/kg, 160 μg/kg, 180 μg/kg, 200 μg/kg, 220 μg/kg, 250 μg/kg, 300 μg/kg, 350 μg/kg, 400 μg/kg, 450 μg/kg, 500 μg/kg (e.g., 5 μg/kg, 10 μg/kg, 20 μg/kg, 30 μg/kg, 40 μg/kg, or 50 μg/kg) a period of time, e.g., every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more days. In some embodiments, the moxetumomab pasudotox is administered daily, every other day, every third, day, or every fourth day for a period of time, e.g., for a 4 day cycle, a 6 day cycle, an 8 day cycle, a 10 day cycle, a 12 day cycle, or a 14 day cycle (e.g., every other day for 6 days). In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or more cycles of the moxetumomab pasudotox are administered. In some embodiments, the moxetumomab pasudotox is administered via intravenous infusion.

In an embodiment, a CD22 antibody molecule comprises six CDRs (e.g., one each of a HC CDR1, HC CDR2, HC CDR3, LC CDR1, LC CDR2, and LC CDR3) of any one of CAR22-1, CAR22-2, CAR22-3, CAR22-4, CAR22-5, CAR22-6, CAR22-7, CAR22-8, CAR22-9, CAR22-10, CAR22-11, CAR22-12, CAR22-13, CAR22-14, CAR22-15, or CAR22-16, CAR22-17, CAR22-18, CAR22-19, CAR22-20, CAR22-21, CAR22-22, CAR22-23, CAR22-24, CAR22-25, CAR22-26, CAR22-27, CAR22-28, CAR22-29, CAR22-30, CAR22-31, CAR22-32, CAR22-33, CAR22-34, CAR22-35, CAR22-36, CAR22-37, or CAR22-38 (e.g., as described in **Table 7A, 7B, 7C, 8A and/or 8B**), or a sequence substantially identical thereto. In an embodiment, a CD22 antibody molecule comprises three CDRs (e.g., one each of a HC CDR1, HC CDR2, and HC CDR3, or one each of a LC CDR1, LC CDR2, and LC CDR3) of any one of CAR22-1, CAR22-2, CAR22-3, CAR22-4, CAR22-5, CAR22-6, CAR22-7, CAR22-8, CAR22-9, CAR22-10, CAR22-11, CAR22-12, CAR22-13, CAR22-14, CAR22-15, or CAR22-16, CAR22-17, CAR22-18, CAR22-19, CAR22-20, CAR22-21, CAR22-22, CAR22-23, CAR22-24, CAR22-25, CAR22-26, CAR22-27, CAR22-28, CAR22-29, CAR22-30, CAR22-31, CAR22-32, CAR22-33, CAR22-34, CAR22-35, CAR22-36, CAR22-37, or CAR22-38 (e.g., as described in **Table 7A, 7B, 7C, 8A, and/or 8B**), or a sequence substantially identical thereto. In an embodiment, a CD22 antibody molecule comprises a heavy chain variable region, a light chain variable region, or both of a heavy chain variable region and light chain variable region, or an scFv, as described in **Table 6A or 6B**, or a sequence substantially identical thereto. In embodiments, the CD22 antibody molecule is an isolated antibody molecule.

ROR1 Inhibitors

As used herein, the term "ROR1" refers to an antigenic determinant known to be detectable on leukemia precursor cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequences of isoforms 1and 2 precursors of human ROR1 can be found at Accession Nos. NP_005003.2 and NP_001077061.1, respectively, and the mRNA sequences encoding them can be found at Accession Nos. NM_005012.3 and NM_001083592.1, respectively. As used herein, "ROR1" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type ROR1. In one aspect the antigen-binding portion of the CAR recognizes and binds an antigen within the extracellular domain of the ROR1 protein. In one aspect, the ROR1 protein is expressed on a cancer cell.

Also provided herein are ROR1 inhibitors and combination therapies. ROR1 inhibitors include but are not limited to anti-ROR1 CAR-expressing cells, e.g. CARTs, and anti-ROR antibodies (e.g., an anti-ROR1 mono- or bispecific antibody) and fragments thereof. In some embodiments, anti-ROR1 inhibitors can be used to treat B-cell malignancies (e.g., leukemias, such as CLL, and B-cell lymphomas, such as mantle cell lymphoma; ALL; small lymphocytic lymphoma; marginal cell B-Cell lymphoma; and Burkett's Lymphoma) or epithelial cancers (e.g., breast cancer, renal cell carcinoma, lung cancer, colorectal cancers, ovarian cancer, and melanoma). In an embodiment, the CD20 inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein, e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

An exemplary anti-ROR1 inhibitor is described in Hudecek, et al. Clin. Cancer Res. 19.12(2013):3153-64, incorporated herein by reference. For example, an anti-ROR1 inhibitor includes the anti-ROR1 CARTs described in Hudecek et al. (for example, generated as described in Hudecek et al. at page 3155, first full paragraph, incorporated herein by reference). In other examples, an anti-ROR1 inhibitor includes an antibody or fragment thereof comprising the VH and/or VL sequences of the 2A2 and R12 anti-ROR1 monoclonal antibodies described in Hudecek et al. at paragraph bridging pages 3154-55; Baskar et al. MAbs 4(2012):349–61; and Yang et al. PLoS ONE 6(2011):e21018, incorporated herein by reference.

In other embodiments, a ROR1 inhibitor includes an antibody or fragment thereof (e.g.,

single chain variable fragment (scFv)) that targets ROR1, including those described in US 2013/0101607, e.g., SEQ ID NOs: 1 or 2 of US 2013/0101607, incorporated herein by reference. In some embodiments, anti-ROR1 antibody fragments (e.g., scFvs) are conjugated or fused to a biologically active molecule, e.g., to form a chimeric antigen receptor (CAR) that directs immune cells, e.g., T cells to respond to ROR1-expressing cells.

In some embodiments, an exemplary ROR1 inhibitor includes an anti-ROR1 monoclonal antibody called UC-961 (Cirmtuzumab). See, e.g., Clinical Trial Identifier No. NCT02222688. Cirmtuzumab can be used to treat cancers, such as chronic lymphocytic leukemia (CLL), ovarian cancer, and melanoma. See, e.g., Hojjat-Farsangi et al. PLoS One. 8(4): e61167; and NCT02222688.

In some embodiments, cirmtuzumab is administered intravenously, e.g., as an intravenous infusion. For example, each infusion provides about 700-7000 μg (e.g., 700-750, 750-800, 800-850, 850-900, 900-950, 950-1000, 1000-1500, 1500-2000, 2000-2500, 2500-3000, 3000-3500, 3500-4000, 4000-4500, 4500-5000, 5000-5500, 5500-6000, 6000-6500, or 6500-7000 μg) of cirmtuzumab. In other embodiments, cirmtuzumab is administered at a dose of 10-100 μg/kg body weight, e.g., 10-15, 15-20, 20-25, 25-30, 30-35, 35-40, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90, 90-95, or 95-100 μg/kg body weight. In one embodiment, cirmtuzumab is administered at a starting dose of 15 μg/kg body weight.

In some embodiments, cirmtuzumab is administered at a dosing interval of at least 7 days, e.g., 7, 14, 21, 28, 35 days, or more. For example, cirmtuzumab is administered at a dosing interval of at least 1 week, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 24, 26, 28, 20, 22, 24, 26, 28, 30 weeks, or more.

In some embodiments, cirmtuzumab is administered at a dose and dosing interval described herein for a period of time, e.g., at least 1 week, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 24, 26, 28, 30, 40, 50, 60 weeks or greater, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 months or greater, or 1, 2, 3, 4, 5 years or greater. For example, cirmtuzumab is administered at a dose and dosing interval described herein for a total of at least 2 doses per treatment cycle (e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, or more doses per treatment cycle).

In some embodiments, the anti-ROR1 antibody is conjugated or otherwise bound to a

therapeutic agent.

In some embodiments, a ROR1 inhibitor includes an anti-ROR1 CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-ROR1 CAR construct or encoded by a ROR1 binding CAR comprising a scFv, CDRs, or VH and VL chains. For example, an anti-ROR1 CAR-expressing cell, e.g., CART is a generated by engineering a ROR1-CAR (that comprises a ROR1 binding domain) into a cell (e.g., a T cell or NK cell), e.g., for administration in combination with a CAR-expressing cell described herein. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In another aspect, provided herein is a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and ROR1 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a ROR1 CAR.

CD123 Inhibitors

CD123 is also called the alpha-chain of the interleukin-3 receptor (IL-3RA). The IL-3 receptor (IL-3R) is a heterodimer composed of alpha and beta chains. IL-3R is a membrane receptor. The IL-3Rα chain is a glycoprotein of 360 amino acid residues. Abnormalities of CD123 are frequently observed in some leukemic disorders. CD123 is overexpressed in multiple hematologic malignancies, e.g., acute myeloid and B-lymphoid leukemias, blastic plasmocytoid dendritic neoplasms (BPDCN) and hairy cell leukemia.

As used herein, the term "CD123" refers to an antigenic determinant known to be detectable on some malignant hematological cancer cells, e.g., leukemia cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequences of human CD123 can be found at Accession Nos. NP_002174.1 (isoform 1 precursor); NP_001254642.1 (isoform 2 precursor), and the mRNA sequences encoding them can be found at Accession Nos. NM_002183.3 (variant 1); NM_001267713.1 (variant 2). As used herein, "CD123" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type CD123. In one aspect the antigen-binding portion of the CAR recognizes and binds an antigen within the extracellular domain of the CD123 protein. In one

aspect, the CD123 protein is expressed on a cancer cell.

Provided herein are CD123 inhibitors and combination therapies. CD123 inhibitors include but are not limited to small molecules, recombinant proteins, anti-CD123 CAR-expressing cells, e.g. CARTs, and anti-CD123 antibodies (e.g., an anti-CD123 mono- or bispecific antibody) and fragments thereof. In some embodiments, anti-CD123 inhibitors can be used to treat a B-cell malignancy described herein. In an embodiment, the CD123 inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein, e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

In one embodiment, the CD123 inhibitor is a recombinant protein, e.g., comprising the natural ligand (or a fragment) of the CD123 receptor. For example, the recombinant protein is SL-401 (also called DT388IL3; University of Texas Southwestern Medical Center), which is a fusion protein comprising human IL-3 fused to a truncated diphtheria toxin. See, e.g., Testa et al. Biomark Res. 2014; 2: 4; and Clinical Trial Identifier No. NCT00397579.

In another embodiment, the CD123 inhibitor is an anti-CD123 antibody or fragment thereof. In one embodiment, the anti-CD123 antibody or fragment thereof comprises a monoclonal antibody, e.g., a monospecific or bispecific antibody or fragment thereof. For example, the anti-CD123 antibody or fragment thereof comprises CSL360 (CSL Limited). CSL360 is a recombinant chimeric monoclonal antibody that binds to CD123. In some embodiments, CSL360 is administered intravenously, e.g., by intravenous infusion. For example, CSL360 is administered at a dose of 0.1-10 mg/kg, e.g., 0.1-0.5 mg/kg, 0.5-1 mg/kg, 1-5 mg/kg, or 5-10 mg/kg. See, e.g., Clinical Trial Identifier No. NCT01632852; and Testa et al.

In another embodiment, the CD123 antibody or fragment thereof comprises CSL362 (CSL Limited). CSL362 is a humanized monoclonal antibody that targets the CD123 and is optimized for enhanced activation of antibody dependent cell-mediated cytotoxicity (ADCC). In some embodiments, CSL362 is administered intravenously, e.g., by intravenous infusion. In some examples, CSL362 is administered at a dose of 0.1-12 mg/kg, e.g., 0.1-0.2 mg/kg, 0.2-0.5 mg/kg, 0.5-1 mg/kg, 1-6 mg/kg, or 6-12 mg/kg. See, e.g., Clinical Trial Identifier No. NCT01632852.

In one embodiment, the CD123 antibody or fragment thereof comprises a bispecific antibody, e.g., MGD006 (MacroGenics). MGD006 is a bispecific antibody that targets CD123 and CD3. See, e.g., Clinical Trial Identifier No. NCT02152956.

In some embodiments, the CD123 inhibitor is conjugated or otherwise bound to a therapeutic agent.

In some embodiments, a CD123 inhibitor includes an anti-CD123 CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-CD123 CAR construct or encoded by a CD123 binding CAR comprising a scFv, CDRs, or VH and VL chains. For example, an anti-CD123 CAR-expressing cell, e.g., CART is a generated by engineering a CD123-CAR (that comprises a CD123 binding domain) into a cell (e.g., a T cell or NK cell), e.g., for administration in combination with a CAR-expressing cell described herein. In an embodiment, the anti-CD123 CAR construct comprises a scFv sequence, e.g., a scFv sequence provided in US 2014/0322212 A1, incorporated herein by reference. In one embodiment, the anti-CD123 binding domain is a scFv described in US 2014/0322212 A1. In an embodiment, the anti-CD123 binding domain is part of a CAR construct provided in US 2014/0322212 A1. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In another aspect, provided herein is a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD123 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD123 CAR.

CD10 Inhibitors

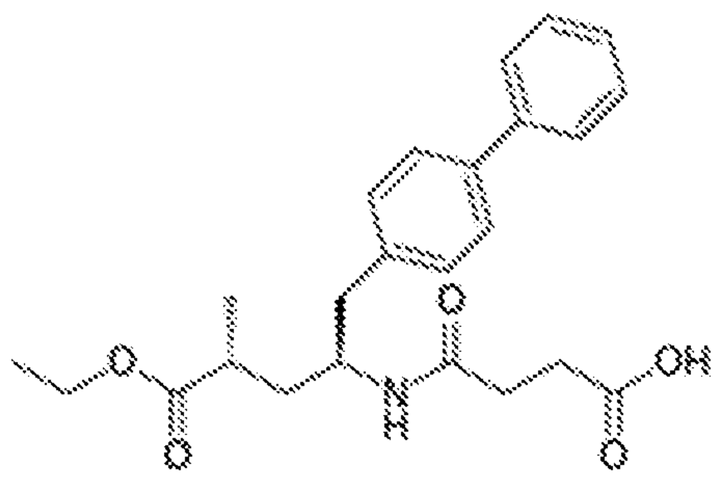
Cluster of differentiation 10 (CD10) is also called Neprilysin, membrane metallo-endopeptidase (MME), neutral endopeptidase (NEP), and common acute lymphoblastic leukemia antigen (CALLA). CD10 is an enzyme encoded by the membrane metallo-endopeptidase (MME) gene. CD10 is expressed on leukemic cells of pre-B phenotype and is a common acute lymphocytic leukemia antigen.

As used herein, the term "CD10" refers to an antigenic determinant known to be detectable on leukemia cells. The human and murine amino acid and nucleic acid sequences can

be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequences of human CD10 can be found at Accession Nos. NP_009218.2; NP_000893.2; NP_009219.2; NP_009220.2, and the mRNA sequences encoding them can be found at Accession Nos. NM_007287.2 (variant 1bis); NM_000902.3 (variant 1); NM_007288.2 (variant 2a); NM_007289.2 (variant 2b). As used herein, "CD10" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type CD10. In one aspect the antigen-binding portion of the CAR recognizes and binds an antigen within the extracellular domain of the CD10 protein. In one aspect, the CD10 protein is expressed on a cancer cell.

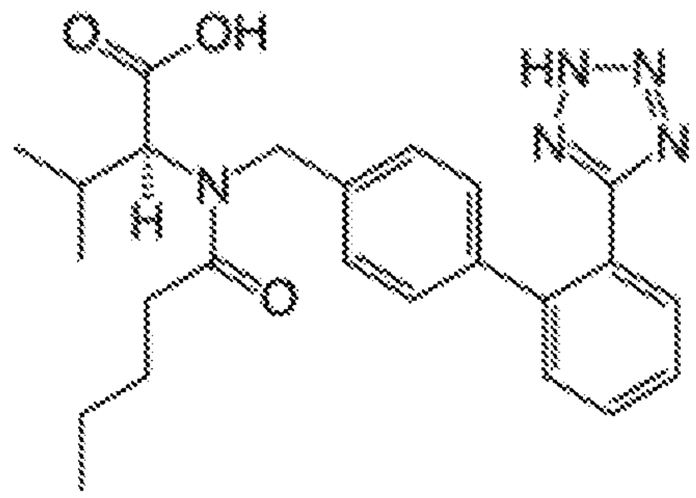
Also provided herein are CD10 inhibitors and combination therapies. CD10 inhibitors include but are not limited to small molecules, recombinant proteins, anti-CD10 CAR-expressing cells, e.g. CARTs, and anti-CD10 antibodies (e.g., an anti-CD10 mono- or bispecific antibody) and fragments thereof. In some embodiments, anti-CD10 inhibitors can be used to treat a B-cell malignancy described herein. In an embodiment, the CD10 inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein, e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

In an embodiment, the CD10 inhibitor comprises sacubitril (AHU-377; Novartis) (4-{[(2*S*,4*R*)-1-(4-Biphenylyl)-5-ethoxy-4-methyl-5-oxo-2-pentanyl]amino}-4-oxobutanoic acid), or a pharmaceutically acceptable salt or a derivative thereof. The structure of sacubitril is shown below.

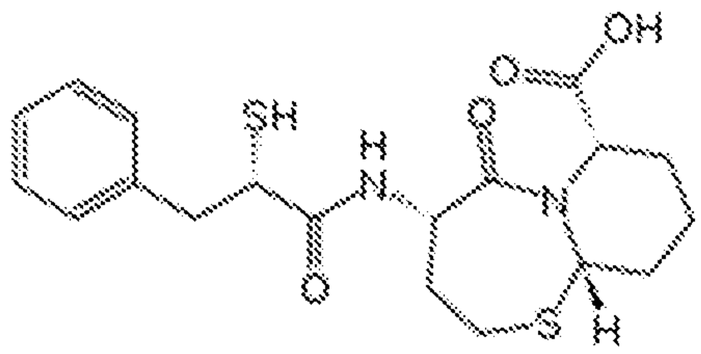


In another embodiment, the CD10 inhibitor comprises valsartan/sacubritril (LCZ696; Novartis) or a pharmaceutically acceptable salt or a derivative thereof. *Valsartan/sacubritril i*s a combination drug comprising a 1:1 mixture of valsartan and sacubitril. The structure of valsartan

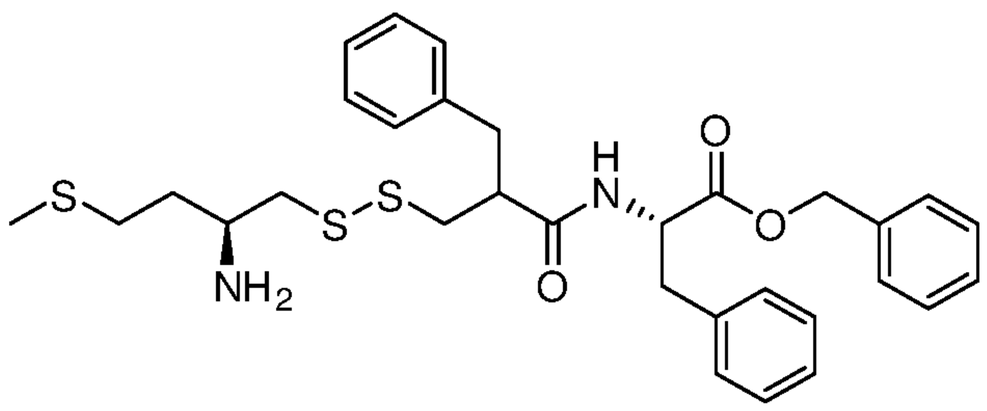
((*S*)-3-methyl-2-(*N*-{[2'-(2*H*-1,2,3,4-tetrazol-5-yl)biphenyl-4-yl]methyl}pentanamido)butanoic acid) is shown below.



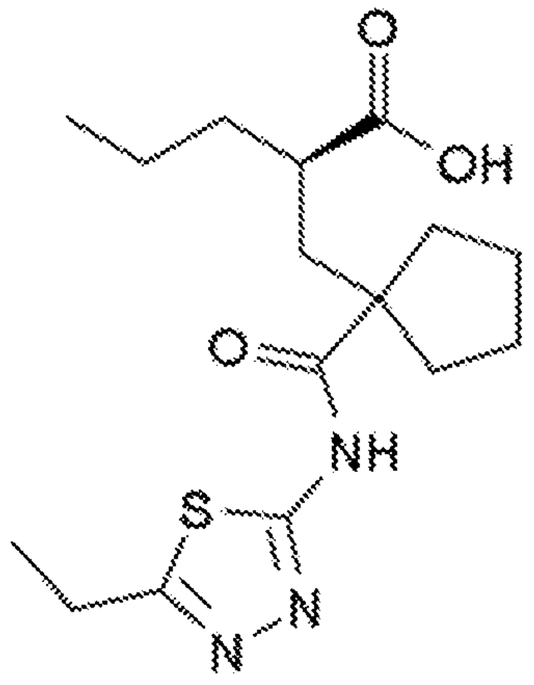
In an embodiment, the CD10 inhibitor comprises omapatrilat (Bristol-Myers Squibb) ((4*S*,7*S*,10a*S*)-5-oxo-4-{[(2*S*)-3-phenyl-2- sulfanylpropanoyl]amino}-2,3,4,7,8,9,10,10a-octahydropyrido[6,1-*b*] [1,3]thiazepine-7-carboxylic acid), or a pharmaceutically acceptable salt or a derivative thereof. The structure of omapatrilat is shown below.



In an embodiment, the CD10 inhibitor comprises RB-101 (benzyl *N*-(3-{[(2*S*)-2-amino-4-(methylthio)butyl]dithio}-2-benzylpropanoyl)-L-phenylalaninate), or a pharmaceutically acceptable salt or a derivative thereof. The structure of RB-101 is shown below.



In an embodiment, the CD10 inhibitor comprises UK-414,495 (Pfizer) ((R)-2-({1-[(5-ethyl-1,3,4-thiadiazol-2-yl)carbamoyl]cyclopentyl}methyl)valeric acid), or a pharmaceutically acceptable salt or a derivative thereof. The structure of UK-414,495 is shown below.



In some embodiments, the CD10 inhibitor is conjugated or otherwise bound to a therapeutic agent.

In some embodiments, a CD10 inhibitor includes an anti-CD10 CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-CD10 CAR construct or encoded by a CD10 binding CAR comprising a scFv, CDRs, or VH and VL chains. For example, an anti-CD10 CAR-expressing cell, e.g., CART is a generated by engineering a CD10-CAR (that comprises a CD10 binding domain) into a cell (e.g., a T cell or NK cell), e.g., for administration in combination with a CAR-expressing cell described herein. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In another aspect, provided herein is a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD10 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD10 CAR.

CD34 Inhibitors

Cluster of differentiation 34 (CD34) is also called hematopoietic progenitor cell antigen CD34 and is a cell surface glycoprotein that functions as a cell-cell adhesion factor. CD34 is sometimes expressed on some cancers/tumors, e.g., alveolar soft part sarcoma, preB-ALL, AML, AML-M7, dermatofibrosarcoma protuberans, gastrointestinal stromal tumors, giant cell fibroblastoma, granulocytic sarcoma, Kaposi's sarcoma, liposarcoma, malignant fibrous histiocytoma, malignant peripheral nerve sheath tumors, mengingeal hemangiopericytomas,

meningiomas, neurofibromas, schwannomas, and papillary thyroid carcinoma.

As used herein, the term "CD34" refers to an antigenic determinant known to be detectable on hematopoietic stem cells and some cancer cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequences of human CD34 can be found at Accession Nos. NP_001020280.1 (isoform a precursor); NP_001764.1 (isoform b precursor), and the mRNA sequences encoding them can be found at Accession Nos. NM_001025109.1 (variant 1); NM_001773.2 (variant 2). As used herein, "CD34" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type CD34. In one aspect the antigen-binding portion of the CAR recognizes and binds an antigen within the extracellular domain of the CD34 protein. In one aspect, the CD34 protein is expressed on a cancer cell.

Also provided herein are CD34 inhibitors and combination therapies. CD34 inhibitors include but are not limited to small molecules, recombinant proteins, anti-CD34 CAR-expressing cells, e.g. CARTs, and anti-CD34 antibodies (e.g., an anti-CD34 mono- or bispecific antibody) and fragments thereof. In some embodiments, anti-CD34 inhibitors can be used to treat a B-cell malignancy described herein. In an embodiment, the CD34 inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein, e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

In an embodiment, the CD34 inhibitor comprises an antibody or fragment thereof, e.g., the My-10 monoclonal antibody or an immunoliposome comprising the My-10 monoclonal antibody, as described in Mercadal et al. Biochim. Biophys. Acta. 1371.1(1998):17-23. In other embodiments, the CD34 inhibitor comprises an immunoliposome containing a cancer drug, e.g., doxorubicin, that is targeted to CD34-expressing cells, as described in Carrion et al. Life Sci. 75.3(2004):313-28. In an embodiment, the CD34 inhibitor comprises a monoclonal antibody against CD34 as described in Maleki et al. Hum. Antibodies. 22(2013):1-8. In another embodiment, the CD34 inhibitor comprises a monoclonal antibody that targets CD34, as described in Maleki et al. Cell J. 16.3(2014):361-66.

In some embodiments, the CD34 inhibitor is conjugated or otherwise bound to a

therapeutic agent.

In some embodiments, a CD34 inhibitor includes an anti-CD34 CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-CD34 CAR construct or encoded by a CD34 binding CAR comprising a scFv, CDRs, or VH and VL chains. For example, an anti-CD34 CAR-expressing cell, e.g., CART is a generated by engineering a CD34-CAR (that comprises a CD34 binding domain) into a cell (e.g., a T cell or NK cell), e.g., for administration in combination with a CAR-expressing cell described herein. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In another aspect, provided herein is a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and CD34 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD34 CAR.

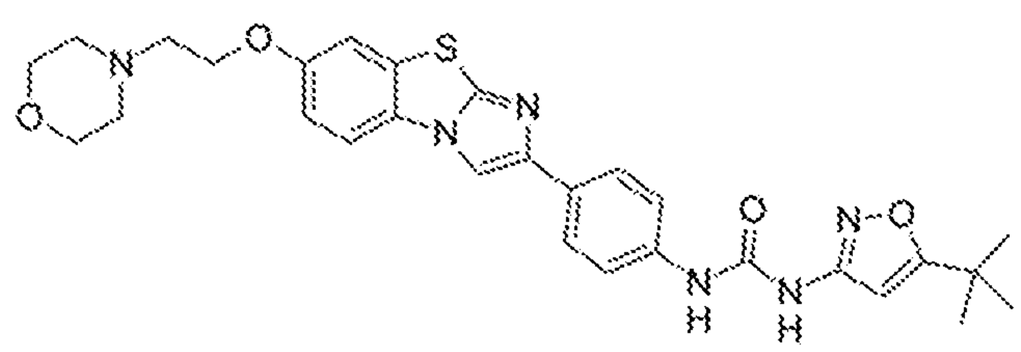
FLT-3 Inhibitors

Fms-like tyrosine kinase 3 (FLT-3), also called Cluster of differentiation antigen 135 (CD135), receptor-type tyrosine-protein kinase FLT3, or fetal liver kinase-2 (Flk2), is a receptor tyrosine kinase. FLT-3 is a cytokine receptor for the ligand, cytokine Flt3 ligand (FLT3L). FLT-3 is expressed on the surface of many hematopoietic progenitor cells and is important for lymphocyte development. The FLT3 gene is commonly mutated in leukemia, e.g., acute myeloid leukemia (AML).

As used herein, the term "FLT-3" refers to an antigenic determinant known to be detectable on hematopoietic progenitor cells and some cancer cells, e.g., leukemia cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequences of human FLT-3 can be found at Accession Nos. NP_004110.2, and the mRNA sequences encoding them can be found at Accession Nos. NM_004119.2. As used herein, "FLT-3" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type FLT-3. In one aspect the antigen-binding portion of the CAR recognizes and binds an antigen within the extracellular domain of the FLT-3 protein. In one aspect, the FLT-3 protein is expressed on a cancer cell.

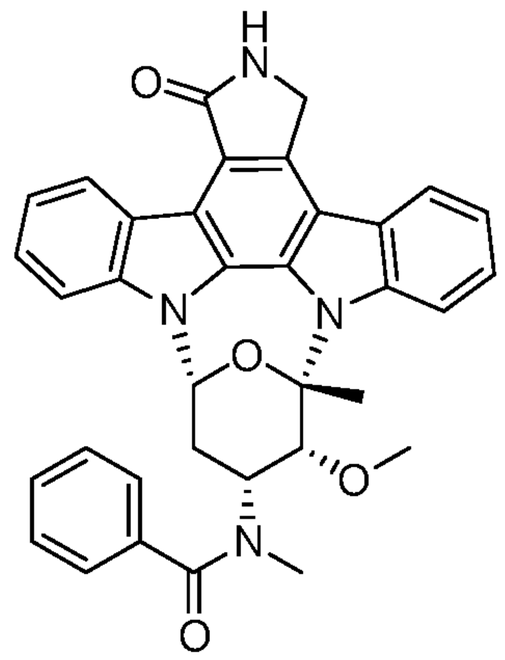
Also provided herein are FLT-3 inhibitors and combination therapies. FLT-3 inhibitors include but are not limited to small molecules, recombinant proteins, anti-FLT-3 CAR-expressing cells, e.g. CARTs, and anti-FLT-3 antibodies (e.g., an anti-FLT-3 mono- or bispecific antibody) and fragments thereof. In some embodiments, anti-FLT-3 inhibitors can be used to treat a B-cell malignancy described herein. In an embodiment, the FLT-3 inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein, e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

In some embodiments, the FLT-3 inhibitor comprises quizartinib (AC220; Ambit Biosciences) or a pharmaceutically acceptable salt or a derivative thereof. Quizartinib is a small molecule receptor tyrosine kinase inhibitor. The structure of quizartinib (1-(5-(*tert*-Butyl)isoxazol-3-yl)-3-(4-(7-(2-morpholinoethoxy)benzo[*d*]imidazo[2,1-b]thiazol-2-yl)phenyl)urea) is shown below.



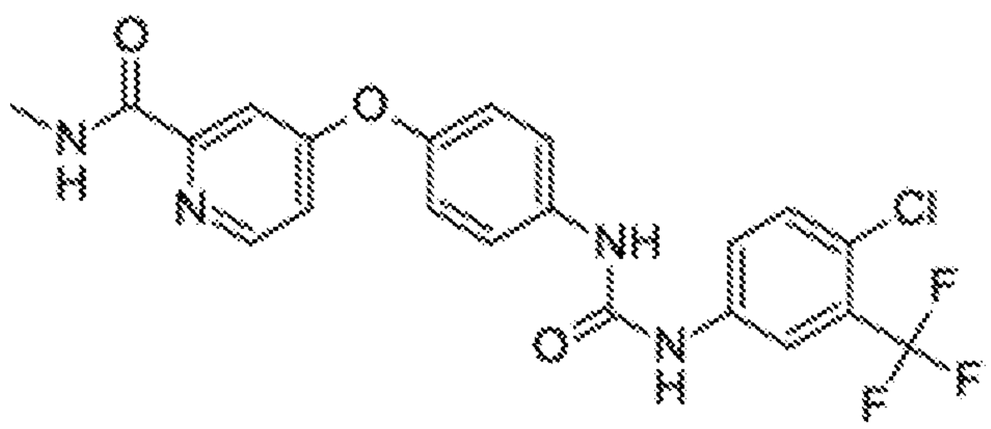
In some embodiments, the FLT-3 inhibitor comprises midostaurin is (PKC412; Technische Universität Dresden) or a pharmaceutically acceptable salt or a derivative thereof. Midostaurin is a protein kinase inhibitor that is a semi-synthetic derivative of staurosporine, an alkaloid from the bacterium Streptomyces staurosporeus.

The structure of midostaurin ((9*S*,10*R*,11*R*,13*R*)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-(methylamino)-9,13-epoxy-1*H*,9*H*-diindolo[1,2,3-gh:3',2',1'-lm]pyrrolo[3,4-j][1,7]benzodiamzonine-1-one) is shown below.



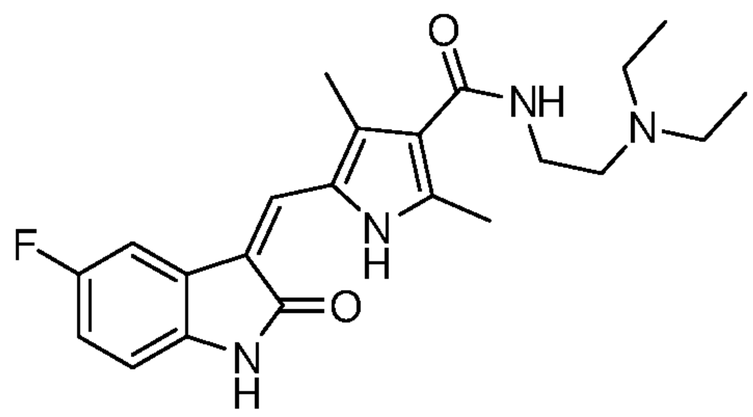
In some embodiments, midostaurin is administered orally, e.g., at a dose of about 25-200 mg, e.g., about 25-50 mg, 50-100 mg, 100-150 mg, or 150-200 mg. For example, midostaurin is administered, e.g., orally, at a dose of about 25-200 mg twice daily, e.g., about 25-50 mg, 50-100 mg, 100-150 mg, or 150-200 mg twice daily. See, e.g., Clinical Trial Identifier No. NCT01830361.

In an embodiment, the FLT-3 inhibitor comprises sorafenib (Bayer and Onyx Pharmaceuticals) or a pharmaceutically acceptable salt or a derivative thereof. Sorafenib is a small molecular inhibitor of multiple tyrosine protein kinases (e.g., VEGFR and PDGFR), Raf kinases (e.g., C-Raf and B-Raf), and some intracellular serine/threonine kinases (e.g. C-Raf, wild-type B-Raf, and mutant B-Raf). See, e.g., labeling.bayerhealthcare.com/html/products/pi/Nexavar_PI.pdf. The structure of sorafenib (4-[4-[[4-chloro-3-(trifluoromethyl)phenyl]carbamoylamino] phenoxy]-*N*-methyl-pyridine-2-carboxamide) is shown below.

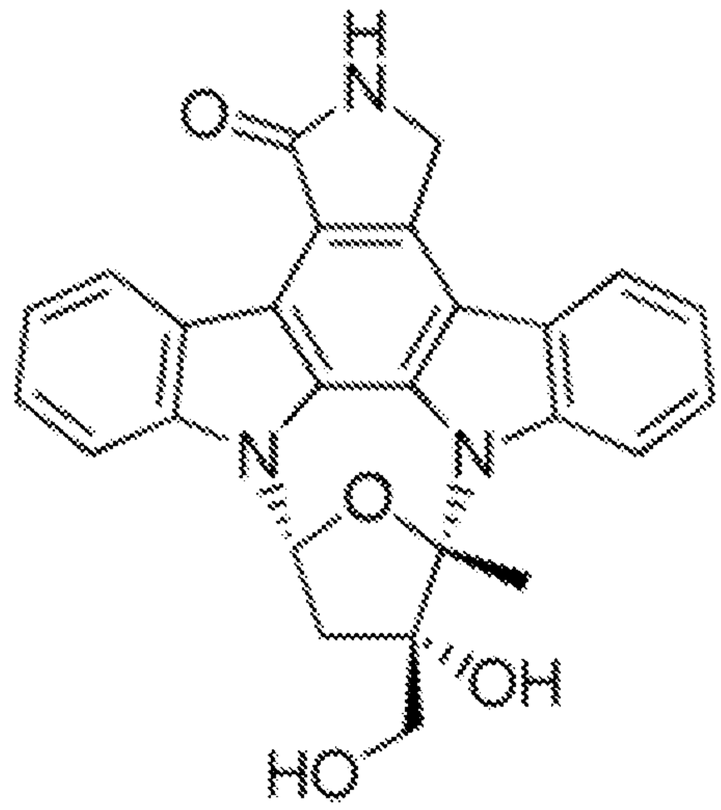


In some embodiments, the FLT-3 inhibitor comprises sunitinib (previously known as SU11248; Pfizer) or a pharmaceutically acceptable salt or derivative thereof. Sunitinib is a small molecule oral drug that inhibits multiple receptor tyrosine kinases, including FLT3. Sunitinib has been approved by the Food and Drug Administration (FDA) for the treatment of renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumor (GIST). The

structure of sunitinib (*N*-(2-diethylaminoethyl)-5-[(*Z*)-(5-fluoro-2-oxo-1H-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxamide) is shown below.



In some embodiments, the FLT-3 inhibitor comprises lestaurtinib (CEP-701; Cephalon) or a pharmaceutically acceptable salt or derivative thereof. Lestaurtinib is a tyrosine kinase inhibitor that is structurally related to staurosporine. The structure of lestaurtinib ((9*S*,10*S*,12*R*)-2,3,9,10,11,12-Hexahydro-10-hydroxy-10-(hydroxymethyl)-9-methyl-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocin-1-one) is shown below.



In some embodiments, lestaurtinib is administered orally, e.g., at a dose of about 40-100 mg twice a day, e.g., about 40-60 mg, 50-70 mg, 60-80 mg, 70-90 mg, or 80-100 mg twice a day. See, e.g., Clinical Trial Identifier No. NCT00079482; or NCT00030186.

In some embodiments, the FLT-3 inhibitor is conjugated or otherwise bound to a therapeutic agent.

In some embodiments, a FLT-3 inhibitor includes an anti-FLT-3 CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-FLT-3 CAR construct or encoded by a FLT-3

binding CAR comprising a scFv, CDRs, or VH and VL chains. For example, an anti-FLT-3 CAR-expressing cell, e.g., CART is a generated by engineering a FLT-3-CAR (that comprises a FLT-3 binding domain) into a cell (e.g., a T cell or NK cell), e.g., for administration in combination with a CAR-expressing cell described herein. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In another aspect, provided herein is a population of CAR-expressing cells, e.g., CART cells, comprising a mixture of cells expressing CD19 CARs and FLT-3 CARs. For example, in one embodiment, the population of CART cells can include a first cell expressing a CD19 CAR and a second cell expressing a FLT-3 CAR.

In one embodiment the antigen binding domain CAR (e.g., a CD19, ROR1, CD20, CD22, CD123, CD10, CD34, or FLT-3 antigen binding domain) comprises an scFv portion, e.g., a human scFv portion. The scFv may be preceded by an optional leader sequence such as provided in SEQ ID NO: 13, and followed by an optional hinge sequence such as provided in SEQ ID NO: 14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49, a transmembrane region such as provided in SEQ ID NO:15, an intracellular signaling domain that includes SEQ ID NO:16 or SEQ ID NO:51 and a CD3 zeta sequence that includes SEQ ID NO:17 or SEQ ID NO:43, e.g., wherein the domains are contiguous with and in the same reading frame to form a single fusion protein.

In some embodiments, the present disclosure encompasses a recombinant nucleic acid construct comprising a nucleic acid molecule encoding a CAR (e.g., a CD19 CAR, a ROR1 CAR, a CD20 CAR, a CD22 CAR, a CD123 CAR, a CD10 CAR, a CD34 CAR, or a FLT-3 CAR), wherein the nucleic acid molecule comprises the nucleic acid sequence encoding an antigen binding domain, e.g., described herein, e.g., that is contiguous with and in the same reading frame as a nucleic acid sequence encoding an intracellular signaling domain. An exemplary intracellular signaling domain that can be used in the CAR includes, but is not limited to, one or more intracellular signaling domains of, e.g., CD3-zeta, CD28, 4-1BB, and the like. In some instances, the CAR can comprise any combination of CD3-zeta, CD28, 4-1BB, and the like.

In one embodiment, the antigen binding domain (e.g., a CD19, ROR1, CD20, CD22, CD123, CD10, CD34, or FLT-3 antigen binding domain) is characterized by particular

functional features or properties of an antibody or antibody fragment. For example, in one embodiment, the portion of a CAR composition of the invention that comprises an antigen binding domain specifically binds a human B-cell antigen (e.g., CD19, ROR1, CD20, CD22, CD123, CD10, CD34, or FLT-3) or a fragment thereof. In certain embodiments, the scFv is contiguous with and in the same reading frame as a leader sequence. In one aspect the leader sequence is the polypeptide sequence provided as SEQ ID NO:13.

In one embodiment, the antigen binding domain is a fragment, e.g., a single chain variable fragment (scFv). In one embodiments, the antigen binding domain is a Fv, a Fab, a (Fab')2, or a bi-functional (e.g. bi-specific) hybrid antibody (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)). In one aspect, the antibodies and fragments thereof of the invention binds a B-cell protein or a fragment thereof with wild-type or enhanced affinity. In some instances, a human scFv can be derived from a display library.

In one embodiment, the antigen binding domain, e.g., scFv comprises at least one mutation such that the mutated scFv confers improved stability to the CAR construct. In another embodiment, the antigen binding domain, e.g., scFv comprises at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 mutations arising from, e.g., the humanization process such that the mutated scFv confers improved stability to the CAR construct.

In one embodiment, the population of CAR-expressing cells includes, e.g., a first cell expressing a CAR (e.g., a CD19 CAR, a ROR1 CAR, a CD20 CAR, a CD22 CAR, a CD123 CAR, a CD10 CAR, a CD34 CAR, or a FLT-3 CAR) that includes a primary intracellular signaling domain, and a second cell expressing a CAR (e.g., a CD19 CAR, a ROR1 CAR, a CD20 CAR, a CD22 CAR, a CD123 CAR, a CD10 CAR, a CD34 CAR, or a FLT-3 CAR)) that includes a secondary signaling domain.

CD79b Inhibitors

As used herein, the term "CD79b" refers to an antigenic determinant known to be detectable on some malignant hematological cancer cells, e.g., leukemia cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequences of human CD79b can be found at Accession Nos. NP_000617.1 (isoform 1 precursor), NP_067613.1 (isoform 2

precursor), or NP_001035022.1 (isoform 3 precursor), and the mRNA sequences encoding them can be found at Accession Nos. NM_000626.2 (transcript variant 1), NM_021602.2 (transcript variant 2), or NM_001039933.1 (transcript variant 3). As used herein, "CD79b" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type CD79b. In one aspect the antigen-binding portion of the CAR recognizes and binds an antigen within the extracellular domain of the CD79b protein. In one aspect, the CD79b protein is expressed on a cancer cell. In embodiments, the CD79b protein is a wild-type CD79b protein; in other embodiments, the CD79b protein is a mutant CD79b protein.

CD79b is also called immunoglobulin-associated beta, which is a component of the B lymphocyte antigen receptor multimeric complex. CD79b forms a heterodimer with another accessory protein called CD79a (immunoglobulin-associated alpha), and the heterodimer complexes with surface immunoglobulins on B cells. CD79b is important for the assembly of and surface expression of the B lymphocyte antigen receptor. CD79b and CD79a are important for pre-B-cell and B-cell development. Mutation and aberrant CD79b expression occurs in many B-CLL cells and may be correlated with the loss of surface expression and/or defective signaling of B lymphocyte antigen receptor in B-CLL. See, e.g., Thompson et al. Blood 90.4(1997):1387-94. In some cases, overexpression of a mutant form or splice variant of CD79b has been correlated with diminished B lymphocyte antigen receptor in B-CLL and other lymphoid malignancies. See, e.g., Cragg et al. Blood 100.9(2002):3068-76.

Provided herein are CD79b inhibitors and combination therapies. CD79b inhibitors include but are not limited to small molecules, recombinant proteins, anti-CD79b CAR-expressing cells, e.g. CARTs, and anti-CD79b antibodies (e.g., an anti-CD79b mono- or bispecific antibody) and fragments thereof. In some embodiments, anti-CD79b inhibitors can be used to treat a B-cell malignancy described herein. In an embodiment, the CD79b inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein, e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

In an embodiment, the CD79b inhibitor is an anti-CD79b antibody or fragment thereof. In one embodiment, the anti-79b antibody or fragment thereof comprises a monoclonal antibody, e.g., a monospecific or bispecific antibody or fragment thereof. For example, the anti-

CD79b antibody or fragment thereof comprises an anti-CD79b antibody drug conjugate such as polatuzumab vedotin (Roche). In embodiments, polatuzumab vedotin is used to treat a cancer, e.g., NHL, e.g., follicular lymphoma or DLBCL, e.g., relapsed or refractory follicular lymphoma or DLBCL. See, e.g., NCT02257567. In embodiments, the anti-CD79b antibody or fragment thereof is a bispecific antibody comprising components that bind to CD32B and D79B, such as MGD010 (MacroGenics),. See, e.g., NCT02376036.

In some embodiments, the CD79b inhibitor is conjugated or otherwise bound to a therapeutic agent.

In some embodiments, a CD79b inhibitor includes an anti-CD79b CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-CD79b CAR construct or encoded by a CD79b binding CAR comprising a scFv, CDRs, or VH and VL chains. For example, an anti-CD79b CAR-expressing cell, e.g., CART is a generated by engineering a CD79b-CAR (that comprises a CD79b binding domain) into a cell (e.g., a T cell or NK cell), e.g., for administration in combination with a CAR-expressing cell described herein. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In another aspect, provided herein is a population of CAR-expressing cells, e.g., CART cells or CAR-expressing NK cells, comprising a mixture of cells expressing CD19 CARs and CD79b CARs. For example, in one embodiment, the population of CAR-expressing cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD79b CAR.

C179b Inhibitors

As used herein, the term "CD179b" refers to an antigenic determinant known to be detectable on some malignant hematological cancer cells, e.g., leukemia cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequences of human CD179b can be found at Accession Nos. NP_064455.1 (isoform a precursor) or NP_690594.1 (isoform b precursor), and the mRNA sequences encoding them can be found at Accession Nos. NM_020070.3 (transcript variant 1) or NM_152855.2 (transcript variant 2). As used herein, "CD179b" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type CD179b. In one aspect the antigen-binding

portion of the CAR recognizes and binds an antigen within the extracellular domain of the CD179b protein. In one aspect, the CD179b protein is expressed on a cancer cell. In embodiments, the CD179b protein is a wild-type CD179b protein; in other embodiments, the CD179b protein is a mutant CD179b protein.

CD179b is also called immunoglobulin lambda-like polypeptide 1 (IGLL1). CD179b is a subunit of a heterodimeric light chain that complexes with a membrane-bound Ig mu heavy chain. Together, the light chain and heavy chain form the preB cell receptor. Mutations in CD179b have been correlated with B cell deficiency and agammaglobulinemia. CD179b is expressed in some cancer cells, e.g., precursor B-cell lymphoblastic lymphoma cells.

Provided herein are CD179b inhibitors and combination therapies. CD179b inhibitors include but are not limited to small molecules, recombinant proteins, anti-CD179b CAR-expressing cells, e.g. CARTs, and anti-CD179b antibodies (e.g., an anti-CD179b mono- or bispecific antibody) and fragments thereof. In some embodiments, anti-CD179b inhibitors can be used to treat a B-cell malignancy described herein. In an embodiment, the CD179b inhibitor is administered in combination with a CD20 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a CAR-expressing cell described herein, e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

In an embodiment, the CD179b inhibitor is an anti-CD179b antibody or fragment thereof. In one embodiment, the anti-179b antibody or fragment thereof comprises a monoclonal antibody, e.g., a monospecific or bispecific antibody or fragment thereof.

In some embodiments, the CD179b inhibitor is conjugated or otherwise bound to a therapeutic agent.

In some embodiments, a CD179b inhibitor includes an anti-CD179b CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-CD179b CAR construct or encoded by a CD179b binding CAR comprising a scFv, CDRs, or VH and VL chains. For example, an anti-CD179b CAR-expressing cell, e.g., CART is a generated by engineering a CD179b-CAR (that comprises a CD179b binding domain) into a cell (e.g., a T cell or NK cell), e.g., for administration in combination with a CAR-expressing cell described herein. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In another aspect, provided herein is a population of CAR-expressing cells, e.g., CART cells or CAR-expressing NK cells, comprising a mixture of cells expressing CD19 CARs and CD179b CARs. For example, in one embodiment, the population of CAR-expressing cells can include a first cell expressing a CD19 CAR and a second cell expressing a CD179b CAR.

CD79a Inhibitors

As used herein, the term "CD79a" refers to an antigenic determinant known to be detectable on some malignant hematological cancer cells, e.g., leukemia cells. The human and murine amino acid and nucleic acid sequences can be found in a public database, such as GenBank, UniProt and Swiss-Prot. For example, the amino acid sequences of human CD79a can be found at Accession Nos. NP_001774.1 (isoform 1 precursor) or NP_067612.1 (isoform 2 precursor), and the mRNA sequences encoding them can be found at Accession Nos. NM_001783.3 (transcript variant 1) or NM_021601.3 (transcript variant 2). As used herein, "CD79a" includes proteins comprising mutations, e.g., point mutations, fragments, insertions, deletions and splice variants of full length wild-type CD79a. In one aspect the antigen-binding portion of the CAR recognizes and binds an antigen within the extracellular domain of the CD79a protein. In one aspect, the CD79a protein is expressed on a cancer cell. In embodiments, the CD79a protein is a wild-type CD79a protein; in other embodiments, the CD79a protein is a mutant CD79a protein.

CD79a is also called immunoglobulin-associated alpha. CD79a heterodimerizes with CD79b to form a component of the B lymphocyte antigen receptor multimeric complex. CD79a is expressed in many hematological cancers, e.g., acute leukemias (e.g., AML), B-cell Lymphomas, and Myelomas.

Provided herein are CD79a inhibitors and combination therapies. CD79a inhibitors include but are not limited to small molecules, recombinant proteins, anti-CD79a CAR-expressing cells, e.g. CARTs, and anti-CD79a antibodies (e.g., an anti-CD79a mono- or bispecific antibody) and fragments thereof. In some embodiments, anti-CD79a inhibitors can be used to treat a B-cell malignancy described herein. In an embodiment, the CD79a inhibitor is administered in combination with a CD19 inhibitor, e.g., a CD19 CAR-expressing cell, e.g., a

CAR-expressing cell described herein, e.g., a cell expressing a CAR comprising an antibody binding domain that is murine, human, or humanized.

In an embodiment, the CD79a inhibitor is an anti-CD79a antibody or fragment thereof. In one embodiment, the anti-CD79a antibody or fragment thereof comprises a monoclonal antibody, e.g., a monospecific or bispecific antibody or fragment thereof. For example, the anti-CD79a antibody or fragment thereof comprises an anti-CD79a antibody or fragment thereof (e.g., variable regions or CDRs) described in Polson et al. Blood 110.2(2007):616-23, incorporated herein by reference. For example, the anti-CD79a antibody or fragment thereof comprises the 7H7, 15E4, or 16C11 antibody or fragment thereof (e.g., variable regions or CDRs) described in Polson et al. See Id.

In some embodiments, the CD79a inhibitor is conjugated or otherwise bound to a therapeutic agent.

In some embodiments, a CD79a inhibitor includes an anti-CD79a CAR-expressing cell, e.g., CART, e.g., a cell expressing an anti-CD79a CAR construct or encoded by a CD79a binding CAR comprising a scFv, CDRs, or VH and VL chains. For example, an anti-CD79a CAR-expressing cell, e.g., CART is a generated by engineering a CD79a-CAR (that comprises a CD79a binding domain) into a cell (e.g., a T cell or NK cell), e.g., for administration in combination with a CAR-expressing cell described herein. Also provided herein are methods of use of the CAR-expressing cells described herein for adoptive therapy.

In another aspect, provided herein is a population of CAR-expressing cells, e.g., CART cells or CAR-expressing NK cells, comprising a mixture of cells expressing CD19 CARs and CD79a CARs. For example, in one embodiment, the population of CAR-expressing cells can include a first cell expressing a CD20 CAR and a second cell expressing a CD79a CAR.

CAR Therapies

The inhibitors herein, e.g., CAR-expressing cells directed against CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a, may comprise one or more of the compositions described herein, e.g., a transmembrane domain, intracellular signaling domain, costimulatory domain, leader sequence, or hinge.

In one aspect, the present invention encompasses a recombinant nucleic acid construct comprising a transgene encoding a CAR. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence encoding an anti-CD19 binding domain selected from one or more of SEQ ID NOS:61-72, wherein the sequence is contiguous with and in the same reading frame as the nucleic acid sequence encoding an intracellular signaling domain. An exemplary intracellular signaling domain that can be used in the CAR includes, but is not limited to, one or more intracellular signaling domains of, e.g., CD3-zeta, CD28, 4-1BB, and the like. In some instances, the CAR can comprise any combination of CD3-zeta, CD28, 4-1BB, and the like.

In one aspect, the present invention contemplates modifications of the starting antibody or fragment (e.g., scFv) amino acid sequence that generate functionally equivalent molecules. For example, the VH or VL of an antigen binding domain, e.g., scFv, comprised in the CAR can be modified to retain at least about 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%,81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity of the starting VH or VL framework region of the antigen binding domain, e.g., scFv. The present invention contemplates modifications of the entire CAR construct, e.g., modifications in one or more amino acid sequences of the various domains of the CAR construct in order to generate functionally equivalent molecules. The CAR construct can be modified to retain at least about 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity of the starting CAR construct. The present invention also contemplates modifications of CDRs, e.g., modifications in one or more amino acid sequences of one or more CDRs of a CAR construct in order to generate functionally equivalent molecules. For instance, the CDR may have, e.g., up to and including 1, 2, 3, 4, 5, or 6 alterations (e.g., substitutions) relative to a CDR sequence provided herein.

The nucleic acid sequences coding for the desired molecules can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the nucleic acid of interest can be produced synthetically, rather than cloned.

The present invention includes, among other things, retroviral and lentiviral vector constructs expressing a CAR that can be directly transduced into a cell.

The present invention also includes an RNA construct that can be directly transfected into a cell. A method for generating mRNA for use in transfection involves in vitro transcription (IVT) of a template with specially designed primers, followed by polyA addition, to produce a construct containing 3' and 5' untranslated sequence ("UTR"), a 5' cap and/or Internal Ribosome Entry Site (IRES), the nucleic acid to be expressed, and a polyA tail, typically 50-2000 bases in length (SEQ ID NO:118). RNA so produced can efficiently transfect different kinds of cells. In one embodiment, the template includes sequences for the CAR. In an embodiment, an RNA CAR vector is transduced into a T cell by electroporation.

Antigen binding domain

In one aspect, the CAR of the invention comprises a target-specific binding element otherwise referred to as an antigen binding domain. The choice of moiety depends upon the type and number of ligands that define the surface of a target cell. For example, the antigen binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus examples of cell surface markers that may act as ligands for the antigen binding domain in a CAR of the invention include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells. The antigen-binding domain can bind, e.g., one or more of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

In one aspect, the CAR-mediated T-cell response can be directed to an antigen of interest by way of engineering an antigen binding domain that specifically binds a desired antigen into the CAR.

The antigen binding domain (e.g., an antigen-binding domain that binds one or more of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a) can be any domain that binds to the antigen including but not limited to a monoclonal antibody, a polyclonal antibody, a recombinant antibody, a murine antibody, a human antibody, a humanized antibody, and a functional fragment thereof, including but not limited to a single-domain antibody such as a heavy chain variable domain (VH), a light chain variable domain (VL) and a variable domain (VHH) of camelid derived nanobody, and to an alternative scaffold known in the art to function as antigen binding domain, such as a recombinant fibronectin

domain, and the like.

In some instances, it is beneficial for the antigen binding domain to be derived from the same species in which the CAR will ultimately be used in. For example, for use in humans, it may be beneficial for the antigen binding domain (e.g., an antigen-binding domain that binds one or more of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a) of the CAR to comprise human or humanized residues for the antigen binding domain of an antibody or antibody fragment.

A humanized antibody can be produced using a variety of techniques known in the art, including but not limited to, CDR-grafting (see, e.g., European Patent No. EP 239,400; International Publication No. WO 91/09967; and U.S. Pat. Nos. 5,225,539, 5,530,101, and 5,585,089, each of which is incorporated herein in its entirety by reference), veneering or resurfacing (see, e.g., European Patent Nos. EP 592,106 and EP 519,596; Padlan, 1991, Molecular Immunology, 28(4/5):489-498; Studnicka et al., 1994, Protein Engineering, 7(6):805-814; and Roguska et al., 1994, PNAS, 91:969-973, each of which is incorporated herein by its entirety by reference), chain shuffling (see, e.g., U.S. Pat. No. 5,565,332, which is incorporated herein in its entirety by reference), and techniques disclosed in, e.g., U.S. Patent Application Publication No. US2005/0042664, U.S. Patent Application Publication No. US2005/0048617, U.S. Pat. No. 6,407,213, U.S. Pat. No. 5,766,886, International Publication No. WO 9317105, Tan et al., J. Immunol., 169:1119-25 (2002), Caldas et al., Protein Eng., 13(5):353-60 (2000), Morea et al., Methods, 20(3):267-79 (2000), Baca et al., J. Biol. Chem., 272(16):10678-84 (1997), Roguska et al., Protein Eng., 9(10):895-904 (1996), Couto et al., Cancer Res., 55 (23 Supp):5973s-5977s (1995), Couto et al., Cancer Res., 55(8):1717-22 (1995), Sandhu J S, Gene, 150(2):409-10 (1994), and Pedersen et al., J. Mol. Biol., 235(3):959-73 (1994), each of which is incorporated herein in its entirety by reference. Often, framework residues in the framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, for example improve, antigen binding. These framework substitutions are identified by methods well-known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Pat. No. 5,585,089; and Riechmann et al., 1988, Nature, 332:323, which are incorporated herein by reference in their entireties.)

A humanized antibody or antibody fragment has one or more amino acid residues remaining in it from a source which is nonhuman. These nonhuman amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. As provided herein, humanized antibodies or antibody fragments comprise one or more CDRs from nonhuman immunoglobulin molecules and framework regions wherein the amino acid residues comprising the framework are derived completely or mostly from human germline. Multiple techniques for humanization of antibodies or antibody fragments are well-known in the art and can essentially be performed following the method of Winter and co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody, i.e., CDR-grafting (EP 239,400; PCT Publication No. WO 91/09967; and U.S. Pat. Nos. 4,816,567; 6,331,415; 5,225,539; 5,530,101; 5,585,089; 6,548,640, the contents of which are incorporated herein by reference herein in their entirety). In such humanized antibodies and antibody fragments, substantially less than an intact human variable domain has been substituted by the corresponding sequence from a nonhuman species. Humanized antibodies are often human antibodies in which some CDR residues and possibly some framework (FR) residues are substituted by residues from analogous sites in rodent antibodies. Humanization of antibodies and antibody fragments can also be achieved by veneering or resurfacing (EP 592,106; EP 519,596; Padlan, 1991, Molecular Immunology, 28(4/5):489-498; Studnicka et al., Protein Engineering, 7(6):805-814 (1994); and Roguska et al., PNAS, 91:969-973 (1994)) or chain shuffling (U.S. Pat. No. 5,565,332), the contents of which are incorporated herein by reference herein in their entirety.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., J. Immunol., 151:2296 (1993); Chothia et al., J. Mol. Biol., 196:901 (1987), the contents of which are incorporated herein by reference herein in their entirety). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different

humanized antibodies (see, e.g., Nicholson et al. Mol. Immun. 34 (16-17): 1157-1165 (1997); Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993), the contents of which are incorporated herein by reference herein in their entirety). In some embodiments, the framework region, e.g., all four framework regions, of the heavy chain variable region are derived from a VH4_4-59 germline sequence. In one embodiment, the framework region can comprise, one, two, three, four or five modifications, e.g., substitutions, e.g., from the amino acid at the corresponding murine sequence (e.g., of SEQ ID NO:59). In one embodiment, the framework region, e.g., all four framework regions of the light chain variable region are derived from a VK3_1.25 germline sequence. In one embodiment, the framework region can comprise, one, two, three, four or five modifications, e.g., substitutions, e.g., from the amino acid at the corresponding murine sequence (e.g., of SEQ ID NO:59).

In some aspects, the portion of a CAR composition of the invention that comprises an antibody fragment is humanized with retention of high affinity for the target antigen and other favorable biological properties. According to one aspect of the invention, humanized antibodies and antibody fragments are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, e.g., the analysis of residues that influence the ability of the candidate immunoglobulin to bind the target antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody or antibody fragment characteristic, such as increased affinity for the target antigen, is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

A humanized antibody or antibody fragment may retain a similar antigenic specificity as the original antibody, e.g., in the present invention, the ability to bind human CD19, CD20, or CD22. In some embodiments, a humanized antibody or antibody fragment may have improved affinity and/or specificity of binding to human CD19, CD20, or CD22.

In one aspect, the binding domain (e.g., an antigen-binding domain that binds one or

more of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a) is a fragment, e.g., a single chain variable fragment (scFv). In one aspect, the binding domain is a Fv, a Fab, a (Fab')2, or a bi-functional (e.g. bi-specific) hybrid antibody (e.g., Lanzavecchia et al., Eur. J. Immunol. 17, 105 (1987)). In one aspect, the antibodies and fragments thereof of the invention binds a CD19, CD20, or CD22 protein with wild-type or enhanced affinity.

In some instances, scFvs can be prepared according to method known in the art (see, for example, Bird et al., (1988) Science 242:423-426 and Huston et al., (1988) Proc. Natl. Acad. Sci. USA 85:5879-5883). ScFv molecules can be produced by linking VH and VL regions together using flexible polypeptide linkers. The scFv molecules comprise a linker (e.g., a Ser-Gly linker) with an optimized length and/or amino acid composition. The linker length can greatly affect how the variable regions of a scFv fold and interact. In fact, if a short polypeptide linker is employed (e.g., between 5-10 amino acids) intrachain folding is prevented. Interchain folding is also required to bring the two variable regions together to form a functional epitope binding site. For examples of linker orientation and size see, e.g., Hollinger et al. 1993 Proc Natl Acad. Sci. U.S.A. 90:6444-6448, U.S. Patent Application Publication Nos. 2005/0100543, 2005/0175606, 2007/0014794, and PCT publication Nos. WO2006/020258 and WO2007/024715, is incorporated herein by reference.

An scFv can comprise a linker of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, or more amino acid residues between its VL and VH regions. The linker sequence may comprise any naturally occurring amino acid. In some embodiments, the linker sequence comprises amino acids glycine and serine. In another embodiment, the linker sequence comprises sets of glycine and serine repeats such as $(Gly_4Ser)n$, where n is a positive integer equal to or greater than 1 (SEQ ID NO:18). In one embodiment, the linker can be $(Gly_4Ser)_4$ (SEQ ID NO:106) or $(Gly_4Ser)_3$(SEQ ID NO:107). Variation in the linker length may retain or enhance activity, giving rise to superior efficacy in activity studies.

In some embodiments, the amino acid sequence of the antigen binding domain (e.g., an antigen-binding domain that binds one or more of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a) or other portions or the entire CAR) can be modified, e.g., an amino acid sequence described herein can be modified, e.g., by a conservative

substitution. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

Percent identity in the context of two or more nucleic acids or polypeptide sequences, refers to two or more sequences that are the same. Two sequences are "substantially identical" if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (e.g., 60% identity, optionally 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%,81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200 or more amino acids) in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman, (1970) Adv. Appl. Math. 2:482c, by the homology alignment algorithm of Needleman and Wunsch, (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson and Lipman, (1988) Proc. Nat'l. Acad. Sci. USA 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science

Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., Brent et al., (2003) Current Protocols in Molecular Biology).

Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al., (1977) Nuc. Acids Res. 25:3389-3402; and Altschul et al., (1990) J. Mol. Biol. 215:403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information.

The percent identity between two amino acid sequences can also be determined using the algorithm of E. Meyers and W. Miller, (1988) Comput. Appl. Biosci. 4:11-17) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch (1970) J. Mol. Biol. 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

In one aspect, the present invention contemplates modifications of the starting antibody or fragment (e.g., scFv) amino acid sequence that generate functionally equivalent molecules. For example, the VH or VL of a binding domain (e.g., an antigen-binding domain that binds one or more of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a), e.g., scFv, comprised in the CAR can be modified to retain at least about 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%,81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity of the starting VH or VL framework region of the anti-CD19 binding domain, e.g., scFv. More broadly, the VH or VL of a B-cell antigen binding domain, to CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1, e.g., scFv, comprised in the CAR can be modified to retain at least about 70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%,81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity of the starting VH or VL framework region of the antigen binding domain, e.g., scFv. The present invention contemplates modifications of the entire CAR construct, e.g., modifications in one or more amino acid sequences of the various domains of the CAR construct in order to generate functionally equivalent molecules. The CAR construct can be modified to retain at least about

70%, 71%. 72%. 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%,81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identity of the starting CAR construct.

BISPECIFIC CARs

In an embodiment a multispecific antibody molecule is a bispecific antibody molecule. A bispecific antibody has specificity for no more than two antigens. A bispecific antibody molecule is characterized by a first immunoglobulin variable domain sequence which has binding specificity for a first epitope and a second immunoglobulin variable domain sequence that has binding specificity for a second epitope. In an embodiment the first and second epitopes are on the same antigen, e.g., the same protein (or subunit of a multimeric protein). In an embodiment the first and second epitopes overlap. In an embodiment the first and second epitopes do not overlap. In an embodiment the first and second epitopes are on different antigens, e.g., different proteins (or different subunits of a multimeric protein). In an embodiment a bispecific antibody molecule comprises a heavy chain variable domain sequence and a light chain variable domain sequence which have binding specificity for a first epitope and a heavy chain variable domain sequence and a light chain variable domain sequence which have binding specificity for a second epitope. In an embodiment a bispecific antibody molecule comprises a half antibody having binding specificity for a first epitope and a half antibody having binding specificity for a second epitope. In an embodiment a bispecific antibody molecule comprises a half antibody, or fragment thereof, having binding specificity for a first epitope and a half antibody, or fragment thereof, having binding specificity for a second epitope. In an embodiment a bispecific antibody molecule comprises a scFv, or fragment thereof, have binding specificity for a first epitope and a scFv, or fragment thereof, have binding specificity for a second epitope. In an embodiment the first epitope is located on CD19 and the second epitope is located on CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1.

In certain embodiments, the antibody molecule is a multi-specific (*e.g.*, a bispecific or a trispecific) antibody molecule. Protocols for generating bispecific or heterodimeric antibody molecules are known in the art; including but not limited to, for example, the "knob in a hole" approach described in, *e.g.*, US 5731168; the electrostatic steering Fc pairing as described in,

e.g., WO 09/089004, WO 06/106905 and WO 2010/129304; Strand Exchange Engineered Domains (SEED) heterodimer formation as described in, *e.g.*, WO 07/110205; Fab arm exchange as described in, *e.g.*, WO 08/119353, WO 2011/131746, and WO 2013/060867; double antibody conjugate, *e.g.*, by antibody cross-linking to generate a bi-specific structure using a heterobifunctional reagent having an amine-reactive group and a sulfhydryl reactive group as described in, *e.g.*, US 4433059; bispecific antibody determinants generated by recombining half antibodies (heavy-light chain pairs or Fabs) from different antibodies through cycle of reduction and oxidation of disulfide bonds between the two heavy chains, as described in, *e.g.*, US 4444878; trifunctional antibodies, *e.g.*, three Fab' fragments cross-linked through sulfhdryl reactive groups, as described in, *e.g.*, US5273743; biosynthetic binding proteins, *e.g.*, pair of scFvs cross-linked through C-terminal tails preferably through disulfide or amine-reactive chemical cross-linking, as described in, *e.g.*, US5534254; bifunctional antibodies, *e.g.*, Fab fragments with different binding specificities dimerized through leucine zippers (*e.g.*, c-fos and c-jun) that have replaced the constant domain, as described in, *e.g.*, US5582996; bispecific and oligospecific mono-and oligovalent receptors, *e.g.*, VH-CH1 regions of two antibodies (two Fab fragments) linked through a polypeptide spacer between the CH1 region of one antibody and the VH region of the other antibody typically with associated light chains, as described in, *e.g.*, US5591828; bispecific DNA-antibody conjugates, *e.g.*, crosslinking of antibodies or Fab fragments through a double stranded piece of DNA, as described in, *e.g.*, US5635602; bispecific fusion proteins, *e.g.*, an expression construct containing two scFvs with a hydrophilic helical peptide linker between them and a full constant region, as described in, *e.g.*, US5637481; multivalent and multispecific binding proteins, *e.g.*, dimer of polypeptides having first domain with binding region of Ig heavy chain variable region, and second domain with binding region of Ig light chain variable region, generally termed diabodies (higher order structures are also encompassed creating for bispecific, trispecific, or tetraspecific molecules, as described in, *e.g.*, US5837242; minibody constructs with linked VL and VH chains further connected with peptide spacers to an antibody hinge region and CH3 region, which can be dimerized to form bispecific/multivalent molecules, as described in, *e.g.*, US5837821; VH and VL domains linked with a short peptide linker (*e.g.*, 5 or 10 amino acids) or no linker at all in either orientation, which can form dimers to form bispecific diabodies; trimers and tetramers, as described in, *e.g.*, US5844094; String of VH domains (or VL domains in family members) connected by peptide

linkages with crosslinkable groups at the C-terminus further associated with VL domains to form a series of FVs (or scFvs), as described in, *e.g.*, US5864019; and single chain binding polypeptides with both a VH and a VL domain linked through a peptide linker are combined into multivalent structures through non-covalent or chemical crosslinking to form, *e.g.*, homobivalent, heterobivalent, trivalent, and tetravalent structures using both scFV or diabody type format, as described in, *e.g.*, US5869620. Additional exemplary multispecific and bispecific molecules and methods of making the same are found, for example, in US5910573, US5932448, US5959083, US5989830, US6005079, US6239259, US6294353, US6333396, US6476198, US6511663, US6670453, US6743896, US6809185, US6833441, US7129330, US7183076, US7521056, US7527787, US7534866, US7612181, US2002004587A1, US2002076406A1, US2002103345A1, US2003207346A1, US2003211078A1, US2004219643A1, US2004220388A1, US2004242847A1, US2005003403A1, US2005004352A1, US2005069552A1, US2005079170A1, US2005100543A1, US2005136049A1, US2005136051A1, US2005163782A1, US2005266425A1, US2006083747A1, US2006120960A1, US2006204493A1, US2006263367A1, US2007004909A1, US2007087381A1, US2007128150A1, US2007141049A1, US2007154901A1, US2007274985A1, US2008050370A1, US2008069820A1, US2008152645A1, US2008171855A1, US2008241884A1, US2008254512A1, US2008260738A1, US2009130106A1, US2009148905A1, US2009155275A1, US2009162359A1, US2009162360A1, US2009175851A1, US2009175867A1, US2009232811A1, US2009234105A1, US2009263392A1, US2009274649A1, EP346087A2, WO0006605A2, WO02072635A2, WO04081051A1, WO06020258A2, WO2007044887A2, WO2007095338A2, WO2007137760A2, WO2008119353A1, WO2009021754A2, WO2009068630A1, WO9103493A1, WO9323537A1, WO9409131A1, WO9412625A2, WO9509917A1, WO9637621A2, WO9964460A1. The contents of the above-referenced applications are incorporated herein by reference in their entireties.

Within each antibody or antibody fragment (e.g., scFv) of a bispecific antibody molecule, the VH can be upstream or downstream of the VL. In some embodiments, the upstream antibody or antibody fragment (e.g., scFv) is arranged with its VH (VH_1) upstream of its VL (VL_1) and the downstream antibody or antibody fragment (e.g., scFv) is arranged with its VL (VL_2) upstream of its VH (VH_2), such that the overall bispecific antibody molecule has the

arrangement VH_1-VL_1-VL_2-VH_2. In other embodiments, the upstream antibody or antibody fragment (e.g., scFv) is arranged with its VL (VL_1) upstream of its VH (VH_1) and the downstream antibody or antibody fragment (e.g., scFv) is arranged with its VH (VH_2) upstream of its VL (VL_2), such that the overall bispecific antibody molecule has the arrangement VL_1-VH_1-VH_2-VL_2. Optionally, a linker is disposed between the two antibodies or antibody fragments (e.g., scFvs), e.g., between VL_1 and VL_2 if the construct is arranged as VH_1-VL_1-VL_2-VH_2, or between VH_1 and VH_2 if the construct is arranged as VL_1-VH_1-VH_2-VL_2. The linker may be a linker as described herein, e.g., a (Gly_4-Ser)n linker, wherein n is 1, 2, 3, 4, 5, or 6, e.g., 4 (SEQ ID NO: 53). In general, the linker between the two scFvs should be long enough to avoid mispairing between the domains of the two scFvs. Optionally, a linker is disposed between the VL and VH of the first scFv. Optionally, a linker is disposed between the VL and VH of the second scFv. In constructs that have multiple linkers, any two or more of the linkers can be the same or different. Accordingly, in some embodiments, a bispecific CAR comprises VLs, VHs, and optionally one or more linkers in an arrangement as described herein.

In certain embodiments the antibody molecule is a bispecific antibody molecule having a first binding specificity for a first B-cell epitope and a second binding specificity for another B-cell antigen. For instance, in some embodiments the bispecific antibody molecule has a first binding specificity for CD19 and a second binding specificity for one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. In some embodiments the bispecific antibody molecule has a first binding specificity for CD19 and a second binding specificity for CD22.

Chimeric TCR

In one aspect, the antibodies and antibody fragments disclosed herein (e.g., those directed against CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a) can be grafted to one or more constant domain of a T cell receptor ("TCR") chain, for example, a TCR alpha or TCR beta chain, to create an chimeric TCR that binds specifically to a cancer associated antigen. Without being bound by theory, it is believed that chimeric TCRs will signal through the TCR complex upon antigen binding. For example, an scFv as disclosed herein, can be grafted to the constant domain, e.g., at least a portion of the extracellular constant domain, the transmembrane domain and the cytoplasmic domain, of a TCR chain, for example,

the TCR alpha chain and/or the TCR beta chain. As another example, an antibody fragment, for example a VL domain as described herein, can be grafted to the constant domain of a TCR alpha chain, and an antibody fragment, for example a VH domain as described herein, can be grafted to the constant domain of a TCR beta chain (or alternatively, a VL domain may be grafted to the constant domain of the TCR beta chain and a VH domain may be grafted to a TCR alpha chain). As another example, the CDRs of an antibody or antibody fragment, e.g., the CDRs of an antibody or antibody fragment as described in any of the Tables herein may be grafted into a TCR alpha and/or beta chain to create a chimeric TCR that binds specifically to a cancer associated antigen. For example, the LC CDRs disclosed herein may be grafted into the variable domain of a TCR alpha chain and the HC CDRs disclosed herein may be grafted to the variable domain of a TCR beta chain, or vice versa. Such chimeric TCRs may be produced by any appropriate method (For example, Willemsen RA et al, Gene Therapy 2000; 7: 1369–1377; Zhang T et al, Cancer Gene Ther 2004; 11: 487–496; Aggen et al, Gene Ther. 2012 Apr;19(4):365-74).

Non-antibody scaffolds

In embodiments, the antigen binding domain comprises a non antibody scaffold, e.g., a fibronectin, ankyrin, domain antibody, lipocalin, small modular immuno-pharmaceutical, maxybody, Protein A, or affilin. The non antibody scaffold has the ability to bind to target antigen on a cell. In embodiments, the antigen binding domain is a polypeptide or fragment thereof of a naturally occurring protein expressed on a cell. In some embodiments, the antigen binding domain comprises a non-antibody scaffold. A wide variety of non-antibody scaffolds can be employed so long as the resulting polypeptide includes at least one binding region which specifically binds to the target antigen on a target cell.

Non-antibody scaffolds include: fibronectin (Novartis, MA), ankyrin (Molecular Partners AG, Zurich, Switzerland), domain antibodies (Domantis, Ltd., Cambridge, MA, and Ablynx nv, Zwijnaarde, Belgium), lipocalin (Pieris Proteolab AG, Freising, Germany), small modular immuno-pharmaceuticals (Trubion Pharmaceuticals Inc., Seattle, WA), maxybodies (Avidia, Inc., Mountain View, CA), Protein A (Affibody AG, Sweden), and affilin (gamma-crystallin or ubiquitin) (Scil Proteins GmbH, Halle, Germany).

Fibronectin scaffolds can be based on fibronectin type III domain (e.g., the tenth module

of the fibronectin type III (^{10} Fn3 domain)). The fibronectin type III domain has 7 or 8 beta strands which are distributed between two beta sheets, which themselves pack against each other to form the core of the protein, and further containing loops (analogous to CDRs) which connect the beta strands to each other and are solvent exposed. There are at least three such loops at each edge of the beta sheet sandwich, where the edge is the boundary of the protein perpendicular to the direction of the beta strands (see US 6,818,418). Because of this structure, this non-antibody scaffold mimics antigen binding properties that are similar in nature and affinity to those of antibodies. These scaffolds can be used in a loop randomization and shuffling strategy in vitro that is similar to the process of affinity maturation of antibodies in vivo.

The ankyrin technology is based on using proteins with ankyrin derived repeat modules as scaffolds for bearing variable regions which can be used for binding to different targets. The ankyrin repeat module is a 33 amino acid polypeptide consisting of two anti-parallel α-helices and a β-turn. Binding of the variable regions is mostly optimized by using ribosome display.

Avimers are derived from natural A-domain containing protein such as HER3. These domains are used by nature for protein-protein interactions and in human over 250 proteins are structurally based on A-domains. Avimers consist of a number of different "A-domain" monomers (2-10) linked via amino acid linkers. Avimers can be created that can bind to the target antigen using the methodology described in, for example, U.S. Patent Application Publication Nos. 20040175756; 20050053973; 20050048512; and 20060008844.

Affibody affinity ligands are small, simple proteins composed of a three-helix bundle based on the scaffold of one of the IgG-binding domains of Protein A. Protein A is a surface protein from the bacterium Staphylococcus aureus. This scaffold domain consists of 58 amino acids, 13 of which are randomized to generate affibody libraries with a large number of ligand variants (See e.g., US 5,831,012). Affibody molecules mimic antibodies, they have a molecular weight of 6 kDa, compared to the molecular weight of antibodies, which is 150 kDa. In spite of its small size, the binding site of affibody molecules is similar to that of an antibody.

Protein epitope mimetics (PEM) are medium-sized, cyclic, peptide-like molecules (MW 1-2kDa) mimicking beta-hairpin secondary structures of proteins, the major secondary structure involved in protein-protein interactions. Antigen binding domains, e.g., those comprising scFv, single domain antibodies, or camelid antibodies, can be directed to any target receptor/ligand

described herein, e.g., the PD1 receptors, PD-L1 or PD-L2.

In an embodiment the antigen binding domain comprises the extracellular domain, or a counter-ligand binding fragment thereof, of molecule that binds a counterligand on the surface of a target cell.

An antigen binding domain can comprise the extracellular domain of an inhibitory receptor. Engagement with a counterligand of the coinhibitory molecule is redirected into an optimization of immune effector response.

An antigen binding domain can comprise the extracellular domain of a costimulatory molecule, referred to as a Costimulatory ECD domain, Engagement with a counter ligand of the costimulatory molecule results in optimization of immune effector response.

Transmembrane domain

With respect to the transmembrane domain, in various embodiments, a CAR can be designed to comprise a transmembrane domain that is attached to the extracellular domain of the CAR. A transmembrane domain can include one or more additional amino acids adjacent to the transmembrane region, e.g., one or more amino acid associated with the extracellular region of the protein from which the transmembrane was derived (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 up to 15 amino acids of the extracellular region) and/or one or more additional amino acids associated with the intracellular region of the protein from which the transmembrane protein is derived (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 up to 15 amino acids of the intracellular region). In one aspect, the transmembrane domain is one that is associated with one of the other domains of the CAR, e.g., in one embodiment, the transmembrane domain may be from the same protein that the signaling domain, costimulatory domain or the hinge domain is derived from. In another aspect, the transmembrane domain is not derived from the same protein that any other domain of the CAR is derived from. In some instances, the transmembrane domain can be selected or modified by amino acid substitution to avoid binding of such domains to the transmembrane domains of the same or different surface membrane proteins, e.g., to minimize interactions with other members of the receptor complex. In one aspect, the transmembrane domain is capable of homodimerization with another CAR on the cell surface of a CAR-expressing cell. In a different aspect the amino acid sequence of the transmembrane domain may be modified or substituted so as to minimize interactions with the binding domains of the native binding partner

present in the same CAR-expressing cell.

The transmembrane domain may be derived either from a natural or from a recombinant source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. In one aspect the transmembrane domain is capable of signaling to the intracellular domain(s) whenever the CAR has bound to a target. A transmembrane domain of particular use in this invention may include at least the transmembrane region(s) of e.g., the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154. In some embodiments, a transmembrane domain may include at least the transmembrane region(s) of, e.g., KIRDS2, OX40, CD2, CD27, LFA-1 (CD11a, CD18), ICOS (CD278), 4-1BB (CD137), GITR, CD40, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD160, CD19, IL2R beta, IL2R gamma, IL7R α, ITGA1, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, TNFR2, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, PAG/Cbp, NKG2D, NKG2C, or CD19.

In some instances, the transmembrane domain can be attached to the extracellular region of the CAR, e.g., the antigen binding domain of the CAR, via a hinge, e.g., a hinge from a human protein. For example, in one embodiment, the hinge can be a human Ig (immunoglobulin) hinge, e.g., an IgG4 hinge, an IgD hinge, a GS linker (e.g., a GS linker described herein), a KIR2DS2 hinge, or a CD8a hinge. In one embodiment, the hinge or spacer comprises (e.g., consists of) the amino acid sequence of SEQ ID NO:14. In one aspect, the transmembrane domain comprises (e.g., consists of) a transmembrane domain of SEQ ID NO: 15.

In one aspect, the hinge or spacer comprises an IgG4 hinge. For example, in one embodiment, the hinge or spacer comprises a hinge of the amino acid sequence

ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWY
VDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTI
SKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP

PVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGKM (SEQ ID NO:45). In some embodiments, the hinge or spacer comprises a hinge encoded by a nucleotide sequence of

GAGAGCAAGTACGGCCCTCCCTGCCCCCCTTGCCCTGCCCCCGAGTTCCTGGGCGGA
CCCAGCGTGTTCCTGTTCCCCCCCAAGCCCAAGGACACCCTGATGATCAGCCGGACC
CCCGAGGTGACCTGTGTGGTGGTGGACGTGTCCCAGGAGGACCCCGAGGTCCAGTT
CAACTGGTACGTGGACGGCGTGGAGGTGCACAACGCCAAGACCAAGCCCCGGGAG
GAGCAGTTCAATAGCACCTACCGGGTGGTGTCCGTGCTGACCGTGCTGCACCAGGA
CTGGCTGAACGGCAAGGAATACAAGTGTAAGGTGTCCAACAAGGGCCTGCCCAGCA
GCATCGAGAAAACCATCAGCAAGGCCAAGGGCCAGCCTCGGGAGCCCCAGGTGTA
CACCCTGCCCCCTAGCCAAGAGGAGATGACCAAGAACCAGGTGTCCCTGACCTGCC
TGGTGAAGGGCTTCTACCCCAGCGACATCGCCGTGGAGTGGGAGAGCAACGGCCAG
CCCGAGAACAACTACAAGACCACCCCCCCTGTGCTGGACAGCGACGGCAGCTTCTT
CCTGTACAGCCGGCTGACCGTGGACAAGAGCCGGTGGCAGGAGGGCAACGTCTTTA
GCTGCTCCGTGATGCACGAGGCCCTGCACAACCACTACACCCAGAAGAGCCTGAGC
CTGTCCCTGGGCAAGATG (SEQ ID NO:46).

In one aspect, the hinge or spacer comprises an IgD hinge. For example, in one embodiment, the hinge or spacer comprises a hinge of the amino acid sequence

RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKEKEKEEQEERET
KTPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFVVGSDLKDAHLTWEVAGKVPTG
GVEEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLNHPSLPPQRLMALREPAAQAP
VKLSLNLLASSDPPEAASWLLCEVSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGST
TFWAWSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSYVTDH (SEQ ID NO:47). In some embodiments, the hinge or spacer comprises a hinge encoded by a nucleotide sequence of

AGGTGGCCCGAAAGTCCCAAGGCCCAGGCATCTAGTGTTCCTACTGCACAGCCCCA
GGCAGAAGGCAGCCTAGCCAAAGCTACTACTGCACCTGCCACTACGCGCAATACTG
GCCGTGGCGGGGAGGAGAAGAAAAAGGAGAAAGAGAAAGAAGAACAGGAAGAGA
GGGAGACCAAGACCCCTGAATGTCCATCCCATACCCAGCCGCTGGGCGTCTATCTCT
TGACTCCCGCAGTACAGGACTTGTGGCTTAGAGATAAGGCCACCTTTACATGTTTCG
TCGTGGGCTCTGACCTGAAGGATGCCCATTTGACTTGGGAGGTTGCCGGAAAGGTA

CCCACAGGGGGGGTTGAGGAAGGGTTGCTGGAGCGCCATTCCAATGGCTCTCAGAG
CCAGCACTCAAGACTCACCCTTCCGAGATCCCTGTGGAACGCCGGGACCTCTGTCAC
ATGTACTCTAAATCATCCTAGCCTGCCCCCACAGCGTCTGATGGCCCTTAGAGAGCC
AGCCGCCCAGGCACCAGTTAAGCTTAGCCTGAATCTGCTCGCCAGTAGTGATCCCCC
AGAGGCCGCCAGCTGGCTCTTATGCGAAGTGTCCGGCTTTAGCCCGCCCAACATCTT
GCTCATGTGGCTGGAGGACCAGCGAGAAGTGAACACCAGCGGCTTCGCTCCAGCCC
GGCCCCCACCCCAGCCGGGTTCTACCACATTCTGGGCCTGGAGTGTCTTAAGGGTCC
CAGCACCACCTAGCCCCCAGCCAGCCACATACACCTGTGTTGTGTCCCATGAAGAT
AGCAGGACCCTGCTAAATGCTTCTAGGAGTCTGGAGGTTTCCTACGTGACTGACCAT
T (SEQ ID NO:48).

In one aspect, the transmembrane domain may be recombinant, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. In one aspect a triplet of phenylalanine, tryptophan and valine can be found at each end of a recombinant transmembrane domain.

Optionally, a short oligo- or polypeptide linker, between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic region of the CAR. A glycine-serine doublet provides a particularly suitable linker. For example, in one aspect, the linker comprises the amino acid sequence of GGGGSGGGGS (SEQ ID NO:49). In some embodiments, the linker is encoded by a nucleotide sequence of GGTGGCGGAGGTTCTGGAGGTGGAGGTTCC (SEQ ID NO:50).

In one aspect, the hinge or spacer comprises a KIR2DS2 hinge.

Cytoplasmic domain

The cytoplasmic domain or region of the CAR includes an intracellular signaling domain. An intracellular signaling domain is generally responsible for activation of at least one of the normal effector functions of the immune cell in which the CAR has been introduced.

Examples of intracellular signaling domains for use in the CAR of the invention include the cytoplasmic sequences of the T cell receptor (TCR) and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivative or

variant of these sequences and any recombinant sequence that has the same functional capability.

It is known that signals generated through the TCR alone are insufficient for full activation of the T cell and that a secondary and/or costimulatory signal is also required. Thus, T cell activation can be said to be mediated by two distinct classes of cytoplasmic signaling sequences: those that initiate antigen-dependent primary activation through the TCR (primary intracellular signaling domains) and those that act in an antigen-independent manner to provide a secondary or costimulatory signal (secondary cytoplasmic domain, e.g., a costimulatory domain).

A primary signaling domain regulates primary activation of the TCR complex either in a stimulatory way, or in an inhibitory way. Primary intracellular signaling domains that act in a stimulatory manner may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs or ITAMs.

Examples of ITAM containing primary intracellular signaling domains that are of particular use in the invention include those of CD3 zeta, common FcR gamma (FCER1G), Fc gamma RIIa, FcR beta (Fc Epsilon R1b), CD3 gamma, CD3 delta, CD3 epsilon, CD79a, CD79b, CD278 (also known as "ICOS"), FcεRI, DAP10, DAP12, and CD66d. In one embodiment, a CAR of the invention comprises an intracellular signaling domain, e.g., a primary signaling domain of CD3-zeta.

In one embodiment, a primary signaling domain comprises a modified ITAM domain, e.g., a mutated ITAM domain which has altered (e.g., increased or decreased) activity as compared to the native ITAM domain. In one embodiment, a primary signaling domain comprises a modified ITAM-containing primary intracellular signaling domain, e.g., an optimized and/or truncated ITAM-containing primary intracellular signaling domain. In an embodiment, a primary signaling domain comprises one, two, three, four or more ITAM motifs.

Further examples of molecules containing a primary intracellular signaling domain that are of particular use in the invention include those of DAP10, DAP12, and CD32.

Costimulatory Signaling Domain

The intracellular signalling domain of the CAR can comprise the CD3-zeta signaling

domain by itself or it can be combined with any other desired intracellular signaling domain(s) useful in the context of a CAR of the invention. For example, the intracellular signaling domain of the CAR can comprise a CD3 zeta chain portion and a costimulatory signaling domain. The costimulatory signaling domain refers to a portion of the CAR comprising the intracellular domain of a costimulatory molecule. In one embodiment, the intracellular domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of CD28. In one aspect, the intracellular domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of ICOS.

A costimulatory molecule can be a cell surface molecule other than an antigen receptor or its ligands that is required for an efficient response of lymphocytes to an antigen. Examples of such molecules include CD27, CD28, 4-1BB (CD137), OX40, CD30, CD40, PD1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83, and the like. For example, CD27 costimulation has been demonstrated to enhance expansion, effector function, and survival of human CART cells in vitro and augments human T cell persistence and antitumor activity in vivo (Song et al. Blood. 2012; 119(3):696-706). Further examples of such costimulatory molecules include MHC class I molecule, TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83.

The intracellular signaling sequences within the cytoplasmic portion of the CAR of the invention may be linked to each other in a random or specified order. Optionally, a short oligo-

or polypeptide linker, for example, between 2 and 10 amino acids (e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acids) in length may form the linkage between intracellular signaling sequence. In one embodiment, a glycine-serine doublet can be used as a suitable linker. In one embodiment, a single amino acid, e.g., an alanine, a glycine, can be used as a suitable linker.

In one aspect, the intracellular signaling domain is designed to comprise two or more, e.g., 2, 3, 4, 5, or more, costimulatory signaling domains. In an embodiment, the two or more, e.g., 2, 3, 4, 5, or more, costimulatory signaling domains, are separated by a linker molecule, e.g., a linker molecule described herein. In one embodiment, the intracellular signaling domain comprises two costimulatory signaling domains. In some embodiments, the linker molecule is a glycine residue. In some embodiments, the linker is an alanine residue.

In one aspect, the intracellular signaling domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of CD28. In one aspect, the intracellular signaling domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of 4-1BB. In one aspect, the signaling domain of 4-1BB is a signaling domain of SEQ ID NO: 16. In one aspect, the signaling domain of CD3-zeta is a signaling domain of SEQ ID NO: 17.

In one aspect, the intracellular signaling domain is designed to comprise the signaling domain of CD3-zeta and the signaling domain of CD27. In one aspect, the signaling domain of CD27 comprises an amino acid sequence of QRRKYRSNKGESPVEPAEPCRYSCPREEEGSTIPIQEDYRKPEPACSP (SEQ ID NO:51). In one aspect, the signalling domain of CD27 is encoded by a nucleic acid sequence of AGGAGTAAGAGGAGCAGGCTCCTGCACAGTGACTACATGAACATGACTCCCCGCCG CCCCGGGCCCACCCGCAAGCATTACCAGCCCTATGCCCCACCACGCGACTTCGCAG CCTATCGCTCC (SEQ ID NO:52).

Natural Killer Cell Receptor (NKR) CARs

In an embodiment, a CAR molecule described herein comprises one or more components of a natural killer cell receptor (NKR), thereby forming an NKR-CAR. The NKR component can be a transmembrane domain, a hinge domain, or a cytoplasmic domain from any of the following natural killer cell receptors: killer cell immunoglobulin-like receptor (KIR), e.g., KIR2DL1, KIR2DL2/L3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3,

KIR2DS4, DIR2DS5, KIR3DL1/S1, KIR3DL2, KIR3DL3, KIR2DP1, and KIR3DP1; natural cytotoxicity receptor (NCR), e.g., NKp30, NKp44, NKp46; signaling lymphocyte activation molecule (SLAM) family of immune cell receptors, e.g., CD48, CD229, 2B4, CD84, NTB-A, CRACC, BLAME, and CD2F-10; Fc receptor (FcR), e.g., CD16, and CD64; and Ly49 receptors, e.g., LY49A, LY49C. The NKR-CAR molecules described herein may interact with an adaptor molecule or intracellular signaling domain, e.g., DAP12. Exemplary configurations and sequences of CAR molecules comprising NKR components are described in International Publication No. WO2014/145252, the contents of which are hereby incorporated by reference.

Strategies for Regulating Chimeric Antigen Receptors

In some embodiments, a regulatable CAR (RCAR) where the CAR activity can be controlled is desirable to optimize the safety and efficacy of a CAR therapy. There are many ways CAR activities can be regulated. For example, inducing apoptosis using, e.g., a caspase fused to a dimerization domain (see, e.g., Di et al., N Engl. J. Med. 2011 Nov. 3; 365(18):1673-1683), can be used as a safety switch in the CAR therapy of the instant invention. In one embodiment, the cells (e.g., T cells or NK cells) expressing a CAR of the present invention further comprise an inducible apoptosis switch, wherein a human caspase (e.g., caspase 9) or a modified version is fused to a modification of the human FKB protein that allows conditional dimerization. In the presence of a small molecule, such as a rapalog (e.g., AP 1903, AP20187), the inducible caspase (e.g., caspase 9) is activated and leads to the rapid apoptosis and death of the cells (e.g., T cells or NK cells) expressing a CAR of the present invention. Examples of a caspase-based inducible apoptosis switch (or one or more aspects of such a switch) have been described in, e.g., US2004040047; US20110286980; US20140255360; WO1997031899; WO2014151960; WO2014164348; WO2014197638; WO2014197638; all of which are incorporated by reference herein.

In another example, CAR-expressing cells can also express an inducible Caspase-9 (iCaspase-9) molecule that, upon administration of a dimerizer drug (e.g., rimiducid (also called AP1903 (Bellicum Pharmaceuticals) or AP20187 (Ariad)) leads to activation of the Caspase-9 and apoptosis of the cells. The iCaspase-9 molecule contains a chemical inducer of dimerization (CID) binding domain that mediates dimerization in the presence of a CID. This results in inducible and selective depletion of CAR-expressing cells. In some cases, the iCaspase-9

molecule is encoded by a nucleic acid molecule separate from the CAR-encoding vector(s). In some cases, the iCaspase-9 molecule is encoded by the same nucleic acid molecule as the CAR-encoding vector. The iCaspase-9 can provide a safety switch to avoid any toxicity of CAR-expressing cells. See, e.g., Song et al. Cancer Gene Ther. 2008; 15(10):667-75; Clinical Trial Id. No. NCT02107963; and Di Stasi et al. N. Engl. J. Med. 2011; 365:1673-83.

Alternative strategies for regulating the CAR therapy of the instant invention include utilizing small molecules or antibodies that deactivate or turn off CAR activity, e.g., by deleting CAR-expressing cells, e.g., by inducing antibody dependent cell-mediated cytotoxicity (ADCC). For example, CAR-expressing cells described herein may also express an antigen that is recognized by molecules capable of inducing cell death, e.g., ADCC or complement-induced cell death. For example, CAR expressing cells described herein may also express a receptor capable of being targeted by an antibody or antibody fragment. Examples of such receptors include EpCAM, VEGFR, integrins (e.g., integrins αvβ3, α4, αI¾β3, α4β7, α5β1, αvβ3, αv), members of the TNF receptor superfamily (e.g., TRAIL-R1 , TRAIL-R2), PDGF Receptor, interferon receptor, folate receptor, GPNMB, ICAM-1 , HLA-DR, CEA, CA-125, MUC1 , TAG-72, IL-6 receptor, 5T4, GD2, GD3, CD2, CD3, CD4, CD5, CD1 1 , CD1 1 a/LFA-1 , CD15, CD18/ITGB2, CD19, CD20, CD22, CD23/lgE Receptor, CD25, CD28, CD30, CD33, CD38, CD40, CD41 , CD44, CD51 , CD52, CD62L, CD74, CD80, CD125, CD147/basigin, CD152/CTLA-4, CD154/CD40L, CD195/CCR5, CD319/SLAMF7, and EGFR, and truncated versions thereof (e.g., versions preserving one or more extracellular epitopes but lacking one or more regions within the cytoplasmic domain).

For example, a CAR-expressing cell described herein may also express a truncated epidermal growth factor receptor (EGFR) which lacks signaling capacity but retains the epitope that is recognized by molecules capable of inducing ADCC, e.g., cetuximab (ERBITUX®), such that administration of cetuximab induces ADCC and subsequent depletion of the CAR-expressing cells (see, e.g., WO2011/056894, and Jonnalagadda et al., Gene Ther. 2013; 20(8)853-860). Another strategy includes expressing a highly compact marker/suicide gene that combines target epitopes from both CD32 and CD20 antigens in the CAR-expressing cells described herein, which binds rituximab, resulting in selective depletion of the CAR-expressing cells, e.g., by ADCC (see, e.g., Philip et al., Blood. 2014; 124(8)1277-1287). Other methods for depleting CAR-expressing cells described herein include administration of CAMPATH, a

monoclonal anti-CD52 antibody that selectively binds and targets mature lymphocytes, e.g., CAR-expressing cells, for destruction, e.g., by inducing ADCC. In other embodiments, the CAR-expressing cell can be selectively targeted using a CAR ligand, e.g., an anti-idiotypic antibody. In some embodiments, the anti-idiotypic antibody can cause effector cell activity, e.g., ADCC or ADC activities, thereby reducing the number of CAR-expressing cells. In other embodiments, the CAR ligand, e.g., the anti-idiotypic antibody, can be coupled to an agent that induces cell killing, e.g., a toxin, thereby reducing the number of CAR-expressing cells. Alternatively, the CAR molecules themselves can be configured such that the activity can be regulated, e.g., turned on and off, as described below.

In other embodiments, a CAR-expressing cell described herein may also express a target protein recognized by the T cell depleting agent. In one embodiment, the target protein is CD20 and the T cell depleting agent is an anti-CD20 antibody, e.g., rituximab. In such embodiment, the T cell depleting agent is administered once it is desirable to reduce or eliminate the CAR-expressing cell, e.g., to mitigate the CAR induced toxicity. In other embodiments, the T cell depleting agent is an anti-CD52 antibody, e.g., alemtuzumab.

In an aspect, a RCAR comprises a set of polypeptides, typically two in the simplest embodiments, in which the components of a standard CAR described herein, e.g., an antigen binding domain and an intracellular signaling domain, are partitioned on separate polypeptides or members. In some embodiments, the set of polypeptides include a dimerization switch that, upon the presence of a dimerization molecule, can couple the polypeptides to one another, e.g., can couple an antigen binding domain to an intracellular signaling domain. In one embodiment, a CAR of the present invention utilizes a dimerization switch as those described in, e.g., WO2014127261, which is incorporated by reference herein. Additional description and exemplary configurations of such regulatable CARs are provided herein and in International Publication No. WO 2015/090229, hereby incorporated by reference in its entirety.

In some embodiments, an RCAR involves a switch domain, e.g., a FKBP switch domain, as set out SEQ ID NO: 122, or comprise a fragment of FKBP having the ability to bind with FRB, e.g., as set out in SEQ ID NO: 123. In some embodiments, the RCAR involves a switch domain comprising a FRB sequence, e.g., as set out in SEQ ID NO: 124, or a mutant FRB sequence, e.g., as set out in any of SEQ ID Nos. 125-130.D V P D Y A S L G G P S S P K K K R K V S R G V Q V E T I S P G D G R T F P K R G Q T C V V H Y T G M L E D G K K

FDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTIS PDYAYGATGHPGIIPPHATLVFDVELLKLETSY (SEQ ID NO: 122)

VQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKFDSSR DRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPDYAY GATGHPGIIPPHATLVFDVELLKLETS (SEQ ID NO: 123)

ILWHEMWHEG LEEASRLYFG ERNVKGMFEV LEPLHAMMER GPQTLKETSF NQAYGRDLME AQEWCRKYMK SGNVKDLTQA WDLYYHVFRR ISK (SEQ ID NO: 124)

Table 1. Exemplary mutant FRB having increased affinity for a dimerization molecule.

FRB mutant	**Amino Acid Sequence**	**SEQ ID NO:**
E2032I mutant	ILWHEMWHEGLIEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISKTS	125
E2032L mutant	ILWHEMWHEGLLEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISKTS	126
T2098L mutant	ILWHEMWHEGLEEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLLQAWDLYYHVFRRISKTS	127
E2032, T2098 mutant	ILWHEMWHEGL**X**EASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDL**X**QAWDLYYHVFRRISKTS	128
E2032I, T2098L mutant	ILWHEMWHEGLIEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLLQAWDLYYHVFRRISKTS	129
E2032L, T2098L mutant	ILWHEMWHEGLLEASRLYFGERNVKGMFEVLEPLHAMMERGPQTLKETSFNQAYGR DLMEAQEWCRKYMKSGNVKDLLQAWDLYYHVFRRISKTS	130

Split CAR

In some embodiments, the CAR-expressing cell uses a split CAR. The split CAR approach is described in more detail in publications WO2014/055442 and WO2014/055657. Briefly, a split CAR system comprises a cell expressing a first CAR having a first antigen binding domain and a costimulatory domain (e.g., 41BB), and the cell also expresses a second CAR having a second antigen binding domain and an intracellular signaling domain (e.g., CD3 zeta). When the cell encounters the first antigen, the costimulatory domain is activated, and the cell proliferates. When the cell encounters the second antigen, the intracellular signaling domain is activated and cell-killing activity begins. Thus, the CAR-expressing cell is only fully activated in the presence of both antigens.

RNA Transfection

Disclosed herein are methods for producing an in vitro transcribed RNA CAR. The present invention also includes (among other things) a CAR encoding RNA construct that can be directly transfected into a cell. A method for generating mRNA for use in transfection can involve in vitro transcription (IVT) of a template with specially designed primers, followed by polyA addition, to produce a construct containing 3' and 5' untranslated sequence ("UTR"), a 5' cap and/or Internal Ribosome Entry Site (IRES), the nucleic acid to be expressed, and a polyA tail, typically 50-2000 bases in length (SEQ ID NO:118). RNA so produced can efficiently transfect different kinds of cells. In one aspect, the template includes sequences for the CAR.

In one aspect the CAR is encoded by a messenger RNA (mRNA). In one aspect the mRNA encoding the CAR is introduced into an immune effector cell, e.g., a T cell or a NK cell, for production of a CAR-expressing cell, e.g., a CART cell or a CAR NK cell.

In one embodiment, the in vitro transcribed RNA CAR can be introduced to a cell as a form of transient transfection. The RNA is produced by in vitro transcription using a polymerase chain reaction (PCR)-generated template. DNA of interest from any source can be directly converted by PCR into a template for in vitro mRNA synthesis using appropriate primers and RNA polymerase. The source of the DNA can be, for example, genomic DNA, plasmid DNA, phage DNA, cDNA, synthetic DNA sequence or any other appropriate source of DNA. A desired temple for in vitro transcription is a CAR of the present invention. For example, the template for the RNA CAR comprises an extracellular region comprising a single chain variable domain of an anti-tumor antibody; a hinge region, a transmembrane domain (e.g., a transmembrane domain of CD8a); and a cytoplasmic region that includes an intracellular signaling domain, e.g., comprising the signaling domain of CD3-zeta and the signaling domain of 4-1BB.

In one embodiment, the DNA to be used for PCR contains an open reading frame. The DNA can be from a naturally occurring DNA sequence from the genome of an organism. In one embodiment, the nucleic acid can include some or all of the 5' and/or 3' untranslated regions (UTRs). The nucleic acid can include exons and introns. In one embodiment, the DNA to be used for PCR is a human nucleic acid sequence. In another embodiment, the DNA to be used for PCR is a human nucleic acid sequence including the 5' and 3' UTRs. The DNA can alternatively

be an artificial DNA sequence that is not normally expressed in a naturally occurring organism. An exemplary artificial DNA sequence is one that contains portions of genes that are ligated together to form an open reading frame that encodes a fusion protein. The portions of DNA that are ligated together can be from a single organism or from more than one organism.

PCR is used to generate a template for in vitro transcription of mRNA which is used for transfection. Methods for performing PCR are well known in the art. Primers for use in PCR are designed to have regions that are substantially complementary to regions of the DNA to be used as a template for the PCR. "Substantially complementary," as used herein, refers to sequences of nucleotides where a majority or all of the bases in the primer sequence are complementary, or one or more bases are non-complementary, or mismatched. Substantially complementary sequences are able to anneal or hybridize with the intended DNA target under annealing conditions used for PCR. The primers can be designed to be substantially complementary to any portion of the DNA template. For example, the primers can be designed to amplify the portion of a nucleic acid that is normally transcribed in cells (the open reading frame), including 5' and 3' UTRs. The primers can also be designed to amplify a portion of a nucleic acid that encodes a particular domain of interest. In one embodiment, the primers are designed to amplify the coding region of a human cDNA, including all or portions of the 5' and 3' UTRs. Primers useful for PCR can be generated by synthetic methods that are well known in the art. "Forward primers" are primers that contain a region of nucleotides that are substantially complementary to nucleotides on the DNA template that are upstream of the DNA sequence that is to be amplified. "Upstream" is used herein to refer to a location 5, to the DNA sequence to be amplified relative to the coding strand. "Reverse primers" are primers that contain a region of nucleotides that are substantially complementary to a double-stranded DNA template that are downstream of the DNA sequence that is to be amplified. "Downstream" is used herein to refer to a location 3' to the DNA sequence to be amplified relative to the coding strand.

Any DNA polymerase useful for PCR can be used in the methods disclosed herein. The reagents and polymerase are commercially available from a number of sources.

Chemical structures with the ability to promote stability and/or translation efficiency may also be used. The RNA in some embodiments has 5' and 3' UTRs. In one embodiment, the 5' UTR is between one and 3000 nucleotides in length. The length of 5' and 3' UTR sequences to be added to the coding region can be altered by different methods, including, but not limited to,

designing primers for PCR that anneal to different regions of the UTRs. Using this approach, one of ordinary skill in the art can modify the 5' and 3' UTR lengths required to achieve optimal translation efficiency following transfection of the transcribed RNA.

The 5' and 3' UTRs can be the naturally occurring, endogenous 5' and 3' UTRs for the nucleic acid of interest. Alternatively, UTR sequences that are not endogenous to the nucleic acid of interest can be added by incorporating the UTR sequences into the forward and reverse primers or by any other modifications of the template. The use of UTR sequences that are not endogenous to the nucleic acid of interest can be useful for modifying the stability and/or translation efficiency of the RNA. For example, it is known that AU-rich elements in 3' UTR sequences can decrease the stability of mRNA. Therefore, 3' UTRs can be selected or designed to increase the stability of the transcribed RNA based on properties of UTRs that are well known in the art.

In one embodiment, the 5' UTR can contain the Kozak sequence of the endogenous nucleic acid. Alternatively, when a 5' UTR that is not endogenous to the nucleic acid of interest is being added by PCR as described above, a consensus Kozak sequence can be redesigned by adding the 5' UTR sequence. Kozak sequences can increase the efficiency of translation of some RNA transcripts, but does not appear to be required for all RNAs to enable efficient translation. The requirement for Kozak sequences for many mRNAs is known in the art. In other embodiments the 5' UTR can be 5'UTR of an RNA virus whose RNA genome is stable in cells. In other embodiments various nucleotide analogues can be used in the 3' or 5' UTR to impede exonuclease degradation of the mRNA.

To enable synthesis of RNA from a DNA template without the need for gene cloning, a promoter of transcription should be attached to the DNA template upstream of the sequence to be transcribed. When a sequence that functions as a promoter for an RNA polymerase is added to the 5' end of the forward primer, the RNA polymerase promoter becomes incorporated into the PCR product upstream of the open reading frame that is to be transcribed. In one embodiment, the promoter is a T7 polymerase promoter, as described elsewhere herein. Other useful promoters include, but are not limited to, T3 and SP6 RNA polymerase promoters. Consensus nucleotide sequences for T7, T3 and SP6 promoters are known in the art.

In an embodiment, the mRNA has both a cap on the 5' end and a 3' poly(A) tail which

determine ribosome binding, initiation of translation and stability mRNA in the cell. On a circular DNA template, for instance, plasmid DNA, RNA polymerase produces a long concatameric product which is not suitable for expression in eukaryotic cells. The transcription of plasmid DNA linearized at the end of the 3' UTR results in normal sized mRNA which is not effective in eukaryotic transfection even if it is polyadenylated after transcription.

On a linear DNA template, phage T7 RNA polymerase can extend the 3' end of the transcript beyond the last base of the template (Schenborn and Mierendorf, Nuc Acids Res., 13:6223-36 (1985); Nacheva and Berzal-Herranz, Eur. J. Biochem., 270:1485-65 (2003).

The conventional method of integration of polyA/T stretches into a DNA template is molecular cloning. However polyA/T sequence integrated into plasmid DNA can cause plasmid instability, which is why plasmid DNA templates obtained from bacterial cells are often highly contaminated with deletions and other aberrations. This makes cloning procedures not only laborious and time consuming but often not reliable. That is why a method which allows construction of DNA templates with polyA/T 3' stretch without cloning highly desirable.

The polyA/T segment of the transcriptional DNA template can be produced during PCR by using a reverse primer containing a polyT tail, such as 100T tail (SEQ ID NO: 29) (size can be 50-5000 T (SEQ ID NO: 30)), or after PCR by any other method, including, but not limited to, DNA ligation or in vitro recombination. Poly(A) tails also provide stability to RNAs and reduce their degradation. Generally, the length of a poly(A) tail positively correlates with the stability of the transcribed RNA. In one embodiment, the poly(A) tail is between 100 and 5000 adenosines (SEQ ID NO: 57).

Poly(A) tails of RNAs can be further extended following in vitro transcription with the use of a poly(A) polymerase, such as E. coli polyA polymerase (E-PAP). In one embodiment, increasing the length of a poly(A) tail from 100 nucleotides to between 300 and 400 nucleotides (SEQ ID NO: 104) results in about a two-fold increase in the translation efficiency of the RNA. Additionally, the attachment of different chemical groups to the 3' end can increase mRNA stability. Such attachment can contain modified/artificial nucleotides, aptamers and other compounds. For example, ATP analogs can be incorporated into the poly(A) tail using poly(A) polymerase. ATP analogs can further increase the stability of the RNA.

5' caps on also provide stability to RNA molecules. In an embodiment, RNAs produced

by the methods disclosed herein include a 5' cap. The 5' cap is provided using techniques known in the art and described herein (Cougot, et al., Trends in Biochem. Sci., 29:436-444 (2001); Stepinski, et al., RNA, 7:1468-95 (2001); Elango, et al., Biochim. Biophys. Res. Commun., 330:958-966 (2005)).

The RNAs produced by the methods disclosed herein can also contain an internal ribosome entry site (IRES) sequence. The IRES sequence may be any viral, chromosomal or artificially designed sequence which initiates cap-independent ribosome binding to mRNA and facilitates the initiation of translation. Any solutes suitable for cell electroporation, which can contain factors facilitating cellular permeability and viability such as sugars, peptides, lipids, proteins, antioxidants, and surfactants can be included.

RNA can be introduced into target cells using any of a number of different methods, for instance, commercially available methods which include, but are not limited to, electroporation (Amaxa Nucleofector-II (Amaxa Biosystems, Cologne, Germany)), (ECM 830 (BTX) (Harvard Instruments, Boston, Mass.) or the Gene Pulser II (BioRad, Denver, Colo.), Multiporator (Eppendort, Hamburg Germany), cationic liposome mediated transfection using lipofection, polymer encapsulation, peptide mediated transfection, or biolistic particle delivery systems such as “gene guns” (see, for example, Nishikawa, et al. Hum Gene Ther., 12(8):861-70 (2001).

Non-viral delivery methods

In some aspects, non-viral methods can be used to deliver a nucleic acid encoding a CAR described herein into a cell or tissue or a subject.

In some embodiments, the non-viral method includes the use of a transposon (also called a transposable element). In some embodiments, a transposon is a piece of DNA that can insert itself at a location in a genome, for example, a piece of DNA that is capable of self-replicating and inserting its copy into a genome, or a piece of DNA that can be spliced out of a longer nucleic acid and inserted into another place in a genome. For example, a transposon comprises a DNA sequence made up of inverted repeats flanking genes for transposition.

Exemplary methods of nucleic acid delivery using a transposon include a Sleeping Beauty transposon system (SBTS) and a piggyBac (PB) transposon system. See, e.g.,

Aronovich et al. Hum. Mol. Genet. 20.R1(2011):R14-20; Singh et al. Cancer Res. 15(2008):2961–2971; Huang et al. Mol. Ther. 16(2008):580–589; Grabundzija et al. Mol. Ther. 18(2010):1200–1209; Kebriaei et al. Blood. 122.21(2013):166; Williams. Molecular Therapy 16.9(2008):1515–16; Bell et al. Nat. Protoc. 2.12(2007):3153-65; and Ding et al. Cell. 122.3(2005):473-83, all of which are incorporated herein by reference.

The SBTS includes two components: 1) a transposon containing a transgene and 2) a source of transposase enzyme. The transposase can transpose the transposon from a carrier plasmid (or other donor DNA) to a target DNA, such as a host cell chromosome/genome. For example, the transposase binds to the carrier plasmid/donor DNA, cuts the transposon (including transgene(s)) out of the plasmid, and inserts it into the genome of the host cell. See, e.g., Aronovich et al.

Exemplary transposons include a pT2-based transposon. See, e.g., Grabundzija et al. Nucleic Acids Res. 41.3(2013):1829-47; and Singh et al. Cancer Res. 68.8(2008): 2961–2971, all of which are incorporated herein by reference. Exemplary transposases include a Tc1/mariner-type transposase, e.g., the SB10 transposase or the SB11 transposase (a hyperactive transposase which can be expressed, e.g., from a cytomegalovirus promoter). See, e.g., Aronovich et al.; Kebriaei et al.; and Grabundzija et al., all of which are incorporated herein by reference.

Use of the SBTS permits efficient integration and expression of a transgene, e.g., a nucleic acid encoding a CAR described herein. Provided herein are methods of generating a cell, e.g., T cell or NK cell, that stably expresses a CAR described herein, e.g., using a transposon system such as SBTS.

In accordance with methods described herein, in some embodiments, one or more nucleic acids, e.g., plasmids, containing the SBTS components are delivered to a cell (e.g., T or NK cell). For example, the nucleic acid(s) are delivered by standard methods of nucleic acid (e.g., plasmid DNA) delivery, e.g., methods described herein, e.g., electroporation, transfection, or lipofection. In some embodiments, the nucleic acid contains a transposon comprising a transgene, e.g., a nucleic acid encoding a CAR described herein. In some embodiments, the nucleic acid contains a transposon comprising a transgene (e.g., a nucleic acid encoding a CAR described herein) as well as a nucleic acid sequence encoding a transposase enzyme. In other

embodiments, a system with two nucleic acids is provided, e.g., a dual-plasmid system, e.g., where a first plasmid contains a transposon comprising a transgene, and a second plasmid contains a nucleic acid sequence encoding a transposase enzyme. For example, the first and the second nucleic acids are co-delivered into a host cell.

In some embodiments, cells, e.g., T or NK cells, are generated that express a CAR described herein by using a combination of gene insertion using the SBTS and genetic editing using a nuclease (e.g., Zinc finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs), the CRISPR/Cas system, or engineered meganuclease re-engineered homing endonucleases).

In some embodiments, use of a non-viral method of delivery permits reprogramming of cells, e.g., T or NK cells, and direct infusion of the cells into a subject. Advantages of non-viral vectors include but are not limited to the ease and relatively low cost of producing sufficient amounts required to meet a patient population, stability during storage, and lack of immunogenicity.

Nucleic Acid Constructs Encoding a CAR, e.g., a CD19 CAR, CD20 CAR, or CD22 CAR

The present invention also provides nucleic acid molecules encoding one or more CAR constructs described herein, e.g., CD19 CAR, CD20 CAR, or CD22 CAR. In one aspect, the nucleic acid molecule is provided as a messenger RNA transcript. In one aspect, the nucleic acid molecule is provided as a DNA construct.

Accordingly, in one aspect, the invention pertains to an isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR), wherein the CAR comprises a binding domain (e.g., that binds CD19, CD20, or CD22) a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, e.g., a costimulatory signaling domain and/or a primary signaling domain, e.g., zeta chain.

In one embodiment, the binding domain is an anti-CD19 binding domain described herein, e.g., an anti-CD19 binding domain which comprises a sequence selected from a group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:59, or a sequence with 95-99% identity thereof.

In one embodiment, the nucleic acid comprises CD22-encoding a nucleic acid set out in **Table 6A** or a sequence with 95-99% identity thereof. In one embodiment, the nucleic acid is a nucleic acid encoding an amino acid sequence set out in any of **Tables 6A-10B** or a sequence with 95-99% identity thereof.

In one embodiment, the nucleic acid comprises CD20-encoding a nucleic acid set out in **Table 11A** or a sequence with 95-99% identity thereof. In one embodiment, the nucleic acid is a nucleic acid encoding an amino acid sequence set out in any of **Tables 11A-15B** or a sequence with 95-99% identity thereof.

In one embodiment, the transmembrane domain is transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. In one embodiment, the transmembrane domain comprises a sequence of SEQ ID NO: 15, or a sequence with 95-99% identity thereof. In one embodiment, the anti-CD19 binding domain is connected to the transmembrane domain by a hinge region, e.g., a hinge described herein. In one embodiment, the hinge region comprises SEQ ID NO:14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49, or a sequence with 95-99% identity thereof. In one embodiment, the isolated nucleic acid molecule further comprises a sequence encoding a costimulatory domain. In one embodiment, the costimulatory domain is a functional signaling domain of a protein selected from the group consisting of OX40, CD27, CD28, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), and 4-1BB (CD137). In one embodiment, the costimulatory domain is a functional signaling domain of a protein selected from the group consisting of MHC class I molecule, TNF receptor proteins, Immunoglobulin-like proteins, cytokine receptors, integrins, signaling lymphocytic activation molecules (SLAM proteins), activating NK cell receptors, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), KIRDS2, SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229),

CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83. In one embodiment, the costimulatory domain comprises a sequence of SEQ ID NO:16, or a sequence with 95-99% identity thereof. In one embodiment, the intracellular signaling domain comprises a functional signaling domain of 4-1BB and a functional signaling domain of CD3 zeta. In one embodiment, the intracellular signaling domain comprises the sequence of SEQ ID NO: 16 or SEQ ID NO:51, or a sequence with 95-99% identity thereof, and the sequence of SEQ ID NO: 17 or SEQ ID NO:43, or a sequence with 95-99% identity thereof, wherein the sequences comprising the intracellular signaling domain are expressed in the same frame and as a single polypeptide chain.

In another aspect, the invention pertains to an isolated nucleic acid molecule encoding a CAR construct comprising a leader sequence of SEQ ID NO: 13, a scFv domain having a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:59, (or a sequence with 95-99% identity thereof), a hinge region of SEQ ID NO:14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49 (or a sequence with 95-99% identity thereof), a transmembrane domain having a sequence of SEQ ID NO: 15 (or a sequence with 95-99% identity thereof), a 4-1BB costimulatory domain having a sequence of SEQ ID NO:16 or a CD27 costimulatory domain having a sequence of SEQ ID NO:51 (or a sequence with 95-99% identity thereof), and a CD3 zeta stimulatory domain having a sequence of SEQ ID NO:17 or SEQ ID NO:43 (or a sequence with 95-99% identity thereof).

In another aspect, the invention pertains to an isolated polypeptide molecule encoded by the nucleic acid molecule. In one embodiment, the isolated polypeptide molecule comprises a sequence selected from the group consisting of SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:59 or a sequence with 95-99% identity thereof.

In another aspect, the invention pertains to a nucleic acid molecule encoding a chimeric antigen receptor (CAR) molecule that comprises an anti-CD19 binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said

anti-CD19 binding domain comprises a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:59, or a sequence with 95-99% identity thereof.

In one embodiment, the encoded CAR molecule (e.g., CD19 CAR, CD20 CAR, or CD22 CAR) further comprises a sequence encoding a costimulatory domain. In one embodiment, the costimulatory domain is a functional signaling domain of a protein selected from the group consisting of OX40, CD27, CD28, CDS, ICAM-1, LFA-1 (CD11a/CD18) and 4-1BB (CD137). In one embodiment, the costimulatory domain comprises a sequence of SEQ ID NO:16. In one embodiment, the transmembrane domain is a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154. In one embodiment, the transmembrane domain comprises a sequence of SEQ ID NO:15. In one embodiment, the intracellular signaling domain comprises a functional signaling domain of 4-1BB and a functional signaling domain of zeta. In one embodiment, the intracellular signaling domain comprises the sequence of SEQ ID NO: 16 and the sequence of SEQ ID NO: 17, wherein the sequences comprising the intracellular signaling domain are expressed in the same frame and as a single polypeptide chain. In one embodiment, the anti-CD19 binding domain is connected to the transmembrane domain by a hinge region. In one embodiment, the hinge region comprises SEQ ID NO:14. In one embodiment, the hinge region comprises SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49.

In another aspect, the invention pertains to an encoded CAR molecule comprising a leader sequence of SEQ ID NO: 13, a scFv domain having a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, and SEQ ID NO:59, or a sequence with 95-99% identity thereof, a hinge region of SEQ ID NO:14 or SEQ ID NO:45 or SEQ ID NO:47 or SEQ ID NO:49, a transmembrane domain having a sequence of SEQ ID NO: 15, a 4-1BB costimulatory domain having a sequence of SEQ ID NO:16 or a CD27 costimulatory domain having a sequence of SEQ ID NO:51, and a CD3 zeta stimulatory domain having a sequence of SEQ ID NO:17 or SEQ ID NO:43. In one embodiment, the encoded CAR molecule comprises a sequence selected from a group consisting of SEQ ID

NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, and SEQ ID NO:59, or a sequence with 95-99% identity thereof.

The nucleic acid sequences coding for the desired molecules can be obtained using recombinant methods known in the art, such as, for example by screening libraries from cells expressing the gene, by deriving the gene from a vector known to include the same, or by isolating directly from cells and tissues containing the same, using standard techniques. Alternatively, the gene of interest can be produced synthetically, rather than cloned.

The present invention also provides vectors in which a DNA of the present invention is inserted. Vectors derived from retroviruses such as the lentivirus are suitable tools to achieve long-term gene transfer since they allow long-term, stable integration of a transgene and its propagation in daughter cells. Lentiviral vectors have the added advantage over vectors derived from onco-retroviruses such as murine leukemia viruses in that they can transduce non-proliferating cells, such as hepatocytes. They also have the added advantage of low immunogenicity. A retroviral vector may also be, e.g., a gammaretroviral vector. A gammaretroviral vector may include, e.g., a promoter, a packaging signal (ψ), a primer binding site (PBS), one or more (e.g., two) long terminal repeats (LTR), and a transgene of interest, e.g., a gene encoding a CAR. A gammaretroviral vector may lack viral structural gens such as gag, pol, and env. Exemplary gammaretroviral vectors include Murine Leukemia Virus (MLV), Spleen-Focus Forming Virus (SFFV), and Myeloproliferative Sarcoma Virus (MPSV), and vectors derived therefrom. Other gammaretroviral vectors are described, e.g., in Tobias Maetzig et al., "Gammaretroviral Vectors: Biology, Technology and Application" Viruses. 2011 Jun; 3(6): 677–713.

In another embodiment, the vector comprising the nucleic acid encoding the desired CAR of the invention is an adenoviral vector (A5/35). In another embodiment, the expression of nucleic acids encoding CARs can be accomplished using of transposons such as sleeping beauty, crispr, CAS9, and zinc finger nucleases. See below June et al. 2009*Nature Reviews Immunology* 9.10: 704-716, is incorporated herein by reference.

A vector may also include, e.g., a signal sequence to facilitate secretion, a polyadenylation signal and transcription terminator (e.g., from Bovine Growth Hormone (BGH)

gene), an element allowing episomal replication and replication in prokaryotes (e.g. SV40 origin and ColE1 or others known in the art) and/or elements to allow selection (e.g., ampicillin resistance gene and/or zeocin marker).

In brief summary, the expression of natural or synthetic nucleic acids encoding CARs is typically achieved by operably linking a nucleic acid encoding the CAR polypeptide or portions thereof to a promoter, and incorporating the construct into an expression vector. The vectors can be suitable for replication and integration eukaryotes. Typical cloning vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the desired nucleic acid sequence.

In some aspects, the expression constructs of the present invention may also be used for nucleic acid immunization and gene therapy, using standard gene delivery protocols. Methods for gene delivery are known in the art. See, e.g., U.S. Pat. Nos. 5,399,346, 5,580,859, 5,589,466, incorporated by reference herein in their entireties. In another embodiment, the invention provides a gene therapy vector.

The nucleic acid can be cloned into a number of types of vectors. For example, the nucleic acid can be cloned into a vector including, but not limited to a plasmid, a phagemid, a phage derivative, an animal virus, and a cosmid. Vectors of particular interest include expression vectors, replication vectors, probe generation vectors, and sequencing vectors.

Further, the expression vector may be provided to a cell in the form of a viral vector. Viral vector technology is well known in the art and is described, for example, in Sambrook et al., 2012, MOLECULAR CLONING: A LABORATORY MANUAL, volumes 1 -4, Cold Spring Harbor Press, NY), and in other virology and molecular biology manuals. Viruses, which are useful as vectors include, but are not limited to, retroviruses, adenoviruses, adeno- associated viruses, herpes viruses, and lentiviruses. In general, a suitable vector contains an origin of replication functional in at least one organism, a promoter sequence, convenient restriction endonuclease sites, and one or more selectable markers, (e.g., WO 01/96584; WO 01/29058; and U.S. Pat. No. 6,326,193).

A number of viral based systems have been developed for gene transfer into mammalian cells. For example, retroviruses provide a convenient platform for gene delivery systems. A selected gene can be inserted into a vector and packaged in retroviral particles using techniques

known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either in vivo or ex vivo. A number of retroviral systems are known in the art. In some embodiments, adenovirus vectors are used. A number of adenovirus vectors are known in the art. In one embodiment, lentivirus vectors are used.

Additional promoter elements, e.g., enhancers, regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the thymidine kinase (tk) promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription. Exemplary promoters include the CMV IE gene, EF-1α, ubiquitin C, or phosphoglycerokinase (PGK) promoters. In an embodiment, the promoter is a PGK promoter, e.g., a truncated PGK promoter as described herein.

An example of a promoter that is capable of expressing a CAR transgene in a mammalian T cell is the EF1a promoter. The native EF1a promoter drives expression of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. The EF1a promoter has been extensively used in mammalian expression plasmids and has been shown to be effective in driving CAR expression from transgenes cloned into a lentiviral vector. See, e.g., Milone et al., Mol. Ther. 17(8): 1453–1464 (2009). In one aspect, the EF1a promoter comprises the sequence provided as SEQ ID NO:100.

Another example of a promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of any polynucleotide sequence operatively linked thereto. However, other constitutive promoter sequences may also be used, including, but not limited to the simian virus 40 (SV40) early promoter, mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) long terminal repeat (LTR) promoter, MoMuLV promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter, as well as human gene promoters such as, but not limited to, the actin promoter,

the myosin promoter, the elongation factor-1α promoter, the hemoglobin promoter, and the creatine kinase promoter. Further, the invention should not be limited to the use of constitutive promoters. Inducible promoters are also contemplated as part of the invention. The use of an inducible promoter provides a molecular switch capable of turning on expression of the polynucleotide sequence which it is operatively linked when such expression is desired, or turning off the expression when expression is not desired. Examples of inducible promoters include, but are not limited to a metallothionine promoter, a glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter.

Another example of a promoter is the phosphoglycerate kinase (PGK) promoter. In embodiments, a truncated PGK promoter (e.g., a PGK promoter with one or more, e.g., 1, 2, 5, 10, 100, 200, 300, or 400, nucleotide deletions when compared to the wild-type PGK promoter sequence) may be desired. The nucleotide sequences of exemplary PGK promoters are provided below.

WT PGK Promoter:

ACCCCTCTCTCCAGCCACTAAGCCAGTTGCTCCCTCGGCTGACGGCTGCACG
CGAGGCCTCCGAACGTCTTACGCCTTGTGGCGCGCCCGTCCTTGTCCCGGGTGTGAT
GGCGGGGTGTGGGGCGGAGGGCGTGGCGGGGAAGGGCCGGCGACGAGAGCCGCGC
GGGACGACTCGTCGGCGATAACCGGTGTCGGGTAGCGCCAGCCGCGCGACGGTAAC
GAGGGACCGCGACAGGCAGACGCTCCCATGATCACTCTGCACGCCGAAGGCAAATA
GTGCAGGCCGTGCGGCGCTTGGCGTTCCTTGGAAGGGCTGAATCCCCGCCTCGTCCT
TCGCAGCGGCCCCCCGGGTGTTCCCATCGCCGCTTCTAGGCCCACTGCGACGCTTGC
CTGCACTTCTTACACGCTCTGGGTCCCAGCCGCGGCGACGCAAAGGGCCTTGGTGC
GGGTCTCGTCGGCGCAGGGACGCGTTTGGGTCCCGACGGAACCTTTTCCGCGTTGG
GGTTGGGGCACCATAAGCT (SEQ ID NO: 1323)

Exemplary truncated PGK Promoters:

PGK100:

ACCCCTCTCTCCAGCCACTAAGCCAGTTGCTCCCTCGGCTGACGGCTGCACG
CGAGGCCTCCGAACGTCTTACGCCTTGTGGCGCGCCCGTCCTTGTCCCGGGTGTGAT
GGCGGGGTG (SEQ ID NO: 1324)

PGK200:

ACCCCTCTCTCCAGCCACTAAGCCAGTTGCTCCCTCGGCTGACGGCTGCACG CGAGGCCTCCGAACGTCTTACGCCTTGTGGCGCGCCCGTCCTTGTCCCGGGTGTGAT GGCGGGGTGTGGGGCGGAGGGCGTGGCGGGGAAGGGCCGGCGACGAGAGCCGCGC GGGACGACTCGTCGGCGATAACCGGTGTCGGGTAGCGCCAGCCGCGCGACGGTAAC G (SEQ ID NO:1325)

PGK300:

ACCCCTCTCTCCAGCCACTAAGCCAGTTGCTCCCTCGGCTGACGGCTGCACG CGAGGCCTCCGAACGTCTTACGCCTTGTGGCGCGCCCGTCCTTGTCCCGGGTGTGAT GGCGGGGTGTGGGGCGGAGGGCGTGGCGGGGAAGGGCCGGCGACGAGAGCCGCGC GGGACGACTCGTCGGCGATAACCGGTGTCGGGTAGCGCCAGCCGCGCGACGGTAAC GAGGGACCGCGACAGGCAGACGCTCCCATGATCACTCTGCACGCCGAAGGCAAATA GTGCAGGCCGTGCGGCGCTTGGCGTTCCTTGGAAGGGCTGAATCCCCG (SEQ ID NO:1326)

PGK400:

ACCCCTCTCTCCAGCCACTAAGCCAGTTGCTCCCTCGGCTGACGGCTGCACG CGAGGCCTCCGAACGTCTTACGCCTTGTGGCGCGCCCGTCCTTGTCCCGGGTGTGAT GGCGGGGTGTGGGGCGGAGGGCGTGGCGGGGAAGGGCCGGCGACGAGAGCCGCGC GGGACGACTCGTCGGCGATAACCGGTGTCGGGTAGCGCCAGCCGCGCGACGGTAAC GAGGGACCGCGACAGGCAGACGCTCCCATGATCACTCTGCACGCCGAAGGCAAATA GTGCAGGCCGTGCGGCGCTTGGCGTTCCTTGGAAGGGCTGAATCCCCGCCTCGTCCT TCGCAGCGGCCCCCCGGGTGTTCCCATCGCCGCTTCTAGGCCCACTGCGACGCTTGC CTGCACTTCTTACACGCTCTGGGTCCCAGCCG (SEQ ID NO:1327)

A vector may also include, e.g., a signal sequence to facilitate secretion, a polyadenylation signal and transcription terminator (e.g., from Bovine Growth Hormone (BGH) gene), an element allowing episomal replication and replication in prokaryotes (e.g. SV40 origin and ColE1 or others known in the art) and/or elements to allow selection (e.g., ampicillin resistance gene and/or zeocin marker).

In order to assess the expression of a CAR polypeptide or portions thereof, the

expression vector to be introduced into a cell can also contain either a selectable marker gene or a reporter gene or both to facilitate identification and selection of expressing cells from the population of cells sought to be transfected or infected through viral vectors. In other aspects, the selectable marker may be carried on a separate piece of DNA and used in a co- transfection procedure. Both selectable markers and reporter genes may be flanked with appropriate regulatory sequences to enable expression in the host cells. Useful selectable markers include, for example, antibiotic-resistance genes, such as neo and the like.

Reporter genes are used for identifying potentially transfected cells and for evaluating the functionality of regulatory sequences. In general, a reporter gene is a gene that is not present in or expressed by the recipient organism or tissue and that encodes a polypeptide whose expression is manifested by some easily detectable property, e.g., enzymatic activity. Expression of the reporter gene is assayed at a suitable time after the DNA has been introduced into the recipient cells. Suitable reporter genes may include genes encoding luciferase, beta-galactosidase, chloramphenicol acetyl transferase, secreted alkaline phosphatase, or the green fluorescent protein gene (e.g., Ui-Tei et al., 2000 FEBS Letters 479: 79-82). Suitable expression systems are well known and may be prepared using known techniques or obtained commercially. In general, the construct with the minimal 5' flanking region showing the highest level of expression of reporter gene is identified as the promoter. Such promoter regions may be linked to a reporter gene and used to evaluate agents for the ability to modulate promoter- driven transcription.

In embodiments, the vector may comprise two or more nucleic acid sequences encoding a CAR, e.g., a first CAR that binds to CD19 and a second CAR, e.g., an inhibitory CAR or a CAR that specifically binds to a second antigen, e.g., CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a. In such embodiments, the two or more nucleic acid sequences encoding the CAR are encoded by a single nucleic molecule in the same frame and as a single polypeptide chain. In this aspect, the two or more CARs, can, e.g., be separated by one or more peptide cleavage sites. (e.g., an auto-cleavage site or a substrate for an intracellular protease). Examples of peptide cleavage sites include the following, wherein the GSG residues are optional:

T2A: (GSG)EGRGSLLTCGDVEENPGP (SEQ ID NO: 1328)

P2A: (GSG)ATNFSLLKQAGDVEENPGP (SEQ ID NO: 1329)

E2A: (GSG)QCTNYALLKLAGDVESNPGP (SEQ ID NO: 1330)

F2A: (GSG)VKQTLNFDLLKLAGDVESNPGP (SEQ ID NO: 1331)

Methods of introducing and expressing genes into a cell are known in the art. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al., 2012, MOLECULAR CLONING: A LABORATORY MANUAL, volumes 1 -4, Cold Spring Harbor Press, NY). A suitable method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle). Other methods of state-of-the-art targeted delivery of nucleic acids are available, such as delivery of polynucleotides with targeted nanoparticles or other suitable sub-micron sized delivery system.

In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (in vitro, ex vivo or in vivo). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a

liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma, St. Louis, MO; dicetyl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, NY); cholesterol ("Choi") can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL.). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20ºC. Chloroform is used as the only solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 1991 Glycobiology 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

Regardless of the method used to introduce exogenous nucleic acids into a host cell or

otherwise expose a cell to the inhibitor of the present invention, in order to confirm the presence of the recombinant DNA sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or by assays described herein to identify agents falling within the scope of the invention.

The present invention further provides a vector comprising a CAR encoding nucleic acid molecule. In one aspect, a CAR vector can be directly transduced into a cell, *e.g.,* a T cell. In one aspect, the vector is a cloning or expression vector, *e.g.,* a vector including, but not limited to, one or more plasmids (*e.g.*, expression plasmids, cloning vectors, minicircles, minivectors, double minute chromosomes), retroviral and lentiviral vector constructs. In one aspect, the vector is capable of expressing the CAR construct in mammalian T cells. In one aspect, the mammalian T cell is a human T cell.

Immune Effector Cells Expressing a CAR

In another aspect, the present invention provides a population of CAR-expressing cells. In some embodiments, the population of CAR-expressing cells comprises a cell that expresses one or more CARs described herein. In some embodiments, the population of CAR-expressing cells comprises a mixture of cells expressing different CARs.

For example, in one embodiment, the population of CART cells can include a first cell expressing a CAR having an antigen binding domain to a tumor antigen described herein, e.g., CD19, and a second cell expressing a CAR having a different antigen binding domain, e.g., an antigen binding domain to a different tumor antigen described herein, e.g., an antigen binding domain to a tumor antigen described herein that differs from the tumor antigen bound by the antigen binding domain of the CAR expressed by the first cell, e.g., CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

As another example, the population of CAR-expressing cells can include a first cell expressing a CAR that includes an antigen binding domain to a tumor antigen described herein, and a second cell expressing a CAR that includes an antigen binding domain to a target other than a tumor antigen as described herein. In one embodiment, the population of CAR-expressing cells includes, e.g., a first cell expressing a CAR that includes a primary intracellular

signaling domain, and a second cell expressing a CAR that includes a secondary signaling domain. Either one or both of the CAR expressing cells can have a truncated PGK promoter, e.g., as described herein, operably linked to the nucleic acid encoding the CAR.

In another aspect, the present invention provides a population of cells wherein at least one cell in the population expresses a CAR having an antigen binding domain to a tumor antigen described herein, and a second cell expressing another agent, e.g., an agent which enhances the activity of a CAR-expressing cell. The CAR expressing cells of the population can have a truncated PGK promoter, e.g., as described herein, operably linked to the nucleic acid encoding the CAR. In one embodiment, the agent can be an agent which inhibits an inhibitory molecule. Inhibitory molecules, e.g., PD-1, can, in some embodiments, decrease the ability of a CAR-expressing cell to mount an immune effector response. Examples of inhibitory molecules include PD-1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (CEACAM-1, CEACAM-3, and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR (e.g., TGFRbeta). In one embodiment, the agent which inhibits an inhibitory molecule comprises a first polypeptide, e.g., an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, e.g., an intracellular signaling domain described herein. In one embodiment, the agent comprises a first polypeptide, e.g., of an inhibitory molecule such as PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3, and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4 or TGFR beta, or a fragment of any of these, and a second polypeptide which is an intracellular signaling domain described herein (e.g., comprising a costimulatory domain (e.g., 41BB, CD27, OX40 or CD28, e.g., as described herein) and/or a primary signaling domain (e.g., a CD3 zeta signaling domain described herein). In one embodiment, the agent comprises a first polypeptide of PD-1 or a fragment thereof, and a second polypeptide of an intracellular signaling domain described herein (e.g., a CD28 signaling domain described herein and/or a CD3 zeta signaling domain described herein).

Co-expression of CAR with Other Molecules or Agents

Co-expression of a Second CAR

In one aspect, the CAR-expressing cell described herein can further comprise a second CAR, e.g., a second CAR that includes a different antigen binding domain, e.g., to the same

target (CD19) or a different target (e.g., CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a). In one embodiment, the second CAR includes an antigen binding domain to a target expressed on acute myeloid leukemia cells, such as, e.g., CD20, CD22, ROR1, CD10, CD33, CLL-1, CD34, CD123, FLT3, CD79b, CD179b, and CD79a. In one embodiment, the CAR-expressing cell comprises a first CAR that targets a first antigen and includes an intracellular signaling domain having a costimulatory signaling domain but not a primary signaling domain, and a second CAR that targets a second, different, antigen and includes an intracellular signaling domain having a primary signaling domain but not a costimulatory signaling domain. While not wishing to be bound by theory, placement of a costimulatory signaling domain, e.g., 4-1BB, CD28, CD27 or OX-40, onto the first CAR, and the primary signaling domain, e.g., CD3 zeta, on the second CAR can limit the CAR activity to cells where both targets are expressed. In one embodiment, the CAR expressing cell comprises a first CD19 CAR that includes a CD19 binding domain, a transmembrane domain and a costimulatory domain and a second CAR that targets an antigen other than CD19 (e.g., an antigen expressed on AML cells, e.g., CD22, CD20, ROR1, CD10, CD33, CLL-1, CD34, CD123, FLT3, CD79b, CD179b, or CD79a) and includes an antigen binding domain, a transmembrane domain and a primary signaling domain. In another embodiment, the CAR expressing cell comprises a first CD19 CAR that includes a CD19 binding domain, a transmembrane domain and a primary signaling domain and a second CAR that targets an antigen other than CD19 (e.g., an antigen expressed on AML cells, e.g., CD22, CD20, ROR1, CD10, CD33, CD123, CLL-1, CD34, FLT3, CD79b, CD179b, or CD79a) and includes an antigen binding domain to the antigen, a transmembrane domain and a costimulatory signaling domain.

In one aspect, the CAR-expressing cell described herein can further comprise a second CAR, e.g., a second CAR that includes a different antigen binding domain, e.g., to the same target (e.g., CD19) or a different target (e.g., a target other than CD19, e.g., CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a). In one embodiment, the CAR-expressing cell comprises a first CAR that targets a first antigen and includes an intracellular signaling domain having a costimulatory signaling domain but not a primary signaling domain, and a second CAR that targets a second, different, antigen and includes an intracellular signaling domain having a primary signaling domain but not a costimulatory signaling domain.

Placement of a costimulatory signaling domain, e.g., 4-1BB, CD28, CD27, OX-40 or ICOS, onto the first CAR, and the primary signaling domain, e.g., CD3 zeta, on the second CAR can limit the CAR activity to cells where both targets are expressed. In one embodiment, the CAR expressing cell comprises a first CAR that includes an antigen binding domain, a transmembrane domain and a costimulatory domain and a second CAR that targets another antigen and includes an antigen binding domain, a transmembrane domain and a primary signaling domain. In another embodiment, the CAR expressing cell comprises a first CAR that includes an antigen binding domain, a transmembrane domain and a primary signaling domain and a second CAR that targets another antigen and includes an antigen binding domain to the antigen, a transmembrane domain and a costimulatory signaling domain.

In one embodiment, the CAR-expressing cell comprises an XCAR described herein (e.g., CD19 CAR, CD20 CAR, or CD22 CAR) and an inhibitory CAR. In one embodiment, the CAR-expressing cell comprises a CD19 CAR described herein and an inhibitory CAR. In one embodiment, the inhibitory CAR comprises an antigen binding domain that binds an antigen found on normal cells but not cancer cells, e.g., normal cells that also express CD19. In one embodiment, the inhibitory CAR comprises the antigen binding domain, a transmembrane domain and an intracellular domain of an inhibitory molecule. For example, the intracellular domain of the inhibitory CAR can be an intracellular domain PD-1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (CEACAM-1, CEACAM-3, and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR (e.g., TGFRbeta).

In one embodiment, when the CAR-expressing cell comprises two or more different CARs, the antigen binding domains of the different CARs can be such that the antigen binding domains do not interact with one another. For example, a cell expressing a first and second CAR can have an antigen binding domain of the first CAR, e.g., as a fragment, e.g., an scFv, that does not form an association with the antigen binding domain of the second CAR, e.g., the antigen binding domain of the second CAR is a VHH.

Co-expression of an Agent that Enhances CAR Activity

In another aspect, the CAR-expressing cell described herein can further express another agent, e.g., an agent that enhances the activity or fitness of a CAR-expressing cell.

For example, in one embodiment, the agent can be an agent which inhibits a molecule that modulates or regulates, e.g., inhibits, T cell function. In some embodiments, the molecule that modulates or regulates T cell function is an inhibitory molecule. Inhibitory molecules, e.g., PD1, can, in some embodiments, decrease the ability of a CAR-expressing cell to mount an immune effector response. Examples of inhibitory molecules include PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR (e.g., TGFR beta).

In one embodiment, an inhibitory nucleic acid, e.g., an inhibitory nucleic acid, e.g., a dsRNA, e.g., an siRNA or shRNA, a clustered regularly interspaced short palindromic repeats (CRISPR), a transcription-activator like effector nuclease (TALEN), or a zinc finger endonuclease (ZFN), e.g., as described herein, can be used to inhibit expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function in the CAR-expressing cell. In an embodiment the agent is an shRNA, e.g., an shRNA described herein. In an embodiment, the agent that modulates or regulates, e.g., inhibits, T-cell function is inhibited within a CAR-expressing cell. For example, a dsRNA molecule that inhibits expression of a molecule that modulates or regulates, e.g., inhibits, T-cell function is linked to the nucleic acid that encodes a component, e.g., all of the components, of the CAR.

In one embodiment, the agent which inhibits an inhibitory molecule comprises a first polypeptide, e.g., an inhibitory molecule, associated with a second polypeptide that provides a positive signal to the cell, e.g., an intracellular signaling domain described herein. In one embodiment, the agent comprises a first polypeptide, e.g., of an inhibitory molecule such as PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, or TGFR (e.g., TGFR beta), or a fragment of any of these (e.g., at least a portion of an extracellular domain of any of these), and a second polypeptide which is an intracellular signaling domain described herein (e.g., comprising a costimulatory domain (e.g., 41BB, CD27 or CD28, e.g., as described herein) and/or a primary signaling domain (e.g., a CD3 zeta signaling domain described herein). In one embodiment, the agent comprises a first

polypeptide of PD1 or a fragment thereof (e.g., at least a portion of an extracellular domain of PD1), and a second polypeptide of an intracellular signaling domain described herein (e.g., a CD28 signaling domain described herein and/or a CD3 zeta signaling domain described herein). PD1 is an inhibitory member of the CD28 family of receptors that also includes CD28, CTLA-4, ICOS, and BTLA. PD-1 is expressed on activated B cells, T cells and myeloid cells (Agata et al. 1996 Int. Immunol 8:765-75). Two ligands for PD1, PD-L1 and PD-L2 have been shown to downregulate T cell activation upon binding to PD1 (Freeman et a. 2000 J Exp Med 192:1027-34; Latchman et al. 2001 Nat Immunol 2:261-8; Carter et al. 2002 Eur J Immunol 32:634-43). PD-L1 is abundant in human cancers (Dong et al. 2003 J Mol Med 81:281-7; Blank et al. 2005 Cancer Immunol. Immunother 54:307-314; Konishi et al. 2004 Clin Cancer Res 10:5094). Immune suppression can be reversed by inhibiting the local interaction of PD1 with PD-L1.

In one embodiment, the agent comprises the extracellular domain (ECD) of an inhibitory molecule, e.g., Programmed Death 1 (PD1), can be fused to a transmembrane domain and intracellular signaling domains such as 41BB and CD3 zeta (also referred to herein as a PD1 CAR). In one embodiment, the PD1 CAR, when used in combinations with a CD19 CAR described herein, improves the persistence of the T cell. In one embodiment, the CAR is a PD1 CAR comprising the extracellular domain of PD1 indicated as underlined in SEQ ID NO: 121. In one embodiment, the PD1 CAR comprises the amino acid sequence of SEQ ID NO:121.

Malpvtalllplalllhaarppgwfldspdrpwnpptfspallvvtegdnatftcsfsntsesfvlnwyrmspsnqtdklaaf
pedrsqpgqdcrfrvtqlpngrdfhmsvvrarrndsgtylcgaislapkaqikeslraelrvterraevptahpspsprpagqfqtlvtttp
aprpptpaptiasqplslrpeacrpaaggavhtrgldfacdiyiwaplagtcgvlllslvitlyckrgrkkllyifkqpfmrpvqttqeedg
cscrfpeeeeggcelrvkfsrsadapaykqgqnqlynelnlgrreeydvldkrrgrdpemggkprrknpqeglynelqkdkmaeay
seigmkgerrrgkghdglyqglstatkdtydalhmqalppr (SEQ ID NO:121).

In one embodiment, the PD1 CAR comprises the amino acid sequence provided below (SEQ ID NO:119).

pgwfldspdrpwnpptfspallvvtegdnatftcsfsntsesfvlnwyrmspsnqtdklaafpedrsqpgqdcrfrvtqlpn
grdfhmsvvrarrndsgtylcgaislapkaqikeslraelrvterraevptahpspsprpagqfqtlvtttpaprpptpaptiasqplslrpe
acrpaaggavhtrgldfacdiyiwaplagtcgvlllslvitlyckrgrkkllyifkqpfmrpvqttqeedgcscrfpeeeeggcelrvkfsr
sadapaykqgqnqlynelnlgrreeydvldkrrgrdpemggkprrknpqeglynelqkdkmaeayseigmkgerrrgkghdgly
qglstatkdtydalhmqalppr (SEQ ID NO:119).

Tin one embodiment, the agent comprises a nucleic acid sequence encoding the PD1 CAR, e.g., the PD1 CAR described herein. In one embodiment, the nucleic acid sequence for the PD1 CAR is shown below, with the PD1 ECD underlined below in SEQ ID NO: 120

atggccctccctgtcactgccctgcttctccccctcgcactcctgctccacgccgctagaccacccggatggtttctggactctcc
ggatcgcccgtggaatcccccaaccttctcaccggcactcttggttgtgactgagggcgataatgcgaccttcacgtgctcgttctccaaca
cctccgaatcattcgtgctgaactggtaccgcatgagcccgtcaaaccagaccgacaagctcgccgcgtttccggaagatcggtcgcaac
cgggacaggattgtcggttccgcgtgactcaactgccgaatggcagagacttccacatgagcgtggtccgcgctaggcgaaacgactcc
gggacctacctgtgcggagccatctcgctggcgcctaaggcccaaatcaaagagagcttgagggccgaactgagagtgaccgagcgca
gagctgaggtgccaactgcacatccatccccatcgcctcggcctgcggggcagtttcagaccctggtcacgaccactccggcgccgcgc
ccaccgactccggccccaactatcgcgagccagcccctgtcgctgaggccggaagcatgccgccctgccgccggaggtgctgtgcata
cccggggattggacttcgcatgcgacatctacatttgggctcctctcgccggaacttgtggcgtgctccttctgtccctggtcatcaccctgta
ctgcaagcggggtcggaaaaagcttctgtacattttcaagcagcccttcatgaggcccgtgcaaaccacccaggaggaggacggttgctc
ctgccggttccccgaagaggaagaaggaggttgcgagctgcgcgtgaagttctcccggagcgccgacgccccgcctataagcagggc
cagaaccagctgtacaacgaactgaacctgggacggcgggaagagtacgatgtgctggacaagcggcgcggccgggaccccgaaat
gggcgggaagcctagaagaaagaaccctcaggaaggcctgtataacgagctgcagaaggacaagatggccgaggcctactccgaaatt
gggatgaagggagagcggcggaggggaaaggggcacgacggcctgtaccaaggactgtccaccgccaccaaggacacatacgatg
ccctgcacatgcaggcccttccccctcgc (SEQ ID NO: 120).

In another example, in one embodiment, the agent which enhances the activity of a CAR-expressing cell can be a costimulatory molecule or costimulatory molecule ligand. Examples of costimulatory molecules include MHC class I molecule, BTLA and a Toll ligand receptor, as well as OX40, CD27, CD28, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), and 4-1BB (CD137). Further examples of such costimulatory molecules include CDS, ICAM-1, GITR, BAFFR, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD160, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE, CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, GADS, SLP-76, PAG/Cbp, CD19a, and a ligand that specifically binds with CD83., e.g., as

described herein. Examples of costimulatory molecule ligands include CD80, CD86, CD40L, ICOSL, CD70, OX40L, 4-1BBL, GITRL, and LIGHT. In embodiments, the costimulatory molecule ligand is a ligand for a costimulatory molecule different from the costimulatory molecule domain of the CAR. In embodiments, the costimulatory molecule ligand is a ligand for a costimulatory molecule that is the same as the costimulatory molecule domain of the CAR. In an embodiment, the costimulatory molecule ligand is 4-1BBL. In an embodiment, the costimulatory ligand is CD80 or CD86. In an embodiment, the costimulatory molecule ligand is CD70. In embodiments, a CAR-expressing immune effector cell described herein can be further engineered to express one or more additional costimulatory molecules or costimulatory molecule ligands.

Co-expression of CAR with a Chemokine Receptor

In embodiments, the CAR-expressing cell described herein further comprises a chemokine receptor molecule. Transgenic expression of chemokine receptors CCR2b or CXCR2 in T cells enhances trafficking to CCL2- or CXCL1-secreting solid tumors including melanoma and neuroblastoma (Craddock et al., *J Immunother*. 2010 Oct; 33(8):780-8 and Kershaw et al., *Hum Gene Ther*. 2002 Nov 1; 13(16):1971-80). Thus, without wishing to be bound by theory, it is believed that chemokine receptors expressed in CAR-expressing cells that recognize chemokines secreted by tumors, e.g., solid tumors, can improve homing of the CAR-expressing cell to the tumor, facilitate the infiltration of the CAR-expressing cell to the tumor, and enhances antitumor efficacy of the CAR-expressing cell. The chemokine receptor molecule can comprise a naturally occurring or recombinant chemokine receptor or a chemokine-binding fragment thereof. A chemokine receptor molecule suitable for expression in a CAR-expressing cell described herein include a CXC chemokine receptor (e.g., CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, or CXCR7), a CC chemokine receptor (e.g., CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, or CCR11), a CX3C chemokine receptor (e.g., CX3CR1), a XC chemokine receptor (e.g., XCR1), or a chemokine-binding fragment thereof. In one embodiment, the chemokine receptor molecule to be expressed with a CAR described herein is selected based on the chemokine(s) secreted by the tumor. In one embodiment, the CAR-expressing cell described herein further comprises, e.g., expresses, a CCR2b receptor or a CXCR2 receptor. In an embodiment, the CAR described herein and the chemokine receptor molecule are on the same vector or are on two different vectors. In embodiments where the CAR

described herein and the chemokine receptor molecule are on the same vector, the CAR and the chemokine receptor molecule are each under control of two different promoters or are under the control of the same promoter.

Conditional Expression of Immune Response-Enhancing Agents

Also provided herein are compositions and methods for conditionally expressing an agent that enhances the immune response or activity of a CAR-expressing cell described herein.

In one aspect, the present disclosure features an immune effector cell that is engineered to constitutively express a CAR, also referred to herein as a nonconditional CAR. In one embodiment, a nonconditional CAR as described herein comprises an antigen binding domain that binds to a cancer associated antigen, e.g., CD19, CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1. In embodiments, the nonconditional CAR-expressing immune effector cell further comprises a conditionally-expressed agent that enhances the therapeutic efficacy, e.g., the immune response, of the CAR-expressing immune effector cell. In such embodiments, the expression of the conditionally expressed agent occurs upon activation of the nonconditional CAR-expressing immune effector cell, e.g., upon binding of the nonconditional CAR molecule to its target, e.g., a cancer associated antigen, e.g., CD19, CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1.

Immune response-enhancing agents as described herein can be characterized by one or more of the following: 1) targets or binds to a different cancer associated antigen than that targeted by the nonconditional CAR; 2) inhibits the expression or activity of an immune checkpoint or inhibitory molecule; and/or 3) activates the expression and/or secretion of a component that enhances immune response or activation of an immune effector cell. The immune response-enhancing agent can be a polypeptide or a nucleic acid, e.g., a nucleic acid that encodes a polypeptide that enhances immune response. Examples of conditionally expressed agents that enhance the immune response include, but are not limited to, an additional CAR (referred to as a conditional CAR); a TCR-based molecule (e.g., a TCR-CAR); an inhibitor of an immune checkpoint or an inhibitory molecule; and/or a cytokine. In embodiments, the conditional CAR binds to a different cancer associated antigen than that targeted by the nonconditional CAR. In embodiments, the inhibitor of an immune checkpoint or inhibitory molecule described herein is an antibody or antigen binding fragment thereof, an inhibitory

nucleic acid (e.g., an siRNA or shRNA), or a small molecule that inhibits or decreases the activity of an immune checkpoint or inhibitory molecule selected from PD1, PD-L1, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, or TGFR beta. In embodiments, the cytokine comprises IL-2, IL-7, IL-15, or IL-21, or functional fragments or derivatives thereof.

In embodiments, the immune effector cell comprises a nonconditional CAR and one or more conditional CARs, where the conditional CAR binds to a different cancer associated antigen than that targeted by the nonconditional CAR. By way of example, in one embodiment, an immune effector cell comprises a nonconditional CAR that binds to CD19 and one or more conditional CARs that bind to CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1, or a combination thereof. In another embodiment, an immune effector cell comprises a nonconditional CAR that binds to CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1 and a conditional CAR that binds to CD19.

Conditional expression of the agent that enhances the immune response upon activation of the CAR-expressing immune effector cell is achieved by operatively linking an activation-conditional control region to the agent that enhances the immune response (e.g., to a nucleic acid sequence encoding such an agent). In one embodiment, the activation conditional control region comprises a promoter sequence that initiates expression, e.g., transcription, of the operatively linked immune response enhancing agent upon activation of the immune effector cell. In one embodiment, the activation conditional control region comprises one or more regulatory sequences (e.g., a transcription factor binding sequence or site) that facilitate the initiation of expression upon activation of the immune effector cell. In embodiments, the activation-conditional control region comprises a promoter sequence and/or one or more transcription factor binding sequences from a promoter or regulatory sequence of a gene that is upregulated upon one or more of the following: immune effector cell (e.g., T cell) activation, T-cell differentiation, T-cell polarization, or helper T cell development. Examples of such genes include, but are not limited to, NFAT (nuclear factor of activated T cells), ATF2 (activating transcription factor 2), NF-κB (nuclear factor-κB), IL-2, IL-2 receptor (IL-2R), IL -3, GM-CSF, IL-4, IL-10, and IFN-γ.

In one embodiment, the activation-conditional control region comprises one or more, e.g., 1, 2, 3, 4, 5, 6, or more, NFAT binding sequences or sites. In embodiments, the NFAT-binding sequence in the promoter comprises (5′-GGAAA-3′) (SEQ ID NO: 1312), optionally situated in a longer consensus sequence of 5' (A/T)GGAAA(A/N)(A/T/C)N 3' (SEQ ID NO: 1313). In embodiments, the NFAT-binding sequence is a κb-like sequence such as GGGACT (SEQ ID NO: 1314). (See, Gibson et al., The Journal of Immunology, 2007, 179: 3831–3840.)

In one embodiment, the activation-conditional control region further comprises an IL-2 promoter (or a minimal IL-2 promoter), an IL-2R promoter, an ATF2 promoter, or a NF-κB promoter, or any functional fragment or derivative thereof. In one embodiment, the activation-conditional control region comprises one or more NFAT-binding sequences, e.g., 3 or 6 NFAT-binding sequences, and an IL-2 promoter, e.g., an IL-2 minimal promoter. In one embodiment, the activation-conditional control region comprises the sequence of AGCTTGGATCCAAGAGGAAAATTTGTTTCATACAGAAGGCGTTAAGAGGAAAATTTGTTTCATACAGAAGGCGTTAAGAGGAAAATTTGTTTCATACAGAAGGCGTTCAAGCTTGTCGAC (SEQ ID NO: 1315).

Sources of Cells

Prior to expansion and genetic modification or other modification, a source of cells, e.g., T cells or natural killer (NK) cells, can be obtained from a subject. Examples of subjects include humans, monkeys, chimpanzees, dogs, cats, mice, rats, and transgenic species thereof. T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors.

In certain aspects of the present disclosure, immune effector cells, e.g., T cells, can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll™ separation. In one aspect, cells from the circulating blood of an individual are obtained by apheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one aspect, the cells collected by apheresis may be washed to remove the plasma fraction and, optionally, to place the cells in an appropriate buffer or media for

subsequent processing steps. In one embodiment, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations.

Initial activation steps in the absence of calcium can lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor, the Baxter CytoMate, or the Haemonetics Cell Saver 5) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca-free, Mg-free PBS, PlasmaLyte A, or other saline solution with or without buffer. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

It is recognized that the methods of the application can utilize culture media conditions comprising 5% or less, for example 2%, human AB serum, and employ known culture media conditions and compositions, for example those described in Smith et al., "Ex vivo expansion of human T cells for adoptive immunotherapy using the novel Xeno-free CTS Immune Cell Serum Replacement" Clinical & Translational Immunology (2015) 4, e31; doi:10.1038/cti.2014.31.

In one aspect, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLLTM gradient or by counterflow centrifugal elutriation.

The methods described herein can include, e.g., selection of a specific subpopulation of immune effector cells, e.g., T cells, that are a T regulatory cell-depleted population, CD25+ depleted cells, using, e.g., a negative selection technique, e.g., described herein. In some embodiments, the population of T regulatory depleted cells contains less than 30%, 25%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1% of CD25+ cells.

In one embodiment, T regulatory cells, e.g., CD25+ T cells, are removed from the population using an anti-CD25 antibody, or fragment thereof, or a CD25-binding ligand, IL-2. In one embodiment, the anti-CD25 antibody, or fragment thereof, or CD25-binding ligand is conjugated to a substrate, e.g., a bead, or is otherwise coated on a substrate, e.g., a bead. In one embodiment, the anti-CD25 antibody, or fragment thereof, is conjugated to a substrate as

described herein.

In one embodiment, the T regulatory cells, e.g., CD25+ T cells, are removed from the population using CD25 depletion reagent from Miltenyi™. In one embodiment, the ratio of cells to CD25 depletion reagent is 1e7 cells to 20 uL, or 1e7 cells to15 uL, or 1e7 cells to 10 uL, or 1e7 cells to 5 uL, or 1e7 cells to 2.5 uL, or 1e7 cells to 1.25 uL. In one embodiment, e.g., for T regulatory cells, e.g., CD25+ depletion, greater than 500 million cells/ml is used. In a further aspect, a concentration of cells of 600, 700, 800, or 900 million cells/ml is used.

In one embodiment, the population of immune effector cells to be depleted includes about 6 x 10^9 CD25+ T cells. In other aspects, the population of immune effector cells to be depleted include about 1 x 10^9 to 1x 10^{10} CD25+ T cell, and any integer value in between. In one embodiment, the resulting population T regulatory depleted cells has 2 x 10^9 T regulatory cells, e.g., CD25+ cells, or less (e.g., 1 x 10^9, 5 x 10^8 , 1 x 10^8, 5 x 10^7, 1 x 10^7, or less CD25+ cells).

In one embodiment, the T regulatory cells, e.g., CD25+ cells, are removed from the population using the CliniMAC system with a depletion tubing set, such as, e.g., tubing 162-01. In one embodiment, the CliniMAC system is run on a depletion setting such as, e.g., DEPLETION2.1.

Without wishing to be bound by a particular theory, decreasing the level of negative regulators of immune cells (e.g., decreasing the number of unwanted immune cells, e.g., T_{REG} cells), in a subject prior to apheresis or during manufacturing of a CAR-expressing cell product significantly reduces the risk of subject relapse. For example, methods of depleting T_{REG} cells are known in the art. Methods of decreasing T_{REG} cells include, but are not limited to, cyclophosphamide, anti-GITR antibody (an anti-GITR antibody described herein), CD25-depletion, mTOR inhibitor, and combinations thereof.

In some embodiments, the manufacturing methods comprise reducing the number of (e.g., depleting) T_{REG} cells prior to manufacturing of the CAR-expressing cell. For example, manufacturing methods comprise contacting the sample, e.g., the apheresis sample, with an anti-GITR antibody and/or an anti-CD25 antibody (or fragment thereof, or a CD25-binding ligand), e.g., to deplete T_{REG} cells prior to manufacturing of the CAR-expressing cell (e.g., T cell, NK cell) product.

Without wishing to be bound by a particular theory, decreasing the level of negative regulators of immune cells (e.g., decreasing the number of unwanted immune cells, e.g., T_{REG} cells), in a subject prior to apheresis or during manufacturing of a CAR-expressing cell product can reduce the risk of a T_{REG} relapse. In an embodiment, a subject is pre-treated with one or more therapies that reduce TREG cells prior to collection of cells for CAR-expressing cell product manufacturing, thereby reducing the risk of subject relapse to CAR-expressing cell treatment. In an embodiment, methods of decreasing T_{REG} cells include, but are not limited to, administration to the subject of one or more of cyclophosphamide, anti-GITR antibody, CD25-depletion, or a combination thereof. In an embodiment, methods of decreasing T_{REG} cells include, but are not limited to, administration to the subject of one or more of cyclophosphamide, anti-GITR antibody, CD25-depletion, mTOR inhibitor, or a combination thereof. Administration of one or more of cyclophosphamide, anti-GITR antibody, CD25-depletion, or a combination thereof, can occur before, during or after an infusion of the CAR-expressing cell product. Administration of one or more of cyclophosphamide, anti-GITR antibody, CD25-depletion, mTOR inhibitor, or a combination thereof, can occur before, during or after an infusion of the CAR-expressing cell product.

In some embodiments, the manufacturing methods comprise reducing the number of (e.g., depleting) T_{REG} cells prior to manufacturing of the CAR-expressing cell. For example, manufacturing methods comprise contacting the sample, e.g., the apheresis sample, with an anti-GITR antibody and/or an anti-CD25 antibody (or fragment thereof, or a CD25-binding ligand), e.g., to deplete T_{REG} cells prior to manufacturing of the CAR-expressing cell (e.g., T cell, NK cell) product.

In an embodiment, a subject is pre-treated with one or more therapies that reduce T_{REG} cells prior to collection of cells for CAR-expressing cell product manufacturing, thereby reducing the risk of subject relapse to CAR-expressing cell treatment. In an embodiment, methods of decreasing T_{REG} cells include, but are not limited to, administration to the subject of one or more of cyclophosphamide, anti-GITR antibody, CD25-depletion, or a combination thereof. Administration of one or more of cyclophosphamide, anti-GITR antibody, CD25-depletion, or a combination thereof, can occur before, during or after an infusion of the CAR-expressing cell product.

In an embodiment, a subject is pre-treated with cyclophosphamide prior to collection of

cells for CAR-expressing cell product manufacturing, thereby reducing the risk of subject relapse to CAR-expressing cell treatment. In an embodiment, a subject is pre-treated with an anti-GITR antibody prior to collection of cells for CAR-expressing cell product manufacturing, thereby reducing the risk of subject relapse to CAR-expressing cell treatment.

In one embodiment, the population of cells to be removed are neither the regulatory T cells or tumor cells, but cells that otherwise negatively affect the expansion and/or function of CART cells, e.g. cells expressing CD14, CD11b, CD33, CD15, or other markers expressed by potentially immune suppressive cells. In one embodiment, such cells are envisioned to be removed concurrently with regulatory T cells and/or tumor cells, or following said depletion, or in another order.

The methods described herein can include more than one selection step, e.g., more than one depletion step. Enrichment of a T cell population by negative selection can be accomplished, e.g., with a combination of antibodies directed to surface markers unique to the negatively selected cells. One method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail can include antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8.

The methods described herein can further include removing cells from the population which express a tumor antigen, e.g., a tumor antigen that does not comprise CD25, e.g., CD19, CD30, CD38, CD123, CD20, CD14 or CD11b, to thereby provide a population of T regulatory depleted, e.g., CD25+ depleted, and tumor antigen depleted cells that are suitable for expression of a CAR, e.g., a CAR described herein. In one embodiment, tumor antigen expressing cells are removed simultaneously with the T regulatory, e.g., CD25+ cells. For example, an anti-CD25 antibody, or fragment thereof, and an anti-tumor antigen antibody, or fragment thereof, can be attached to the same substrate, e.g., bead, which can be used to remove the cells or an anti-CD25 antibody, or fragment thereof, or the anti-tumor antigen antibody, or fragment thereof, can be attached to separate beads, a mixture of which can be used to remove the cells. In other embodiments, the removal of T regulatory cells, e.g., CD25+ cells, and the removal of the tumor antigen expressing cells is sequential, and can occur, e.g., in either order.

Also provided are methods that include removing cells from the population which express a check point inhibitor, e.g., a check point inhibitor described herein, e.g., one or more of PD1+ cells, LAG3+ cells, and TIM3+ cells, to thereby provide a population of T regulatory depleted, e.g., CD25+ depleted cells, and check point inhibitor depleted cells, e.g., PD1+, LAG3+ and/or TIM3+ depleted cells. Exemplary check point inhibitors include PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR (e.g., TGFRbeta), e.g., as described herein. In one embodiment, check point inhibitor expressing cells are removed simultaneously with the T regulatory, e.g., CD25+ cells. For example, an anti-CD25 antibody, or fragment thereof, and an anti-check point inhibitor antibody, or fragment thereof, can be attached to the same bead which can be used to remove the cells, or an anti-CD25 antibody, or fragment thereof, and the anti-check point inhibitor antibody, or fragment thereof, can be attached to separate beads, a mixture of which can be used to remove the cells. In other embodiments, the removal of T regulatory cells, e.g., CD25+ cells, and the removal of the check point inhibitor expressing cells is sequential, and can occur, e.g., in either order.

Methods described herein can include a positive selection step For example, T cells can be isolated by incubation with anti-CD3/anti-CD28 (e.g., 3x28)-conjugated beads, such as DYNABEADS® M-450 CD3/CD28 T, for a time period sufficient for positive selection of the desired T cells. In one aspect, the time period is about 30 minutes. In a further aspect, the time period ranges from 30 minutes to 36 hours or longer and all integer values there between. In a further aspect, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In yet another aspect, the time period is 10 to 24 hours. In one aspect, the incubation time period is 24 hours. Longer incubation times may be used to isolate T cells in any situation where there are few T cells as compared to other cell types, such in isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immunocompromised individuals. Further, use of longer incubation times can increase the efficiency of capture of CD8+ T cells. Thus, by simply shortening or lengthening the time T cells are allowed to bind to the CD3/CD28 beads and/or by increasing or decreasing the ratio of beads to T cells (as described further herein), subpopulations of T cells can be preferentially selected for or against at culture initiation or at other time points during the process.

Additionally, by increasing or decreasing the ratio of anti-CD3 and/or anti-CD28 antibodies on the beads or other surface, subpopulations of T cells can be preferentially selected for or against at culture initiation or at other desired time points.

In one embodiment, a T cell population can be selected that expresses one or more of IFN-γ, TNFα, IL-17A, IL-2, IL-3, IL-4, GM-CSF, IL-10, IL-13, granzyme B, and perforin, or other appropriate molecules, e.g., other cytokines. Methods for screening for cell expression can be determined, e.g., by the methods described in PCT Publication No.: WO 2013/126712.

For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (e.g., particles such as beads) can be varied. In certain aspects, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (e.g., increase the concentration of cells), to ensure maximum contact of cells and beads. For example, in one aspect, a concentration of about 10 billion cells/ml, 9 billion/ml, 8 billion/ml, 7 billion/ml, 6 billion/ml, or 5 billion/ml is used. In one aspect, a concentration of 1 billion cells/ml is used. In one aspect, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further aspects, concentrations of 125 or 150 million cells/ml can be used.

Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells, or from samples where there are many tumor cells present (e.g., leukemic blood, tumor tissue, etc.). Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

In a related aspect, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (e.g., particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4+ T cells express higher levels of CD28 and are more efficiently captured than CD8+ T cells in dilute concentrations. In one aspect, the concentration of cells used is 5×10^6/ml. In other aspects, the concentration used can be from about 1×10^5/ml to 1×10^6/ml, and any integer value in

between.

In other aspects, the cells may be incubated on a rotator for varying lengths of time at varying speeds at either 2-10°C or at room temperature.

T cells for stimulation can also be frozen after a washing step. Wishing not to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After the washing step that removes plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or culture media containing 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin and 7.5% DMSO, or 31.25% Plasmalyte-A, 31.25% Dextrose 5%, 0.45% NaCl, 10% Dextran 40 and 5% Dextrose, 20% Human Serum Albumin, and 7.5% DMSO or other suitable cell freezing media containing for example, Hespan and PlasmaLyte A, the cells then are frozen to -80°C at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled freezing immediately at -20° C or in liquid nitrogen.

In certain aspects, cryopreserved cells are thawed and washed as described herein and allowed to rest for one hour at room temperature prior to activation using the methods of the present invention.

Also contemplated in the context of the invention is the collection of blood samples or apheresis product from a subject at a time period prior to when the expanded cells as described herein might be needed. As such, the source of the cells to be expanded can be collected at any time point necessary, and desired cells, such as T cells, isolated and frozen for later use in immune effector cell therapy for any number of diseases or conditions that would benefit from immune effector cell therapy, such as those described herein. In one aspect a blood sample or an apheresis is taken from a generally healthy subject. In certain aspects, a blood sample or an apheresis is taken from a generally healthy subject who is at risk of developing a disease, but who has not yet developed a disease, and the cells of interest are isolated and frozen for later use. In certain aspects, the T cells may be expanded, frozen, and used at a later time. In certain aspects, samples are collected from a patient shortly after diagnosis of a particular disease as

described herein but prior to any treatments. In a further aspect, the cells are isolated from a blood sample or an apheresis from a subject prior to any number of relevant treatment modalities, including but not limited to treatment with agents such as natalizumab, efalizumab, antiviral agents, chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies, cytoxan, fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, and irradiation.

In a further aspect of the present invention, T cells are obtained from a patient directly following treatment that leaves the subject with functional T cells. In this regard, it has been observed that following certain cancer treatments, in particular treatments with drugs that damage the immune system, shortly after treatment during the period when patients would normally be recovering from the treatment, the quality of T cells obtained may be optimal or improved for their ability to expand ex vivo. Likewise, following ex vivo manipulation using the methods described herein, these cells may be in a preferred state for enhanced engraftment and in vivo expansion. Thus, it is contemplated within the context of the present invention to collect blood cells, including T cells, dendritic cells, or other cells of the hematopoietic lineage, during this recovery phase. Further, in certain aspects, mobilization (for example, mobilization with GM-CSF) and conditioning regimens can be used to create a condition in a subject wherein repopulation, recirculation, regeneration, and/or expansion of particular cell types is favored, especially during a defined window of time following therapy. Illustrative cell types include T cells, B cells, dendritic cells, and other cells of the immune system.

In one embodiment, the immune effector cells expressing a CAR molecule, e.g., a CAR molecule described herein, are obtained from a subject that has received a low, immune enhancing dose of an mTOR inhibitor. In an embodiment, the population of immune effector cells, e.g., T cells, to be engineered to express a CAR, are harvested after a sufficient time, or after sufficient dosing of the low, immune enhancing, dose of an mTOR inhibitor, such that the level of PD1 negative immune effector cells, e.g., T cells, or the ratio of PD1 negative immune effector cells, e.g., T cells/ PD1 positive immune effector cells, e.g., T cells, in the subject or harvested from the subject has been, at least transiently, increased.

In other embodiments, population of immune effector cells, e.g., T cells, which have, or will be engineered to express a CAR, can be treated ex vivo by contact with an amount of an

mTOR inhibitor that increases the number of PD1 negative immune effector cells, e.g., T cells or increases the ratio of PD1 negative immune effector cells, e.g., T cells/ PD1 positive immune effector cells, e.g., T cells.

In one embodiment, a T cell population is diacylglycerol kinase (DGK)-deficient. DGK-deficient cells include cells that do not express DGK RNA or protein, or have reduced or inhibited DGK activity. DGK-deficient cells can be generated by genetic approaches, e.g., administering RNA-interfering agents, e.g., siRNA, shRNA, miRNA, to reduce or prevent DGK expression. Alternatively, DGK-deficient cells can be generated by treatment with DGK inhibitors described herein.

In one embodiment, a T cell population is Ikaros-deficient. Ikaros-deficient cells include cells that do not express Ikaros RNA or protein, or have reduced or inhibited Ikaros activity, Ikaros-deficient cells can be generated by genetic approaches, e.g., administering RNA-interfering agents, e.g., siRNA, shRNA, miRNA, to reduce or prevent Ikaros expression. Alternatively, Ikaros-deficient cells can be generated by treatment with Ikaros inhibitors, e.g., lenalidomide.

In embodiments, a T cell population is DGK-deficient and Ikaros-deficient, e.g., does not express DGK and Ikaros, or has reduced or inhibited DGK and Ikaros activity. Such DGK and Ikaros-deficient cells can be generated by any of the methods described herein.

In an embodiment, the NK cells are obtained from the subject. In another embodiment, the NK cells are an NK cell line, e.g., NK-92 cell line (Conkwest).

Allogeneic CAR

In embodiments described herein, the immune effector cell can be an allogeneic immune effector cell, e.g., T cell or NK cell. For example, the cell can be an allogeneic T cell, e.g., an allogeneic T cell lacking expression of a functional T cell receptor (TCR) and/or human leukocyte antigen (HLA), e.g., HLA class I and/or HLA class II.

A T cell lacking a functional TCR can be, e.g., engineered such that it does not express any functional TCR on its surface, engineered such that it does not express one or more subunits that comprise a functional TCR (e.g., engineered such that it does not express (or exhibits reduced expression) of TCR alpha, TCR beta, TCR gamma, TCR delta, TCR epsilon, and/or

TCR zeta) or engineered such that it produces very little functional TCR on its surface (e.g., engineered such that it does not express (or exhibits reduced expression) of TCR alpha, TCR beta, TCR gamma, TCR delta, TCR epsilon, and/or TCR zeta). Alternatively, the T cell can express a substantially impaired TCR, e.g., by expression of mutated or truncated forms of one or more of the subunits of the TCR. The term "substantially impaired TCR" means that this TCR will not elicit an adverse immune reaction in a host.

A T cell described herein can be, e.g., engineered such that it does not express a functional HLA on its surface. For example, a T cell described herein, can be engineered such that cell surface expression HLA, e.g., HLA class 1 and/or HLA class II, is downregulated. In some embodiments, downregulation of HLA may be accomplished by reducing or eliminating expression of beta-2 microglobulin (B2M).

In some embodiments, the T cell can lack a functional TCR and a functional HLA, e.g., HLA class I and/or HLA class II.

Modified T cells that lack expression of a functional TCR and/or HLA can be obtained by any suitable means, including a knock out or knock down of one or more subunit of TCR or HLA. For example, the T cell can include a knock down of TCR and/or HLA using siRNA, shRNA, clustered regularly interspaced short palindromic repeats (CRISPR) transcription-activator like effector nuclease (TALEN), or zinc finger endonuclease (ZFN).

In some embodiments, the allogeneic cell can be a cell which does not express or expresses at low levels an inhibitory molecule, e.g. a cell engineered by any method described herein. For example, the cell can be a cell that does not express or expresses at low levels an inhibitory molecule, e.g., that can decrease the ability of a CAR-expressing cell to mount an immune effector response. Examples of inhibitory molecules include PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR (e.g., TGFR beta). Inhibition of an inhibitory molecule, e.g., by inhibition at the DNA, RNA or protein level, can optimize a CAR-expressing cell performance. In embodiments, an inhibitory nucleic acid, e.g., an inhibitory nucleic acid, e.g., a dsRNA, e.g., an siRNA or shRNA, a clustered regularly interspaced short palindromic repeats (CRISPR), a transcription-activator

like effector nuclease (TALEN), or a zinc finger endonuclease (ZFN), e.g., as described herein, can be used.

siRNA and shRNA to inhibit TCR or HLA

In some embodiments, TCR expression and/or HLA expression can be inhibited using siRNA or shRNA that targets a nucleic acid encoding a TCR and/or HLA, and/or an inhibitory molecule described herein (e.g., PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR beta), in a T cell.

Expression systems for siRNA and shRNAs, and exemplary shRNAs, are described, e.g., in paragraphs 649 and 650 of International Application WO2015/142675, filed March 13, 2015, which is incorporated by reference in its entirety

CRISPR to inhibit TCR or HLA

"CRISPR" or "CRISPR to TCR and/or HLA" or "CRISPR to inhibit TCR and/or HLA" as used herein refers to a set of clustered regularly interspaced short palindromic repeats, or a system comprising such a set of repeats. "Cas", as used herein, refers to a CRISPR-associated protein. A "CRISPR/Cas" system refers to a system derived from CRISPR and Cas which can be used to silence or mutate a TCR and/or HLA gene, and/or an inhibitory molecule described herein (e.g., PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR beta),.

The CRISPR/Cas system, and uses thereof, are described, e.g., in paragraphs 651-658 of International Application WO2015/142675, filed March 13, 2015, which is incorporated by reference in its entirety.

TALEN to inhibit TCR and/or HLA

"TALEN" or "TALEN to HLA and/or TCR" or "TALEN to inhibit HLA and/or TCR" refers to a transcription activator-like effector nuclease, an artificial nuclease which can be used to edit the HLA and/or TCR gene, and/or an inhibitory molecule described herein (e.g., PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-

5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR beta).

TALENs, TALEs, and uses thereof, are described, e.g., in paragraphs 659-665 of International Application WO2015/142675, filed March 13, 2015, which is incorporated by reference in its entirety.

Zinc finger nuclease to inhibit HLA and/or TCR

"ZFN" or "Zinc Finger Nuclease" or "ZFN to HLA and/or TCR" or "ZFN to inhibit HLA and/or TCR" refer to a zinc finger nuclease, an artificial nuclease which can be used to edit the HLA and/or TCR gene, and/or an inhibitory molecule described herein (e.g., PD1, PD-L1, PD-L2, CTLA4, TIM3, CEACAM (e.g., CEACAM-1, CEACAM-3 and/or CEACAM-5), LAG3, VISTA, BTLA, TIGIT, LAIR1, CD160, 2B4, CD80, CD86, B7-H3 (CD276), B7-H4 (VTCN1), HVEM (TNFRSF14 or CD270), KIR, A2aR, MHC class I, MHC class II, GAL9, adenosine, and TGFR beta).

ZFNs, and uses thereof, are described, e.g., in paragraphs 666-671 of International Application WO2015/142675, filed March 13, 2015, which is incorporated by reference in its entirety.

Telomerase Expression

While not wishing to be bound by any particular theory, in some embodiments, a therapeutic T cell has short term persistence in a patient, due to shortened telomeres in the T cell; accordingly, transfection with a telomerase gene can lengthen the telomeres of the T cell and improve persistence of the T cell in the patient. See Carl June, "Adoptive T cell therapy for cancer in the clinic", Journal of Clinical Investigation, 117:1466-1476 (2007). Thus, in an embodiment, an immune effector cell, e.g., a T cell, ectopically expresses a telomerase subunit, e.g., the catalytic subunit of telomerase, e.g., TERT, e.g., hTERT. In some aspects, this disclosure provides a method of producing a CAR-expressing cell, comprising contacting a cell with a nucleic acid encoding a telomerase subunit, e.g., the catalytic subunit of telomerase, e.g., TERT, e.g., hTERT. The cell may be contacted with the nucleic acid before, simultaneous with, or after being contacted with a construct encoding a CAR.

In one aspect, the disclosure features a method of making a population of immune

effector cells (e.g., T cells or NK cells). In an embodiment, the method comprises: providing a population of immune effector cells (e.g., T cells or NK cells), contacting the population of immune effector cells with a nucleic acid encoding a CAR; and contacting the population of immune effector cells with a nucleic acid encoding a telomerase subunit, e.g., hTERT, under conditions that allow for CAR and telomerase expression.

In an embodiment, the nucleic acid encoding the telomerase subunit is DNA. In an embodiment, the nucleic acid encoding the telomerase subunit comprises a promoter capable of driving expression of the telomerase subunit.

In an embodiment, hTERT has the amino acid sequence of GenBank Protein ID AAC51724.1 (Meyerson et al., "hEST2, the Putative Human Telomerase Catalytic Subunit Gene, Is Up-Regulated in Tumor Cells and during Immortalization" Cell Volume 90, Issue 4, 22 August 1997, Pages 785–795) as follows:

MPRAPRCRAVRSLLRSHYREVLPLATFVRRLGPQGWRLVQRGDPAAFRALVAQ
CLVCVPWDARPPPAAPSFRQVSCLKELVARVLQRLCERGAKNVLAFGFALLDGARGGP
PEAFTTSVRSYLPNTVTDALRGSGAWGLLLRRVGDDVLVHLLARCALFVLVAPSCAYQ
VCGPPLYQLGAATQARPPPHASGPRRRLGCERAWNHSVREAGVPLGLPAPGARRRGGS
ASRSLPLPKRPRRGAAPEPERTPVGQGSWAHPGRTRGPSDRGFCVVSPARPAEEATSLE
GALSGTRHSHPSVGRQHHAGPPSTSRPPRPWDTPCPPVYAETKHFLYSSGDKEQLRPSFL
LSSLRPSLTGARRLVETIFLGSRPWMPGTPRRLPRLPQRYWQMRPLFLELLGNHAQCPY
GVLLKTHCPLRAAVTPAAGVCAREKPQGSVAAPEEEDTDPRRLVQLLRQHSSPWQVYG
FVRACLRRLVPPGLWGSRHNERRFLRNTKKFISLGKHAKLSLQELTWKMSVRGCAWLR
RSPGVGCVPAAEHRLREEILAKFLHWLMSVYVVELLRSFFYVTETTFQKNRLFFYRKSV
WSKLQSIGIRQHLKRVQLRELSEAEVRQHREARPALLTSRLRFIPKPDGLRPIVNMDYVV
GARTFRREKRAERLTSRVKALFSVLNYERARRPGLLGASVLGLDDIHRAWRTFVLRVR
AQDPPPELYFVKVDVTGAYDTIPQDRLTEVIASIIKPQNTYCVRRYAVVQKAAHGHVRK
AFKSHVSTLTDLQPYMRQFVAHLQETSPLRDAVVIEQSSSLNEASSGLFDVFLRFMCHH
AVRIRGKSYVQCQGIPQGSILSTLLCSLCYGDMENKLFAGIRRDGLLLRLVDDFLLVTPH
LTHAKTFLRTLVRGVPEYGCVVNLRKTVVNFPVEDEALGGTAFVQMPAHGLFPWCGL
LLDTRTLEVQSDYSSYARTSIRASLTFNRGFKAGRNMRRKLFGVLRLKCHSLFLDLQVN
SLQTVCTNIYKILLLQAYRFHACVLQLPFHQQVWKNPTFFLRVISDTASLCYSILKAKNA
GMSLGAKGAAGPLPSEAVQWLCHQAFLLKLTRHRVTYVPLLGSLRTAQTQLSRKLPGT

TLTALEAAANPALPSDFKTILD (SEQ ID NO: 1332)

In an embodiment, the hTERT has a sequence at least 80%, 85%, 90%, 95%, 96^, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 1332. In an embodiment, the hTERT has a sequence of SEQ ID NO: 1332. In an embodiment, the hTERT comprises a deletion (e.g., of no more than 5, 10, 15, 20, or 30 amino acids) at the N-terminus, the C-terminus, or both. In an embodiment, the hTERT comprises a transgenic amino acid sequence (e.g., of no more than 5, 10, 15, 20, or 30 amino acids) at the N-terminus, the C-terminus, or both.

In an embodiment, the hTERT is encoded by the nucleic acid sequence of GenBank Accession No. AF018167 (Meyerson et al., "hEST2, the Putative Human Telomerase Catalytic Subunit Gene, Is Up-Regulated in Tumor Cells and during Immortalization" Cell Volume 90, Issue 4, 22 August 1997, Pages 785–795):

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   1 caggcagcgt ggtcctgctg cgcacgtggg aagccctggc cccggccacc cccgcgatgc
  61 cgcgcgctcc ccgctgccga gccgtgcgct ccctgctgcg cagccactac cgcgaggtgc
 121 tgccgctggc cacgttcgtg cggcgcctgg ggccccaggg ctggcggctg gtgcagcgcg
 181 gggacccggc ggctttccgc gcgctggtgg cccagtgcct ggtgtgcgtg ccctgggacg
 241 cacggccgcc ccccgccgcc ccctccttcc gccaggtgtc ctgcctgaag gagctggtgg
 301 cccgagtgct gcagaggctg tgcgagcgcg gcgcgaagaa cgtgctggcc ttcggcttcg
 361 cgctgctgga cggggcccgc ggggggcccc ccgaggcctt caccaccagc gtgcgcagct
 421 acctgcccaa cacggtgacc gacgcactgc gggggagcgg ggcgtggggg ctgctgttgc
 481 gccgcgtggg cgacgacgtg ctggttcacc tgctggcacg ctgcgcgctc tttgtgctgg
 541 tggctcccag ctgcgcctac caggtgtgcg ggccgccgct gtaccagctc ggcgctgcca
 601 ctcaggcccg gcccccgcca cacgctagtg gaccccgaag gcgtctggga tgcgaacggg
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 961 agcaccacgc gggcccccca tccacatcgc ggccaccacg tccctgggac acgccttgtc
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1321 cagccggtgt ctgtgcccgg gagaagcccc agggctctgt ggcggccccc gaggaggagg
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1441 acggcttcgt gcgggcctgc ctgcgccggc tggtgccccc aggcctctgg ggctccaggc
1501 acaacgaacg ccgcttcctc aggaacacca agaagttcat ctccctgggg aagcatgcca
1561 agctctcgct gcaggagctg acgtggaaga tgagcgtgcg gggctgcgct tggctgcgca
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1681 ccaagttcct gcactggctg atgagtgtgt acgtcgtcga gctgctcagg tctttctttt
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1801 gcaagttgca aagcattgga atcagacagc acttgaagag ggtgcagctg cgggagctgt
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2101 gcctggacga tatccacagg gcctggcgca ccttcgtgct gcgtgtgcgg gcccaggacc
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2461 gcagtggcct cttcgacgtc ttcctacgct tcatgtgcca ccacgccgtg cgcatcaggg
2521 gcaagtccta cgtccagtgc caggggatcc cgcagggctc catcctctcc acgctgctct
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2881 tgcagagcga ctactccagc tatgcccgga cctccatcag agccagtctc accttcaacc
2941 gcggcttcaa ggctgggagg aacatgcgtc gcaaactctt tggggtcttg cggctgaagt
3001 gtcacagcct gtttctggat ttgcaggtga acagcctcca gacggtgtgc accaacatct
3061 acaagatcct cctgctgcag gcgtacaggt ttcacgcatg tgtgctgcag ctcccatttc
3121 atcagcaagt ttggaagaac cccacatttt tcctgcgcgt catctctgac acggcctccc
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3241 ccggccctct gccctccgag gccgtgcagt ggctgtgcca ccaagcattc ctgctcaagc
3301 tgactcgaca ccgtgtcacc tacgtgccac tcctggggtc actcaggaca gcccagacgc
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3421 cactgccctc agacttcaag accatcctgg actgatggcc acccgcccac agccaggccg
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3541 ccacacccag gcccgcaccg ctgggagtct gaggcctgag tgagtgtttg gccgaggcct
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3721 agggccagct tttcctcacc aggagcccgg cttccactcc ccacatagga atagtccatc
3781 cccagattcg ccattgttca cccctcgccc tgccctcctt tgccttccac ccccaccatc
3841 caggtggaga ccctgagaag gaccctggga gctctgggaa tttggagtga ccaaaggtgt
3901 gccctgtaca caggcgagga ccctgcacct ggatgggggt ccctgtgggt caaattgggg
3961 ggaggtgctg tgggagtaaa atactgaata tatgagtttt tcagttttga aaaaaaaaaa
4021 aaaaaaa (SEQ ID NO: 1333)

In an embodiment, the hTERT is encoded by a nucleic acid having a sequence at least 80%, 85%, 90%, 95%, 96, 97%, 98%, or 99% identical to the sequence of SEQ ID NO: 1333.

In an embodiment, the hTERT is encoded by a nucleic acid of SEQ ID NO: 1333.

Activation and Expansion of Immune Effector Cells (e.g., T Cells)

Immune effector cells such as T cells may be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005.

The procedure for *ex vivo* expansion of hematopoietic stem and progenitor cells is described in U.S. Pat. No. 5,199,942, incorporated herein by reference, can be applied to the cells of the present invention. Other suitable methods are known in the art, therefore the present invention is not limited to any particular method of ex vivo expansion of the cells. Briefly, ex vivo culture and expansion of T cells can comprise: (1) collecting CD34+ hematopoietic stem and progenitor cells from a mammal from peripheral blood harvest or bone marrow explants; and (2) expanding such cells ex vivo. In addition to the cellular growth factors described in U.S. Pat. No. 5,199,942, other factors such as flt3-L, IL-1, IL-3 and c-kit ligand, can be used for culturing and expansion of the cells.

Generally, a population of immune effector cells may be expanded by contact with a surface having attached thereto an agent that stimulates a CD3/TCR complex associated signal and a ligand that stimulates a costimulatory molecule on the surface of the T cells. In particular, T cell populations may be stimulated as described herein, such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. To stimulate proliferation of either CD4+ T cells or CD8+ T cells, an anti-CD3 antibody and an anti-CD28 antibody may be used. Examples of an anti-CD28 antibody include 9.3, B-T3, XR-CD28 (Diaclone, Besançon, France) can be used as can other methods commonly known in the art (Berg et al., Transplant Proc. 30(8):3975-3977,

1998; Haanen et al., J. Exp. Med. 190(9):13191328, 1999; Garland et al., J. Immunol Meth. 227(1-2):53-63, 1999).

In some embodiments, immune effector cells (such as PBMCs or T cells) are expanded and stimulated by contacting the cells to one or both of an anti-CD3 antibody and IL-2. In embodiments, the cells are expanded without anti-CD3 or anti-CD28 beads.

In certain aspects, the primary stimulatory signal and the costimulatory signal for the T cell may be provided by different protocols. For example, the agents providing each signal may be in solution or coupled to a surface. When coupled to a surface, the agents may be coupled to the same surface (i.e., in "cis" formation) or to separate surfaces (i.e., in "trans" formation). Alternatively, one agent may be coupled to a surface and the other agent in solution. In one aspect, the agent providing the costimulatory signal is bound to a cell surface and the agent providing the primary activation signal is in solution or coupled to a surface. In certain aspects, both agents can be in solution. In one aspect, the agents may be in soluble form, and then cross-linked to a surface, such as a cell expressing Fc receptors or an antibody or other binding agent which will bind to the agents. In this regard, see for example, U.S. Patent Application Publication Nos. 20040101519 and 20060034810 for artificial antigen presenting cells (aAPCs) that are contemplated for use in activating and expanding T cells in the present invention.

In one aspect, the two agents are immobilized on beads, either on the same bead, i.e., "cis," or to separate beads, i.e., "trans." By way of example, the agent providing the primary activation signal is an anti-CD3 antibody or an antigen-binding fragment thereof and the agent providing the costimulatory signal is an anti-CD28 antibody or antigen-binding fragment thereof; and both agents are co-immobilized to the same bead in equivalent molecular amounts. In one aspect, a 1:1 ratio of each antibody bound to the beads for CD4+ T cell expansion and T cell growth is used. In certain aspects, a ratio of anti CD3:CD28 antibodies bound to the beads is used such that an increase in T cell expansion is observed as compared to the expansion observed using a ratio of 1:1. In one particular aspect an increase of from about 1 to about 3 fold is observed as compared to the expansion observed using a ratio of 1:1. In one aspect, the ratio of CD3:CD28 antibody bound to the beads ranges from 100:1 to 1:100 and all integer values there between. In one aspect, more anti-CD28 antibody is bound to the particles than anti-CD3 antibody, i.e., the ratio of CD3:CD28 is less than one. In certain aspects, the ratio of anti CD28 antibody to anti CD3 antibody bound to the beads is greater than 2:1. In one particular aspect, a

1:100 CD3:CD28 ratio of antibody bound to beads is used. In one aspect, a 1:75 CD3:CD28 ratio of antibody bound to beads is used. In a further aspect, a 1:50 CD3:CD28 ratio of antibody bound to beads is used. In one aspect, a 1:30 CD3:CD28 ratio of antibody bound to beads is used. In one aspect, a 1:10 CD3:CD28 ratio of antibody bound to beads is used. In one aspect, a 1:3 CD3:CD28 ratio of antibody bound to the beads is used. In yet one aspect, a 3:1 CD3:CD28 ratio of antibody bound to the beads is used.

Ratios of particles to cells from 1:500 to 500:1 and any integer values in between may be used to stimulate T cells or other target cells. As those of ordinary skill in the art can readily appreciate, the ratio of particles to cells may depend on particle size relative to the target cell. For example, small sized beads could only bind a few cells, while larger beads could bind many. In certain aspects the ratio of cells to particles ranges from 1:100 to 100:1 and any integer values in-between and in further aspects the ratio comprises 1:9 to 9:1 and any integer values in between, can also be used to stimulate T cells. The ratio of anti-CD3- and anti-CD28-coupled particles to T cells that result in T cell stimulation can vary as noted above, however certain suitable values include 1:100, 1:50, 1:40, 1:30, 1:20, 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, and 15:1 with one suitable ratio being at least 1:1 particles per T cell. In one aspect, a ratio of particles to cells of 1:1 or less is used. In one particular aspect, a suitable particle: cell ratio is 1:5. In further aspects, the ratio of particles to cells can be varied depending on the day of stimulation. For example, in one aspect, the ratio of particles to cells is from 1:1 to 10:1 on the first day and additional particles are added to the cells every day or every other day thereafter for up to 10 days, at final ratios of from 1:1 to 1:10 (based on cell counts on the day of addition). In one particular aspect, the ratio of particles to cells is 1:1 on the first day of stimulation and adjusted to 1:5 on the third and fifth days of stimulation. In one aspect, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:5 on the third and fifth days of stimulation. In one aspect, the ratio of particles to cells is 2:1 on the first day of stimulation and adjusted to 1:10 on the third and fifth days of stimulation. In one aspect, particles are added on a daily or every other day basis to a final ratio of 1:1 on the first day, and 1:10 on the third and fifth days of stimulation. One of skill in the art will appreciate that a variety of other ratios may be suitable for use in the present invention. In particular, ratios will vary depending on particle size and on cell size and type. In one aspect, the most typical ratios for use are in the neighborhood of 1:1, 2:1 and 3:1 on the first

day.

In further aspects of the present invention, the cells, such as T cells, are combined with agent-coated beads, the beads and the cells are subsequently separated, and then the cells are cultured. In an alternative aspect, prior to culture, the agent-coated beads and cells are not separated but are cultured together. In a further aspect, the beads and cells are first concentrated by application of a force, such as a magnetic force, resulting in increased ligation of cell surface markers, thereby inducing cell stimulation.

By way of example, cell surface proteins may be ligated by allowing paramagnetic beads to which anti-CD3 and anti-CD28 are attached (3x28 beads) to contact the T cells. In one aspect the cells (for example, 10^4 to 10^9 T cells) and beads (for example, DYNABEADS® M-450 CD3/CD28 T paramagnetic beads at a ratio of 1:1) are combined in a buffer, for example PBS (without divalent cations such as, calcium and magnesium). Again, those of ordinary skill in the art can readily appreciate any cell concentration may be used. For example, the target cell may be very rare in the sample and comprise only 0.01% of the sample or the entire sample (i.e., 100%) may comprise the target cell of interest. Accordingly, any cell number is within the context of the present invention. In certain aspects, it may be desirable to significantly decrease the volume in which particles and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and particles. For example, in one aspect, a concentration of about 10 billion cells/ml, 9 billion/ml, 8 billion/ml, 7 billion/ml, 6 billion/ml, or 5 billion/ml or 2 billion cells/ml is used. In one aspect, greater than 100 million cells/ml is used. In a further aspect, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet one aspect, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further aspects, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T cells. Such populations of cells may have therapeutic value and would be desirable to obtain in certain aspects. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

In one embodiment, cells transduced with a nucleic acid encoding a CAR, e.g., a CAR described herein, are expanded, e.g., by a method described herein. In one embodiment, the

cells are expanded in culture for a period of several hours (e.g., about 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 18, 21 hours) to about 14 days (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14 days). In one embodiment, the cells are expanded for a period of 4 to 9 days. In one embodiment, the cells are expanded for a period of 8 days or less, e.g., 7, 6 or 5 days. In one embodiment, the cells, e.g., a CAR cell described herein, are expanded in culture for 5 days, and the resulting cells are more potent than the same cells expanded in culture for 9 days under the same culture conditions. Potency can be defined, e.g., by various T cell functions, e.g. proliferation, target cell killing, cytokine production, activation, migration, or combinations thereof. In one embodiment, the cells, e.g., a CD19 CAR cell described herein, expanded for 5 days show at least a one, two, three or four fold increase in cells doublings upon antigen stimulation as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, e.g., the cells expressing a CAR described herein, are expanded in culture for 5 days, and the resulting cells exhibit higher proinflammatory cytokine production, e.g., IFN-γ and/or GM-CSF levels, as compared to the same cells expanded in culture for 9 days under the same culture conditions. In one embodiment, the cells, e.g., a CAR cell described herein, expanded for 5 days show at least a one, two, three, four, five, ten fold or more increase in pg/ml of proinflammatory cytokine production, e.g., IFN-γ and/or GM-CSF levels, as compared to the same cells expanded in culture for 9 days under the same culture conditions.

In one aspect of the present invention, the mixture may be cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In one aspect, the mixture may be cultured for 21 days. In one aspect of the invention the beads and the T cells are cultured together for about eight days. In one aspect, the beads and T cells are cultured together for 2-3 days.

Several cycles of stimulation may also be desired such that culture time of T cells can be 60 days or more. Conditions appropriate for T cell culture include an appropriate media (e.g., Minimal Essential Media or RPMI Media 1640 or, X-vivo 15, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-γ, IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGFβ, and TNF-α or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, AIM-V,

DMEM, MEM, α-MEM, F-12, X-Vivo 15, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, e.g., penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (e.g., 37° C) and atmosphere (e.g., air plus 5% CO_2).

In one embodiment, the cells are expanded in an appropriate media (e.g., media described herein) that includes one or more interleukin that result in at least a 200-fold (e.g., 200-fold, 250-fold, 300-fold, 350-fold) increase in cells over a 14 day expansion period, e.g., as measured by a method described herein such as flow cytometry. In one embodiment, the cells are expanded in the presence IL-15 and/or IL-7 (e.g., IL-15 and IL-7).

In some embodiments a CAR-expressing cell described herein (e.g., a T cell such as a CD4+ T cell or a CD8+ T cell) is contacted with a composition comprising a interleukin-15 (IL-15) polypeptide, a interleukin-15 receptor alpha (IL-15Ra) polypeptide, or a combination of both a IL-15 polypeptide and a IL-15Ra polypeptide e.g., hetIL-15, during the manufacturing of the CAR-expressing cell, e.g., ex vivo. In embodiments, a CAR-expressing cell described herein is contacted with a composition comprising a IL-15 polypeptide during the manufacturing of the CAR-expressing cell, e.g., ex vivo. In embodiments, a CAR-expressing cell described herein is contacted with a composition comprising a combination of both a IL-15 polypeptide and a IL-15 Ra polypeptide during the manufacturing of the CAR-expressing cell, e.g., ex vivo. In embodiments, a CAR-expressing cell described herein is contacted with a composition comprising hetIL-15 during the manufacturing of the CAR-expressing cell, e.g., ex vivo.

In one embodiment the CAR-expressing cell (e.g., a T cell or NK cell) described herein is contacted with a composition comprising hetIL-15 during ex vivo expansion. In an embodiment, the CAR-expressing cell described herein is contacted with a composition comprising an IL-15 polypeptide during ex vivo expansion. In an embodiment, the CAR-expressing cell described herein is contacted with a composition comprising both an IL-15 polypeptide and an IL-15Ra polypeptide during ex vivo expansion. In one embodiment the contacting results in the survival and proliferation of a lymphocyte subpopulation, e.g., CD8+ T cells.

In an embodiments, the method of making disclosed herein further comprises contacting the population of immune effector cells (e.g., T cells or NK cells) with a nucleic acid encoding a telomerase subunit, e.g., hTERT. The nucleic acid encoding the telomerase subunit can be DNA.

T cells that have been exposed to varied stimulation times may exhibit different characteristics. For example, typical blood or apheresed peripheral blood mononuclear cell products have a helper T cell population (TH, CD4+) that is greater than the cytotoxic or suppressor T cell population (TC, CD8+). Ex vivo expansion of T cells by stimulating CD3 and CD28 receptors produces a population of T cells that prior to about days 8-9 consists predominately of TH cells, while after about days 8-9, the population of T cells comprises an increasingly greater population of TC cells. Accordingly, depending on the purpose of treatment, infusing a subject with a T cell population comprising predominately of TH cells may be advantageous. Similarly, if an antigen-specific subset of TC cells has been isolated it may be beneficial to expand this subset to a greater degree.

Further, in addition to CD4 and CD8 markers, other phenotypic markers vary significantly, but in large part, reproducibly during the course of the cell expansion process. Thus, such reproducibility enables the ability to tailor an activated T cell product for specific purposes.

Once a CAR, e.g., CD19 CAR is constructed, various assays can be used to evaluate the activity of the molecule, such as but not limited to, the ability to expand T cells following antigen stimulation, sustain T cell expansion in the absence of re-stimulation, and anti-cancer activities in appropriate in vitro and animal models. Assays to evaluate the effects of a CAR, e.g., CD19 CAR are described in further detail below

Western blot analysis of CAR expression in primary T cells can be used to detect the presence of monomers and dimers, e.g., as described in paragraph 695 of International Application WO2015/142675, filed March 13, 2015, which is herein incorporated by reference in its entirety.

In vitro expansion of CAR^+ T cells following antigen stimulation can be measured by flow cytometry. For example, a mixture of $CD4^+$ and $CD8^+$ T cells are stimulated with αCD3/αCD28 beads followed by transduction with lentiviral vectors expressing GFP under the

control of the promoters to be analyzed. Exemplary promoters include the CMV IE gene, EF-1α, ubiquitin C, or phosphoglycerokinase (PGK) promoters. GFP fluorescence is evaluated on day 6 of culture in the $CD4^+$ and/or $CD8^+$ T cell subsets by flow cytometry. See, *e.g.*, Milone *et al.*, Molecular Therapy 17(8): 1453-1464 (2009). Alternatively, a mixture of $CD4^+$ and $CD8^+$ T cells are stimulated with αCD3/αCD28 coated magnetic beads on day 0, and transduced with CAR on day 1 using a bicistronic lentiviral vector expressing CAR along with eGFP using a 2A ribosomal skipping sequence. Cultures are re-stimulated with either $CD19^+$ K562 cells (K562-CD19), wild-type K562 cells (K562 wild type) or K562 cells expressing hCD32 and 4-1BBL in the presence of anti-CD3 and anti-CD28 antibody (K562-BBL-3/28) following washing. Exogenous IL-2 is added to the cultures every other day at 100 IU/ml. GFP^+ T cells are enumerated by flow cytometry using bead-based counting. See, *e.g.*, Milone *et al.*, Molecular Therapy 17(8): 1453-1464 (2009).

Sustained CAR^+ T cell expansion in the absence of re-stimulation can also be measured. See, *e.g.*, Milone *et al.*, Molecular Therapy 17(8): 1453-1464 (2009). Briefly, mean T cell volume (fl) is measured on day 8 of culture using a Coulter Multisizer particle counter, a Nexcelom Cellometer Vision, or Millipore Scepter following stimulation with αCD3/αCD28 coated magnetic beads on day 0, and transduction with the indicated CAR on day 1.

Animal models can also be used to measure a CAR-expressing cell activity, e.g., as described in paragraph 698 of International Application WO2015/142675, filed March 13, 2015, which is herein incorporated by reference in its entirety.

Dose dependent CAR treatment response can be evaluated, e.g., as described in paragraph 699 of International Application WO2015/142675, filed March 13, 2015, which is herein incorporated by reference in its entirety. Assessment of cell proliferation and cytokine production has been previously described, e.g., as described in paragraph 700 of International Application WO2015/142675, filed March 13, 2015, which is herein incorporated by reference in its entirety. Cytotoxicity can be assessed by a standard ^{51}Cr-release assay, e.g., as described in paragraph 701 of International Application WO2015/142675, filed March 13, 2015, which is herein incorporated by reference in its entirety. Imaging technologies can be used to evaluate specific trafficking and proliferation of CARs in tumor-bearing animal models, e.g., as described in paragraph 702 of International Application WO2015/142675, filed March 13, 2015, which is herein incorporated by reference in its entirety.

Other assays, including those described in the Example section herein as well as those that are known in the art can also be used to evaluate the CARs described herein.

Alternatively, or in combination to the methods disclosed herein, methods and compositions for one or more of detection and/or quantification of CAR-expressing cells (e.g., in vitro or in vivo (e.g., clinical monitoring)), immune cell expansion and/or activation, and/or CAR-specific selection, that involve the use of a CAR ligand, are disclosed. In one exemplary embodiment, the CAR ligand is an antibody that binds to the CAR molecule, e.g., binds to the extracellular antigen binding domain of CAR (e.g., an antibody that binds to the antigen binding domain, e.g., an anti-idiotypic antibody; or an antibody that binds to a constant region of the extracellular binding domain). In other embodiments, the CAR ligand is a CAR antigen molecule (e.g., a CAR antigen molecule as described herein).

In one aspect, a method for detecting and/or quantifying CAR-expressing cells is disclosed. For example, the CAR ligand can be used to detect and/or quantify CAR-expressing cells in vitro or in vivo (e.g., clinical monitoring of CAR-expressing cells in a patient, or dosing a patient). The method includes:

providing the CAR ligand (optionally, a labelled CAR ligand, e.g., a CAR ligand that includes a tag, a bead, a radioactive or fluorescent label);

acquiring the CAR-expressing cell (e.g., acquiring a sample containing CAR-expressing cells, such as a manufacturing sample or a clinical sample);

contacting the CAR-expressing cell with the CAR ligand under conditions where binding occurs, thereby detecting the level (e.g., amount) of the CAR-expressing cells present. Binding of the CAR-expressing cell with the CAR ligand can be detected using standard techniques such as FACS, ELISA and the like.

In another aspect, a method of expanding and/or activating cells (e.g., immune effector cells) is disclosed. The method includes:

providing a CAR-expressing cell (e.g., a first CAR-expressing cell or a transiently expressing CAR cell);

contacting said CAR-expressing cell with a CAR ligand, e.g., a CAR ligand as described herein), under conditions where immune cell expansion and/or proliferation occurs, thereby

producing the activated and/or expanded cell population.

In certain embodiments, the CAR ligand is present on (e.g., is immobilized or attached to a substrate, e.g., a non-naturally occurring substrate). In some embodiments, the substrate is a non-cellular substrate. The non-cellular substrate can be a solid support chosen from, e.g., a plate (e.g., a microtiter plate), a membrane (e.g., a nitrocellulose membrane), a matrix, a chip or a bead. In embodiments, the CAR ligand is present in the substrate (e.g., on the substrate surface). The CAR ligand can be immobilized, attached, or associated covalently or non-covalently (e.g., cross-linked) to the substrate. In one embodiment, the CAR ligand is attached (e.g., covalently attached) to a bead. In the aforesaid embodiments, the immune cell population can be expanded in vitro or ex vivo. The method can further include culturing the population of immune cells in the presence of the ligand of the CAR molecule, e.g., using any of the methods described herein.

In other embodiments, the method of expanding and/or activating the cells further comprises addition of a second stimulatory molecule, e.g., CD28. For example, the CAR ligand and the second stimulatory molecule can be immobilized to a substrate, e.g., one or more beads, thereby providing increased cell expansion and/or activation.

In other embodiments, a method for selecting or enriching for a CAR expressing cell is provided. The method includes contacting the CAR expressing cell with a CAR ligand as described herein; and selecting the cell on the basis of binding of the CAR ligand.

In yet other embodiments, a method for depleting (e.g., reducing and/or killing) a CAR expressing cell is provided. The method includes contacting the CAR expressing cell with a CAR ligand as described herein; and targeting the cell on the basis of binding of the CAR ligand thereby reducing the number, and/or killing, the CAR-expressing cell. In one embodiment, the CAR ligand is coupled to a toxic agent (e.g., a toxin or a cell ablative drug). In another embodiment, the anti-idiotypic antibody can cause effector cell activity, e.g., ADCC or ADC activities.

Exemplary anti-CAR antibodies that can be used in the methods disclosed herein are described, e.g., in WO 2014/190273 and by Jena et al., "Chimeric Antigen Receptor (CAR)-Specific Monoclonal Antibody to Detect CD19-Specific T cells in Clinical Trials", PLOS March 2013 8:3 e57838, the contents of which are incorporated by reference. In some aspects and

embodiments, the compositions and methods herein are optimized for a specific subset of T cells, e.g., as described in US Serial No. PCT/US2015/043219 filed July 31, 2015, the contents of which are incorporated herein by reference in their entirety. In some embodiments, the optimized subsets of T cells display an enhanced persistence compared to a control T cell, e.g., a T cell of a different type (e.g., CD8+ or CD4+) expressing the same construct.

In some embodiments, a CD4+ T cell comprises a CAR described herein, which CAR comprises an intracellular signaling domain suitable for (e.g., optimized for, e.g., leading to enhanced persistence in) a CD4+ T cell, e.g., an ICOS domain. In some embodiments, a CD8+ T cell comprises a CAR described herein, which CAR comprises an intracellular signaling domain suitable for (e.g., optimized for, e.g., leading to enhanced persistence of) a CD8+ T cell, e.g., a 4-1BB domain, a CD28 domain, or another costimulatory domain other than an ICOS domain. In some embodiments, the CAR described herein comprises an antigen binding domain described herein, e.g., a CAR comprising an antigen binding domain.

In an aspect, described herein is a method of treating a subject, e.g., a subject having cancer. The method includes administering to said subject, an effective amount of:

1) a CD4+ T cell comprising a CAR (the CARCD4+) comprising:

an antigen binding domain, e.g., an antigen binding domain described herein;

a transmembrane domain; and

an intracellular signaling domain, e.g., a first costimulatory domain, e.g., an ICOS domain; and

2) a CD8+ T cell comprising a CAR (the CARCD8+) comprising:

an antigen binding domain, e.g., an antigen binding domain described herein;

a transmembrane domain; and

an intracellular signaling domain, e.g., a second costimulatory domain, e.g., a 4-1BB domain, a CD28 domain, or another costimulatory domain other than an ICOS domain;

wherein the CARCD4+ and the CARCD8+ differ from one another.

Optionally, the method further includes administering:

3) a second CD8+ T cell comprising a CAR (the second CARCD8+) comprising:

an antigen binding domain, e.g., an antigen binding domain described herein;

a transmembrane domain; and

an intracellular signaling domain, wherein the second CARCD8+ comprises an

intracellular signaling domain, e.g., a costimulatory signaling domain, not present on the CARCD8+, and, optionally, does not comprise an ICOS signaling domain.

Methods of Manufacture/Production

In some embodiments, the methods disclosed herein further include administering a T cell depleting agent after treatment with the cell (e.g., an immune effector cell as described herein, e.g., an immune effector cell expressing CAR driven by a truncated PGK1 promoter), thereby reducing (e.g., depleting) the CAR-expressing cells (e.g., the CD19CAR-expressing cells). Such T cell depleting agents can be used to effectively deplete CAR-expressing cells (e.g., CD19CAR-expressing cells) to mitigate toxicity. In some embodiments, the CAR-expressing cells were manufactured according to a method herein, e.g., assayed (e.g., before or after transfection or transduction) according to a method herein.

In some embodiments, the T cell depleting agent is administered one, two, three, four, or five weeks after administration of the cell, e.g., the population of immune effector cells, described herein.

In one embodiment, the T cell depleting agent is an agent that depletes CAR-expressing cells, e.g., by inducing antibody dependent cell-mediated cytotoxicity (ADCC) and/or complement-induced cell death. For example, CAR-expressing cells described herein may also express an antigen (e.g., a target antigen) that is recognized by molecules capable of inducing cell death, e.g., ADCC or complement-induced cell death. For example, CAR expressing cells described herein may also express a target protein (e.g., a receptor) capable of being targeted by an antibody or antibody fragment. Examples of such target proteins include, but are not limited to, EpCAM, VEGFR, integrins (e.g., integrins αvβ3, α4, αI3/4β3, α4β7, α5β1, αvβ3, αv), members of the TNF receptor superfamily (e.g., TRAIL-R1 , TRAIL-R2), PDGF Receptor, interferon receptor, folate receptor, GPNMB, ICAM-1, HLA-DR, CEA, CA-125, MUC1, TAG-72, IL-6 receptor, 5T4, GD2, GD3, CD2, CD3, CD4, CD5, CD11 , CD11a/LFA-1, CD15, CD18/ITGB2, CD19, CD20, CD22, CD23/lgE Receptor, CD25, CD28, CD30, CD33, CD38, CD40, CD41 , CD44, CD51 , CD52, CD62L, CD74, CD80, CD125, CD147/basigin, CD152/CTLA-4, CD154/CD40L, CD195/CCR5, CD319/SLAMF7, and EGFR, and truncated versions thereof (e.g., versions preserving one or more extracellular epitopes but lacking one or more regions within the cytoplasmic domain).

In some embodiments, the CAR expressing cell co-expresses the CAR and the target

protein, e.g., naturally expresses the target protein or is engineered to express the target protein. For example, the cell, e.g., the population of immune effector cells, can include a nucleic acid (e.g., vector) comprising the CAR nucleic acid (e.g., a CAR nucleic acid as described herein) and a nucleic acid encoding the target protein.

In one embodiment, the T cell depleting agent is a CD52 inhibitor, e.g., an anti-CD52 antibody molecule, e.g., alemtuzumab.

In other embodiments, the cell, e.g., the population of immune effector cells, expresses a CAR molecule as described herein (e.g., CD19CAR) and the target protein recognized by the T cell depleting agent. In one embodiment, the target protein is CD20. In embodiments where the target protein is CD20, the T cell depleting agent is an anti-CD20 antibody, e.g., rituximab.

In further embodiments of any of the aforesaid methods, the methods further include transplanting a cell, e.g., a hematopoietic stem cell, or a bone marrow, into the mammal.

In another aspect, the invention features a method of conditioning a mammal prior to cell transplantation. The method includes administering to the mammal an effective amount of the cell comprising a CAR nucleic acid or polypeptide, e.g., a CD19 CAR nucleic acid or polypeptide. In some embodiments, the cell transplantation is a stem cell transplantation, e.g., a hematopoietic stem cell transplantation, or a bone marrow transplantation. In other embodiments, conditioning a subject prior to cell transplantation includes reducing the number of target-expressing cells in a subject, e.g., CD19-expressing normal cells or CD19-expressing cancer cells.

Biopolymer delivery methods

In some embodiments, one or more CAR-expressing cells as disclosed herein can be administered or delivered to the subject via a biopolymer scaffold, e.g., a biopolymer implant. Biopolymer scaffolds can support or enhance the delivery, expansion, and/or dispersion of the CAR-expressing cells described herein. A biopolymer scaffold comprises a biocompatible (e.g., does not substantially induce an inflammatory or immune response) and/or a biodegradable polymer that can be naturally occurring or synthetic. Exemplary biopolymers are described, e.g., in paragraphs 1004-1006 of International Application WO2015/142675, filed March 13, 2015, which is herein incorporated by reference in its entirety.

Therapeutic Applications

CD19 Associated Diseases and/or Disorders

In one aspect, the invention provides methods for treating a disease associated with CD19 expression. In one aspect, the invention provides methods for treating a disease wherein part of the cancer is negative for CD19 and part of the cancer is positive for CD19. For example, the methods and compositions of the invention are useful for treating subjects that have undergone treatment for a disease associated with expression of CD19, wherein the subject that has undergone treatment related to CD19 expression, e.g., treatment with a CD19 CAR, exhibits a disease associated with expression of CD19.

In another aspect, the invention provides methods for treating a disease associated with expression of a B-cell antigen, e.g., one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1. In one aspect, the invention provides methods for treating a disease wherein part of the tumor is negative for the B-cell antigen and part of the tumor is positive for B-cell antigen. For example, the compositions and methods of the invention are useful for treating subjects that have undergone treatment for a disease associated with expression of the B-cell antigen, wherein the subject that has undergone treatment related to expression of a B-cell antigen, e.g., treatment with a CAR targeting a B-cell antigen, exhibits a disease associated with expression of the B-cell antigen. In a third aspect, the invention provides methods for treating a disease associated with expression of the B-cell antigen, e.g., associated with the expression of CD19 and one or more other B-cell antigens.

In one aspect, the invention pertains to a vector comprising CD19 CAR operably linked to promoter for expression in mammalian cells, e.g., T cells or NK cells. In one aspect, the invention provides a recombinant cell, e.g., a T cell or NK cell, expressing the CD19 CAR for use in treating CD19-expressing cancers, wherein the recombinant T cell expressing the CD19 CAR is termed a CD19 CART. In one aspect, the CD19 CART described herein, is capable of contacting a cancer cell with at least one CD19 CAR expressed on its surface such that the CART targets the cancer cell and growth of the cancer is inhibited.

In one aspect the invention pertains to a CD22 inhibitor which is a CD22 CART, e.g., a

T cell, expressing the CD22 CAR for use in treating CD22-expressing tumors in combination with CD19 CARTS, wherein the recombinant T cell expressing the CD22 CAR is termed a CD22 CART. In one aspect, the CD22 CART described herein, is capable of contacting a tumor cell with at least one CD22 CAR expressed on its surface such that the CD22 CART targets the tumor cell and growth of the tumor is inhibited.

In one aspect, the invention pertains to a method of inhibiting growth of a CD19-expressing cancer cell, comprising contacting the cancer cell with a CD19 CAR expressing cell, e.g., a CD19 CART cell, described, and one or more other CAR expressing cells, e.g., as described herein, such that the CART is activated in response to the antigen and targets the cancer cell, wherein the growth of the cancer is inhibited. The CD19 CAR-expressing cell, e.g., T cell, is administered in combination with a B-cell inhibitor, e.g., a B-cell inhibitor described herein.

In some embodiments, the CD19 inhibitor (e.g., one or more cells that express a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein) and the B cell inhibitor (e.g., one or more inhibitors of CD10, CD19, CD20, CD22, CD34, CD123, FLT-3, or ROR1, e.g., as described herein) are administered simultaneously. In some embodiments, the CD19 inhibitor and the B cell inhibitor are infused into a subject simultaneously, e.g., are admixed in the same infusion volume. In other embodiments, the simultaneous administration comprises separate administration of the CD19 inhibitor and the B cell inhibitor, e.g., administration of each is initiated within a predetermined time interval (e.g., within 15, 30, or 45 minutes of each other).

In some embodiments, the start of CD19 inhibitor delivery and the start of B cell inhibitor delivery are within 1, 2, 3, 4, 6, 12, 18, or 24 hours of each other, or within 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 60, 80, or 100 days of each other. In some embodiments, the end of CD19 inhibitor delivery and the end of B cell inhibitor delivery are within 1, 2, 3, 4, 6, 12, 18, or 24 hours of each other, or within 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 35, 40, 60, 80, or 100 days of each other. In some embodiments, the overlap in terms of administration between the CD19 inhibitor delivery (e.g., infusion) and the end of B cell inhibitor delivery (e.g., infusion) is at least 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, or 45 minutes.

In some embodiments, the B cell inhibitor is administered while the one or more cells

that express a CAR molecule that binds CD19 are present (e.g., undergoing expansion) in the subject. In some embodiments, the CD19 inhibitor is administered while the one or more cells that express a CAR molecule that binds one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a are present (e.g., undergoing expansion) in the subject.

The invention includes (among other things) a type of cellular therapy where T cells are genetically modified to express a chimeric antigen receptor (CAR) and the CAR T cell is infused to a recipient in need thereof. The infused cell is able to kill tumor cells in the recipient. Unlike antibody therapies, CAR-modified T cells are able to replicate in vivo resulting in long-term persistence that can lead to sustained tumor control. In various aspects, the T cells administered to the patient, or their progeny, persist in the patient for at least four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, twelve months, thirteen months, fourteen month, fifteen months, sixteen months, seventeen months, eighteen months, nineteen months, twenty months, twenty-one months, twenty-two months, twenty-three months, two years, three years, four years, or five years after administration of the T cell to the patient.

The invention also includes a type of cellular therapy where immune effector cells, e.g., NK cells or T cells are modified, e.g., by *in vitro* transcribed RNA, to transiently express a chimeric antigen receptor (CAR) and the CAR-expressing (e.g., CAR T) cell is infused to a recipient in need thereof. The infused cell is able to kill cancer cells in the recipient. Thus, in various aspects, the CAR-expressing cells, e.g., T cells, administered to the patient, is present for less than one month, e.g., three weeks, two weeks, one week, after administration of the CAR-expressing cell, e.g., T cell, to the patient.

Without wishing to be bound by any particular theory, the anti-cancer immunity response elicited by the CAR-modified T cells may be an active or a passive immune response, or alternatively may be due to a direct vs indirect immune response. In one aspect, the CAR (e.g., CD19-CAR) transduced T cells exhibit specific proinflammatory cytokine secretion and potent cytolytic activity in response to human cancer cells expressing the target antigen (e.g., CD19), resist soluble target antigen inhibition, mediate bystander killing and mediate regression of an established human cancer. For example, antigen-less cancer cells within a heterogeneous field of target antigen-expressing cancer may be susceptible to indirect destruction by target antigen-

redirected T cells that has previously reacted against adjacent antigen-positive cancer cells.

In one aspect, the CAR-modified cells of the invention, e.g., fully human CAR T cells, may be a type of vaccine for *ex vivo* immunization and/or in vivo therapy in a mammal. In one aspect, the mammal is a human.

With respect to *ex vivo* immunization, at least one of the following occurs *in vitro* prior to administering the cell into a mammal: i) expansion of the cells, ii) introducing a nucleic acid encoding a CAR to the cells or iii) cryopreservation of the cells.

Ex vivo procedures are well known in the art and are discussed more fully below. Briefly, cells are isolated from a mammal (e.g., a human) and genetically modified (i.e., transduced or transfected in vitro) with a vector expressing a CAR disclosed herein. The CAR-modified cell can be administered to a mammalian recipient to provide a therapeutic benefit. The mammalian recipient may be a human and the CAR-modified cell can be autologous with respect to the recipient. Alternatively, the cells can be allogeneic, syngeneic or xenogeneic with respect to the recipient.

The procedure for *ex vivo* expansion of hematopoietic stem and progenitor cells is described in U.S. Pat. No. 5,199,942, incorporated herein by reference, can be applied to the cells of the present invention. Other suitable methods are known in the art, therefore the present invention is not limited to any particular method of *ex vivo* expansion of the cells. Briefly, *ex vivo* culture and expansion of T cells can comprise: (1) collecting CD34+ hematopoietic stem and progenitor cells from a mammal from peripheral blood harvest or bone marrow explants; and (2) expanding such cells *ex vivo*. In addition to the cellular growth factors described in U.S. Pat. No. 5,199,942, other factors such as flt3-L, IL-1, IL-3 and c-kit ligand, can be used for culturing and expansion of the cells.

In addition to using a cell-based vaccine in terms of *ex vivo* immunization, also included in the methods described herein are compositions and methods for *in vivo* immunization to elicit an immune response directed against an antigen in a patient.

Generally, the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised. In particular, the CAR-expressing cells described herein are used in the treatment of diseases, disorders and conditions associated with expression of one or more B-cell antigen. In certain

aspects, the cells are used in the treatment of patients at risk for developing diseases, disorders and conditions associated with expression of one or more B-cell antigen. Thus, the present invention provides (among other things) methods for the treatment or prevention of diseases, disorders and conditions associated with expression of a B-cell antigen comprising administering to a subject in need thereof, a therapeutically effective amount of the CD19 CAR-expressing cells described herein, in combination with one or more of B-cell inhibitor described herein.

The present invention also provides methods for inhibiting the proliferation or reducing a CD19-expressing cell population, the methods comprising contacting a population of cells comprising a CD19-expressing cell with an anti-CD19 CAR-expressing cell described herein that binds to the CD19-expressing cell, and contacting the population of CD19-expressing cells with one or more of a B-cell inhibitor described herein. In a specific aspect, the present invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing CD19, the methods comprising contacting the CD19-expressing cancer cell population with an anti-CD19 CAR-expressing cell described herein that binds to the CD19-expressing cell, and contacting the CD19-expressing cell with one or more B-cell described herein. In one aspect, the present invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing CD19, the methods comprising contacting the CD19-expressing cancer cell population with an anti-CD19 CAR-expressing cell described herein that binds to the CD19-expressing cell and contacting the CD19-expressing cell with one or more B-cell described herein. In certain aspects, the combination of the anti-CD19 CAR-expressing cell described herein and one or more B-cell described herein reduces the quantity, number, amount or percentage of cells and/or cancer cells by at least 25%, at least 30%, at least 40%, at least 50%, at least 65%, at least 75%, at least 85%, at least 95%, or at least 99% in a subject with or animal model for a hematological cancer or another cancer associated with CD19-expressing cells relative to a negative control. In one aspect, the subject is a human.

The present invention also provides methods for inhibiting the proliferation or reducing a cell population comprising CD19-expressing cells and cells expressing a second B-cell antigen. In one aspect, CD19 and second B-cell antigen are expressed by the same cells within the population. In another aspect, CD19 and second B-cell antigen are expressed by distinct subsets of cells within the population. In another aspect, CD19 and second B-cell antigen are expressed

by overlapping subsets of cells within the population, such that some cells express CD19 and second B-cell antigen, some cells express CD19, and some cells express the second B-cell antigen.

The present invention also provides methods for inhibiting the proliferation or reducing a cell population expressing CD19 and a second B-cell antigen, the methods comprising (i) contacting a population of cells comprising a CD19-expressing cell with an anti-CD19 CAR-expressing cell described herein that binds to the CD19-expressing cell, and (ii) contacting the second B-cell antigen-expressing cell with a second CAR-expressing cell described herein that binds to the second B-cell antigen -expressing cell. In a specific aspect, the present invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing CD19 and a second B-cell antigen, the methods comprising (i) contacting the CD19-expressing cancer cell population with an anti-CD19 CAR-expressing cell described herein that binds to the CD19-expressing cell, and (ii) contacting the second B-cell antigen-expressing cell population with a second CAR-expressing cell described herein that binds to the cell expressing the second B-cell antigen. In one aspect, the present invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing CD19 and/or a second B-cell antigen, the methods comprising (i) contacting the CD19-expressing cancer cell population with an anti-CD19 CAR-expressing cell described herein that binds to the CD19-expressing cell and (ii) contacting the second B-cell antigen-expressing cell population with a second CAR-expressing cell described herein that binds to the cell expressing the second B-cell antigen . In certain aspects, the combination of the anti-CD19 CAR-expressing cell described herein and the second CAR-expressing cell described herein, reduces the quantity, number, amount or percentage of cells and/or cancer cells by at least 25%, at least 30%, at least 40%, at least 50%, at least 65%, at least 75%, at least 85%, at least 95%, or at least 99% in a subject with or animal model for a hematological cancer or another cancer associated with CD19 and/or second B-cell antigen-expressing cells relative to a negative control. In one aspect, the subject is a human.

The present invention also provides methods for preventing, treating and/or managing a disease associated with CD19-expressing cells (e.g., a hematologic cancer or atypical cancer expressing CD19), the methods comprising administering to a subject in need an anti-CD19 CAR-expressing cell that binds to the CD19-expressing cell and administering one or B-cell inhibitor described herein. In one aspect, the subject is a human. Non-limiting examples of

disorders associated with CD19-expressing cells include autoimmune disorders (such as lupus), inflammatory disorders (such as allergies and asthma) and cancers (such as hematological cancers or atypical cancers expressing CD19).

The present invention also provides methods for preventing, treating and/or managing a disease associated with CD19 and/or a second B-cell antigen-expressing cells (e.g., a hematologic cancer or atypical cancer expressing CD19 and/or second B-cell antigen), the methods comprising administering to a subject in need an anti-CD19 CAR-expressing cell that binds to the CD19-expressing cell and a B-cell inhibitor.

The present invention also provides methods for preventing, treating and/or managing a disease associated with CD19-expressing cells, the methods comprising administering to a subject in need an anti-CD19 CART cell of the invention that binds to the CD19-expressing cell. In one aspect, the subject is a human.

The present invention also provides methods for preventing relapse of cancer associated with CD19-expressing cells, the methods comprising administering to a subject in need thereof an anti-CD19 CART cell of the invention that binds to the CD19-expressing cell. In one aspect, the methods comprise administering to the subject in need thereof an effective amount of an anti-CD19 CART cell described herein that binds to the CD19-expressing cell in combination with an effective amount of another therapy.

In one aspect, the invention pertains to a method of treating cancer in a subject. The method comprises administering to the subject a CD19 CAR-expressing cell, e.g., T cell, described herein, in combination with a B-cell inhibitor, such that the cancer is treated in the subject. An example of a cancer that is treatable by the methods described herein is a cancer associated with expression of CD19. In one embodiment, the disease is a solid or liquid tumor. In one embodiment, the disease is a hematologic cancer, e.g., as described herein.

Non-cancer related indications associated with expression of CD19 include, but are not limited to, e.g., autoimmune disease, (e.g., lupus), inflammatory disorders (allergy and asthma) and transplantation.

In some embodiments, a cancer that can be treated with the combination described herein is multiple myeloma. Multiple myeloma is a cancer of the blood, characterized by accumulation of a plasma cell clone in the bone marrow. Current therapies for multiple myeloma include, but

are not limited to, treatment with lenalidomide, which is an analog of thalidomide. Lenalidomide has activities which include anti-tumor activity, angiogenesis inhibition, and immunomodulation. In some embodiments, a CD19 CAR, e.g., as described herein, may be used to target myeloma cells. In some embodiments, the combination described herein can be used with one or more additional therapies, e.g., lenalidomide treatment.

The CAR-expressing cells described herein may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as IL-2 or other cytokines or cell populations.

Hematologic Cancers

Hematological cancer conditions are the types of cancer such as leukemia, lymphoma and malignant lymphoproliferative conditions that affect blood, bone marrow and the lymphatic system.

In one embodiment, the hematologic cancer is leukemia. In one embodiment, the cancer is selected from the group consisting of one or more acute leukemias including but not limited to B-cell acute lymphoid leukemia (BALL), T-cell acute lymphoid leukemia (TALL), small lymphocytic leukemia (SLL), acute lymphoid leukemia (ALL); one or more chronic leukemias including but not limited to chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL); additional hematologic cancers or hematologic conditions including, but not limited to mantle cell lymphoma (MCL), B cell prolymphocytic leukemia, blastic plasmacytoid dendritic cell neoplasm, Burkitt's lymphoma, diffuse large B cell lymphoma, follicular lymphoma, hairy cell leukemia, small cell- or a large cell-follicular lymphoma, malignant lymphoproliferative conditions, MALT lymphoma, Marginal zone lymphoma, multiple myeloma, myelodysplasia and myelodysplastic syndrome, non-Hodgkin lymphoma, Hodgkin lymphoma, plasmablastic lymphoma, plasmacytoid dendritic cell neoplasm, Waldenstrom macroglobulinemia, and “preleukemia” which are a diverse collection of hematological conditions united by ineffective production (or dysplasia) of myeloid blood cells. Diseases associated with CD19, CD20, or CD22 expression include, but not limited to atypical and/or non-classical cancers, malignancies, precancerous conditions or proliferative diseases expressing CD19, CD20, or CD22; and any combination thereof.

Leukemia can be classified as acute leukemia and chronic leukemia. Acute leukemia can

be further classified as acute myelogenous leukemia (AML) and acute lymphoid leukemia (ALL). Chronic leukemia includes chronic myelogenous leukemia (CML) and chronic lymphoid leukemia (CLL). Other related conditions include myelodysplastic syndromes (MDS, formerly known as "preleukemia") which are a diverse collection of hematological conditions united by ineffective production (or dysplasia) of myeloid blood cells and risk of transformation to AML.

Lymphoma is a group of blood cell tumors that develop from lymphocytes. Exemplary lymphomas include non-Hodgkin lymphoma and Hodgkin lymphoma.

In an aspect, the invention pertains to a method of treating a mammal having Hodgkin lymphoma, comprising administering to the mammal an effective amount of the cells expressing a CD19 CAR molecule, e.g., a CD19 CAR molecule described herein and a B-cell inhibitor.

In one aspect, the compositions and CART cells or CAR expressing NK cells of the present invention are particularly useful for treating B cell malignancies, such as non-Hodgkin lymphomas, e.g., DLBCL, Follicular lymphoma, or CLL.

Non-Hodgkin lymphoma (NHL) is a group of cancers of lymphocytes, formed from either B or T cells. NHLs occur at any age and are often characterized by lymph nodes that are larger than normal, weight loss, and fever. Different types of NHLs are categorized as aggressive (fast-growing) and indolent (slow-growing) types. B-cell non-Hodgkin lymphomas include Burkitt lymphoma, chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, immunoblastic large cell lymphoma, precursor B-lymphoblastic lymphoma, and mantle cell lymphoma. Examples of T-cell non-Hodgkin lymphomas include mycosis fungoides, anaplastic large cell lymphoma, and precursor T-lymphoblastic lymphoma. Lymphomas that occur after bone marrow or stem cell transplantation are typically B-cell non-Hodgkin lymphomas. See, e.g., Maloney. NEJM. 366.21(2012):2008-16.

Diffuse large B-cell lymphoma (DLBCL) is a form of NHL that develops from B cells. DLBCL is an aggressive lymphoma that can arise in lymph nodes or outside of the lymphatic system, e.g., in the gastrointestinal tract, testes, thyroid, skin, breast, bone, or brain. Three variants of cellular morphology are commonly observed in DLBCL: centroblastic, immunoblastic, and anaplastic. Centroblastic morphology is most common and has the

appearance of medium-to-large-sized lymphocytes with minimal cytoplasm. There are several subtypes of DLBCL. For example, primary central nervous system lymphoma is a type of DLBCL that only affects the brain is called and is treated differently than DLBCL that affects areas outside of the brain. Another type of DLBCL is primary mediastinal B-cell lymphoma, which often occurs in younger patients and grows rapidly in the chest. Symptoms of DLBCL include a painless rapid swelling in the neck, armpit, or groin, which is caused by enlarged lymph nodes. For some subjects, the swelling may be painful. Other symptoms of DLBCL include night sweats, unexplained fevers, and weight loss. Although most patients with DLBCL are adults, this disease sometimes occurs in children. Treatment for DLBCL includes chemotherapy (e.g., cyclophosphamide, doxorubicin, vincristine, prednisone, etoposide), antibodies (e.g., Rituxan), radiation, or stem cell transplants.

Follicular lymphoma a type of non-Hodgkin lymphoma and is a lymphoma of follicle center B-cells (centrocytes and centroblasts), which has at least a partially follicular pattern. Follicular lymphoma cells express the B-cell markers CD10, CD19, CD20, and CD22. Follicular lymphoma cells are commonly negative for CD5. Morphologically, a follicular lymphoma tumor is made up of follicles containing a mixture of centrocytes (also called cleaved follicle center cells or small cells) and centroblasts (also called large noncleaved follicle center cells or large cells). The follicles are surrounded by non-malignant cells, mostly T-cells. The follicles contain predominantly centrocytes with a minority of centroblasts.The World Health Organization (WHO) morphologically grades the disease as follows: grade 1 (<5 centroblasts per high-power field (hpf); grade 2 (6–15 centroblasts/hpf); grade 3 (>15 centroblasts/hpf). Grade 3 is further subdivided into the following grades: grade 3A (centrocytes still present); grade 3B (the follicles consist almost entirely of centroblasts). Treatment of follicular lymphoma includes chemotherapy, e.g., alkyating agents, nucleoside analogs, anthracycline-containing regimens, e.g., a combination therapy called CHOP—cyclophosphamide, doxorubicin, vincristine, prednisone/prednisolone, antibodies (e.g., rituximab), radioimmunotherapy, and hematopoietic stem cell transplantation.

CLL is a B-cell malignancy characterized by neoplastic cell proliferation and accumulation in bone morrow, blood, lymph nodes, and the spleen. The median age at time of diagnosis of CLL is about 65 years. Current treatments include chemotherapy, radiation therapy, biological therapy, or bone marrow transplantation. Sometimes symptoms are treated surgically

(e.g., splenectomy removal of enlarged spleen) or by radiation therapy (e.g., de-bulking swollen lymph nodes). Chemotherapeutic agents to treat CLL include, e.g., fludarabine, 2-chlorodeoxyadenosine (cladribine), chlorambucil, vincristine, pentostatin, cyclophosphamide, alemtuzumab (Campath-1H), doxorubicin, and prednisone. Biological therapy for CLL includes antibodies, e.g., alemtuzumab, rituximab, and ofatumumab; as well as tyrosine kinase inhibitor therapies. A number of criteria can be used to classify stage of CLL, e.g., the Rai or Binet system. The Rai system describes CLL has having five stages: stage 0 where only lymphocytosis is present; stage I where lymphadenopathy is present; stage II where splenomegaly, lymphadenopathy, or both are present; stage III where anemia, organomegaly, or both are present (progression is defined by weight loss, fatigue, fever, massive organomegaly, and a rapidly increasing lymphocyte count); and stage IV where anemia, thrombocytopenia, organomegaly, or a combination thereof are present. Under the Binet staging system, there are three categories: stage A where lymphocytosis is present and less than three lymph nodes are enlarged (this stage is inclusive of all Rai stage 0 patients, one-half of Rai stage I patients, and one-third of Rai stage II patients); stage B where three or more lymph nodes are involved; and stage C wherein anemia or thrombocytopenia, or both are present. These classification systems can be combined with measurements of mutation of the immunoglobulin genes to provide a more accurate characterization of the state of the disease. The presence of mutated immunoglobulin genes correlates to improved prognosis.

In another embodiment, the CAR expressing cells of the present invention are used to treat cancers or leukemias, e.g., with leukemia stem cells. For example, the leukemia stem cells are $CD34^+/CD38^-$ leukemia cells.

CD20 and CD22 Associated Diseases and/or Disorders

The present invention provides, among other things, compositions and methods for treating a disease associated with expression of CD20 or CD22 or condition associated with cells which express CD20 or CD22 including, e.g., a proliferative disease such as a cancer or malignancy or a precancerous condition; or a noncancer related indication associated with cells which express CD20 or CD22. In one aspect, a cancer associated with expression of CD22 is a hematological cancer, e.g., a hematological cancer described herein.

Non-cancer related indications associated with expression of CD20 or CD22 may also be included. Non-cancer related indications associated with expression of CD20 or CD22 include, but are not limited to, e.g., autoimmune disease, (e.g., lupus, rheumatoid arthritis, multiple sclerosis autoimmune hemolytic anemia, pure red cell aplasia, idiopathic thrombocytopenic purpura, Evans syndrome, vasculitis, bullous skin disorders, type 1 diabetes mellitus, Sjogren's syndrome, anti-NMDA receptor encephalitis and Devic's disease, Graves' ophthalmopathy, and autoimmune pancreatitis), inflammatory disorders (allergy and asthma) and solid-organ or hematopoietic cell transplantation.

Compositions and methods disclosed herein may be used to treat hematologic diseases including, but not limited to myelodysplasia, anemia, paroxysmal nocturnal hemoglobinuria, aplastic anemia, acquired pure red cell anemia, Diamon-Blackfan anemia, Fanconi anemia, cytopenia, amegakaryotic thrombocytopenia, myeloproliferative disorders, polycythemia vera, essential thrombocytosis, myelofibrosis, hemoglobinopathies, sickle cell disease, β thalassemia major, among others.

In one aspect, the invention provides methods for treating a disease associated with CD22 expression. In one aspect, the invention provides methods for treating a disease wherein part of the tumor is negative for CD20 or CD22 and part of the tumor is positive for CD20 or CD22. For example, the CAR of the invention is useful for treating subjects that have undergone treatment for a disease associated with expression of CD20 or CD22, wherein the subject that has undergone treatment related to expression of CD20 or CD22 exhibits a disease associated with expression of CD20 or CD22.

In one aspect, the invention pertains to a method of inhibiting growth of a CD20 or CD22-expressing tumor cell, comprising contacting the tumor cell with a CD20 or CD22 CAR cell (e.g., T cell or NK cell) of the present invention such that the CART is activated in response to the antigen and targets the cancer cell, wherein the growth of the tumor is inhibited.

In one aspect, the invention pertains to a method of treating cancer in a subject. The method comprises administering to the subject a CD20 or CD22 CAR expressing cell (e.g., T cell or NK cell) of the present invention such that the cancer is treated in the subject. An example of a cancer that is treatable by the CD20 or CD22 CAR expressing cell (e.g., T cell or NK cell) of the invention is a cancer associated with expression of CD20 or CD22. An example

of a cancer that is treatable by the CD20 or CD22 CAR expressing cell (e.g., T cell or NK cell) of the invention includes but is not limited to a hematological cancer described herein.

The invention includes a type of cellular therapy where cells (e.g., T cells or NK cells) are genetically modified to express a chimeric antigen receptor (CAR) and the CAR expressing cell (e.g., T cell or NK cells) is infused to a recipient in need thereof. The infused cell is able to kill tumor cells in the recipient. Unlike antibody therapies, CAR-modified cells (e.g., T cells or NK cells) are able to replicate in vivo resulting in long-term persistence that can lead to sustained tumor control. In various aspects, the cells (e.g., T cells or NK cells) administered to the patient, or their progeny, persist in the patient for at least four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, twelve months, thirteen months, fourteen month, fifteen months, sixteen months, seventeen months, eighteen months, nineteen months, twenty months, twenty-one months, twenty-two months, twenty-three months, two years, three years, four years, or five years after administration of the T cell to the patient.

The invention also includes a type of cellular therapy where immune effector cells, e.g., NK cells or T cells are modified, e.g., by *in vitro* transcribed RNA, to transiently express a chimeric antigen receptor (CAR) and the CAR-expressing (e.g., CART or CAR expressing NK cell) cell is infused to a recipient in need thereof. The infused cell is able to kill cancer cells in the recipient. Thus, in various aspects, the CAR-expressing cells, e.g., T cells or NK cells, are administered to the patient, is present for less than one month, e.g., three weeks, two weeks, one week, after administration of the CAR-expressing cell, e.g., T cells or NK cell, to the patient.

Without wishing to be bound by any particular theory, the anti-tumor immunity response elicited by the CAR-modified cells (e.g., T cells or NK cells) may be an active or a passive immune response, or alternatively may be due to a direct vs indirect immune response. In one aspect, the CAR transduced cells (e.g., T cells or NK cells) exhibit specific proinflammatory cytokine secretion and potent cytolytic activity in response to human cancer cells expressing CD20 or CD22, resist soluble CD20 or CD22 inhibition, mediate bystander killing and mediate regression of an established human tumor. For example, antigen-less tumor cells within a heterogeneous field of CD20 or CD22-expressing tumor may be susceptible to indirect destruction by CD20 or CD22-redirected T cells that has previously reacted against adjacent antigen-positive cancer cells.

In one aspect, the CAR-modified cells (e.g., T cells or NK cells) of the invention, e.g., fully human CAR-expressing cells, may be a type of vaccine for ex vivo immunization and/or in vivo therapy in a mammal. In one aspect, the mammal is a human.

With respect to ex vivo immunization, at least one of the following occurs in vitro prior to administering the cell into a mammal: i) expansion of the cells, ii) introducing a nucleic acid encoding a CAR to the cells or iii) cryopreservation of the cells.

Ex vivo procedures are well known in the art and are discussed more fully below. Briefly, cells are isolated from a mammal (e.g., a human) and genetically modified (i.e., transduced or transfected in vitro) with a vector expressing a CAR disclosed herein. The CAR-modified cell can be administered to a mammalian recipient to provide a therapeutic benefit. The mammalian recipient may be a human and the CAR-modified cell can be autologous with respect to the recipient. Alternatively, the cells can be allogeneic, syngeneic or xenogeneic with respect to the recipient.

The procedure for ex vivo expansion of hematopoietic stem and progenitor cells is described in U.S. Pat. No. 5,199,942, incorporated herein by reference, can be applied to the cells of the present invention. Other suitable methods are known in the art, therefore the present invention is not limited to any particular method of ex vivo expansion of the cells. Briefly, ex vivo culture and expansion of T cells comprises: (1) collecting CD34+ hematopoietic stem and progenitor cells from a mammal from peripheral blood harvest or bone marrow explants; and (2) expanding such cells ex vivo. In addition to the cellular growth factors described in U.S. Pat. No. 5,199,942, other factors such as flt3-L, IL-1, IL-3 and c-kit ligand, can be used for culturing and expansion of the cells.

In addition to using a cell-based vaccine in terms of ex vivo immunization, the present invention also provides compositions and methods for in vivo immunization to elicit an immune response directed against an antigen in a patient.

Generally, the cells activated and expanded as described herein may be utilized in the treatment and prevention of diseases that arise in individuals who are immunocompromised. In particular, the CAR-modified cells (e.g., T cells or NK cells) of the invention are used in the treatment of diseases, disorders and conditions associated with expression of CD20 or CD22. In certain aspects, the cells of the invention are used in the treatment of patients at risk for

developing diseases, disorders and conditions associated with expression of CD20 or CD22. Thus, the present invention provides methods for the treatment or prevention of diseases, disorders and conditions associated with expression of CD20 or CD22 comprising administering to a subject in need thereof, a therapeutically effective amount of the CAR-modified cells (e.g., T cells or NK cells) of the invention.

In one aspect the CAR expressing cells of the inventions may be used to treat a proliferative disease such as a cancer or malignancy or is a precancerous condition. In one aspect, a cancer associated with expression of CD20 or CD22 is a hematological cancer preleukemia, hyperproliferative disorder, hyperplasia or a dysplasia, which is characterized by abnormal growth of cells.

The CAR-modified cells of the present invention may be administered either alone, or as a pharmaceutical composition in combination with diluents and/or with other components such as IL-2 or other cytokines or cell populations.

The present invention also methods for inhibiting the proliferation or reducing a CD20 or CD22-expressing cell population, the methods comprising contacting a population of cells comprising a CD20 or CD22-expressing cell with a CD20 or CD22 CAR expressing cell of the invention that binds to the CD20 or CD22-expressing cell. In a specific aspect, the present invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing CD20 or CD22, the methods comprising contacting the CD20 or CD22-expressing cancer cell population with a CD20 or CD22 CAR expressing cell of the invention that binds to the CD20 or CD22-expressing cell. In one aspect, the present invention provides methods for inhibiting the proliferation or reducing the population of cancer cells expressing CD20 or CD22, the methods comprising contacting the CD20 or CD22-expressing cancer cell population with a CD20 or CD22 CART of the invention that binds to the CD20 or CD22-expressing cell. In certain aspects, the CD20 or CD22 CAR expressing cell of the invention reduces the quantity, number, amount or percentage of cells and/or cancer cells by at least 25%, at least 30%, at least 40%, at least 50%, at least 65%, at least 75%, at least 85%, at least 95%, or at least 99% in a subject with or animal model for B-cell malignancy or another cancer associated with CD20 or CD22-expressing cells relative to a negative control. In one aspect, the subject is a human.

The present invention provides methods for preventing relapse of cancer associated with CD20 or CD22-expressing cells, the methods comprising administering to a subject in need thereof a CD20 or CD22 CAR expressing cell of the invention that binds to the CD20 or CD22-expressing cell. In one aspect, the methods comprise administering to the subject in need thereof an effective amount of a CD20 or CD22 CAR expressing cell described herein that binds to the CD20 or CD22-expressing cell in combination with an effective amount of another therapy.

In some embodiments, the CD22 expressing cell expresses CD19, CD123, FLT-3, ROR-1, CD79b, CD179b, CD79a, CD10, CD34, and/or CD20. In certain embodiments, the CD22 expressing cell expresses CD19. In some embodiments, the CD22-expressing cell does not express CD19. In some embodiments, the CD20 expressing cell expresses CD19, CD123, FLT-3, ROR-1, CD79b, CD179b, CD79a, CD10, CD34, and/or CD22. In certain embodiments, the CD20 expressing cell expresses CD19. In some embodiments, the CD20-expressing cell does not express CD19.

In some embodiments, the subject is a non-responder to CD19 CAR therapy. In some embodiments, the subject is a partial responder to CD19 CAR therapy. In some embodiments, the subject is a complete responder to CD19 CAR therapy. In some embodiments, the subject is a non-relapser to CD19 CAR therapy. In some embodiments, the subject is a partial relapser to CD19 CAR therapy. In some embodiments, the subject is a complete relapser to CD19 CAR therapy.

In some embodiments, a cancer or other condition that was previously responsive to treatment with CD19 CAR-expressing cells does not express CD19. In some embodiments, a cancer or other condition that was previously responsive to treatment with CD19 CAR-expressing cells has a 10%, 20%, 30%, 40%, 50% or more reduction in CD19 expression levels relative to when the cancer or other condition was responsive to treatment with CD19 CAR-expressing cells. In some embodiments, a cancer or other condition that was previously responsive to treatment with CD19 CAR-expressing cells expresses CD20, CD22, CD123, or any combination thereof.

In some embodiments, the CD20 or CD22 CAR-expressing cell of the invention is administered post-relapse of a cancer or other condition previously treated with CD19 CAR-

expressing cell. In some embodiments, a CD19 CAR-expressing cell and a CD20 or CD22 CAR-expressing cell are administered concurrently, as described herein.

Bone Marrow Ablation

In one aspect, the present invention provides compositions and methods for bone marrow ablation. For example, in one aspect, the invention provides compositions and methods for eradication of at least a portion of existing bone marrow in a subject. It is described herein that, in certain instances, the CD19, C20, or CD22 CAR expressing cell comprising a CAR of the present invention eradicates CD19, C20, or CD22 positive bone marrow myeloid progenitor cells.

In one aspect, the invention provides a method of bone marrow ablation comprising administering a CD19, C20, or CD22 expressing CAR cell (e.g., T cell or NK cell) of the invention to a subject in need of bone marrow ablation. For example, the present method may be used to eradicate some or all of the existing bone marrow of a subject having a disease or disorder in which bone marrow transplantation or bone marrow reconditioning is a beneficial treatment strategy. In one aspect, the bone marrow ablation method of the invention, comprising the administration of a CD19, C20, or CD22 expressing CAR cell (e.g., T cell or NK cell) described elsewhere herein, is performed in a subject prior to bone marrow transplantation. Thus, in one aspect, the method of the invention provides a cellular conditioning regimen prior to bone marrow or stem cell transplantation. In one aspect, bone marrow transplantation comprises transplantation of a stem cell. The bone marrow transplantation may comprise transplantation of autologous or allogeneic cells.

The present invention provides a method of treating a disease or disorder comprising administering a CD19, C20, or CD22 expressing CAR cell (e.g., T cell or NK cell) of the invention to eradicate at least a portion of existing bone marrow. The method may be used as at least a portion of a treatment regimen for treating any disease or disorder where bone marrow transplantation is beneficial. That is, the present method may be used in any subject in need of a bone marrow transplant. In one aspect, bone marrow ablation comprising administration of a CD19, C20, or CD22 expressing CAR cell (e.g., T cell or NK cell) is useful in the treatment of AML. In certain aspects, bone marrow ablation by way of the present method is useful in

treating a hematological cancer, a solid tumor, a hematologic disease, a metabolic disorder, HIV, HTLV, a lysosomal storage disorder, and an immunodeficiency.

Compositions and methods disclosed herein may be used to eradicate at least a portion of existing bone marrow to treat hematological cancers including, but not limited to, cancers described herein, e.g., leukemia, lymphoma, myeloma, ALL, AML, CLL, CML, Hodgkin lymphoma, Non-Hodgkin lymphoma (e.g., DLBCL or follicular lymphoma), and multiple myeloma.

In one aspect, the present invention provides a method of treating cancer comprising bone marrow conditioning, where at least a portion of bone marrow of the subject is eradicated by the CD22 CAR cell (e.g., T cell or NK cell) of the invention. For example, in certain instances, the bone marrow of the subject comprises a malignant precursor cell that can be targeted and eliminated by the activity of the C20 or CD22 CAR cell (e.g., T cell or NK cell). In one aspect, a bone marrow conditioning therapy comprises administering a bone marrow or stem cell transplant to the subject following the eradication of native bone marrow. In one aspect, the bone marrow reconditioning therapy is combined with one or more other anti-cancer therapies, including, but not limited to anti-tumor CAR therapies, chemotherapy, radiation, and the like.

In one aspect, eradication of the administered CD19, CD20, or CD22 CAR expressing cells may be required prior to infusion of bone marrow or stem cell transplant. Eradication of the CD19, CD20, or CD22 expressing CAR cell (e.g., T cell or NK cell) may be accomplished using any suitable strategy or treatment, including, but not limited to, use of a suicide gene, limited CAR persistence using RNA encoded CARs, or anti-T cell modalities including antibodies or chemotherapy.

Combination Therapies

The combination of a CAR as described herein (e.g., a CD20 CAR, a CD22 CAR, or a CD19 CAR-expressing cell described herein e.g., and one or more B-cell inhibitors, e.g., as described herein) may be used in combination with other known agents and therapies.

A CAR-expressing cell described herein (e.g., a CD20 CAR, a CD22 CAR, or a CD19 CAR-expressing cell), optionally the one or more B-cell inhibitors, and/or the at least one

additional therapeutic agent can be administered simultaneously, in the same or in separate compositions, or sequentially. For sequential administration, the CAR-expressing cell described herein (e.g., a CD20 CAR, a CD22 CAR, or a CD19 CAR-expressing cell) can be administered first, and the additional agent can be administered second, or the order of administration can be reversed.

The CAR therapy and/or other therapeutic agents (such as a second CAR therapy), procedures or modalities can be administered during periods of active disorder, or during a period of remission or less active disease. The CAR therapy can be administered before the other treatment, concurrently with the treatment, post-treatment, or during remission of the disorder.

For instance, in some embodiments, CAR therapy is administered to a subject having a disease associated with CD19, CD20, or CD22 expression, e.g., a cancer. The subject can be assayed for indicators of responsiveness or relapse. In some embodiments, when the subject shows one or more signs of relapse, e.g., a frameshift and/or premature stop codon in CD19, an additional therapy is administered. In embodiments, the additional therapy is a B-cell inhibitor. The CD19 therapy may be continued (for instance, when there are still some CD19-expressing cancer cells detectable in the subject) or may be discontinued (for instance, when a risk-benefit analysis favors discontinuing the therapy).

When administered in combination, the CAR therapy and the additional agent (e.g., second or third agent), or all, can be administered in an amount or dose that is higher, lower or the same than the amount or dosage of each agent used individually, e.g., as a monotherapy. In certain embodiments, the administered amount or dosage of the CAR therapy, the additional agent (e.g., second or third agent), or all, is lower (e.g., at least 20%, at least 30%, at least 40%, or at least 50%) than the amount or dosage of each agent used individually, e.g., as a monotherapy. In other embodiments, the amount or dosage of the CAR therapy, the additional agent (e.g., second or third agent), or all, that results in a desired effect (e.g., treatment of cancer) is lower (e.g., at least 20%, at least 30%, at least 40%, or at least 50% lower) than the amount or dosage of each agent used individually, e.g., as a monotherapy, required to achieve the same therapeutic effect.

In embodiments, one or more of the therapeutics in the combination therapy is an

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What is claimed is:

1. A cell that expresses a CAR molecule that binds CD19, e.g., a CD19 CAR, for use in the treatment of a subject having a disease associated with expression of CD19, and wherein said subject has received, is receiving or is about to receive one or more B-cell inhibitors, wherein the B-cell inhibitor comprises an inhibitor of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

2. A method of treating a subject having a disease associated with expression of CD19, comprising administering to the subject an effective number of one or more cells that express a CAR molecule that binds CD19, e.g., a CD19 CAR, in combination with one or more B-cell inhibitors, wherein the B-cell inhibitor comprises an inhibitor of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

3. A B-cell inhibitor, e.g., an inhibitor of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a, for use in the treatment of a subject who is, or is identified as being, a non-responder, partial responder, or relapser to a CD19 inhibitor, e.g., a CD19 CAR therapy.

4. A method of treating a patient who is, or is identified as being, a non-responder, partial responder, or relapser to a CD19 inhibitor, e.g., a CD19 CAR therapy, comprising administering to the subject a B-cell inhibitor, e.g., an inhibitor of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

5. The composition for use or the method of any of the preceding claims, wherein the B-cell inhibitor comprises an inhibitor of one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, or ROR1.

6. The composition for use or the method of any of the preceding claims, wherein the B-cell

inhibitor comprises an effective number of one or more cells that express a CAR molecule that binds one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

7. The composition for use or the method of any of the preceding claims, wherein the one or more cells that express a CAR molecule that binds CD19 are administered concurrently with, before, or after the one or more B-cell inhibitors.

8. The composition for use or the method of any of the preceding claims, wherein the subject has relapsed or is identified as having relapsed after treatment with the one or more cells that express a CAR molecule that binds CD19, e.g., a CD19 CAR.

9. The composition for use or the method of claim 11, wherein the subject has relapsed or is identified as having relapsed based on one or more of reappearance of blasts in the blood, bone marrow (> 5%), or any extramedullary site, after a complete response; or

wherein the subject has relapsed or is identified as having relapsed based on detection of CD19- blasts above a predetermined threshold, e.g., over 1%, 2%, 3%, 4%, 5%, or 10%; or

wherein the subject has relapsed or is identified as having relapsed based on an increase in RNA levels of one or more of MIR199A1, MIR1203, uc021ovp, ITM2C, or HLA-DQB1; or

wherein the subject has relapsed or is identified as having relapsed based on a decrease in RNA levels of one or more of PPIAL4D, TTTY10, TXLNG2P, MIR4650-1, KDM5D, USP9Y, PRKY, RPS4Y2, RPS4Y1, NCRNA00185, SULT1E1, and EIF1AY.

10. The composition for use or the method of any of the preceding claims, further comprising performing lymphodepletion on that subject, e.g., prior to administering the one or more cells that express a CAR molecule that binds CD19, wherein optionally the lymphodepletion comprises administering one or more of melphalan, cytoxan, cyclophosphamide, and fludarabine.

11. The composition for use or the method of any of the preceding claims, wherein the B-cell inhibitor comprises an effective number of one or more cells that express: a CAR molecule that binds CD10, e.g., as described herein; a CAR molecule that binds CD20, e.g., as described herein; a CAR molecule that binds CD22, e.g., as described herein; a CAR molecule that binds CD34, e.g., as described herein; a CAR molecule that binds CD123, e.g., as described herein; a CAR molecule that binds FLT-3, e.g., as described herein; or a CAR molecule that binds ROR1, e.g., as described herein.

12. The composition for use or the method of any of the preceding claims, wherein the CD19 CAR comprises an antibody or antibody fragment which includes a CD19 binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said CD19 binding domain comprises one or more of (e.g., all three of) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD19 scFv or light chain binding domain amino acid sequence listed in **Tables 2 or 3**, and one or more of (e.g., all three of) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD19 scFv or heavy chain binding domain amino acid sequence listed in **Tables 2 or 3**.

13. The composition for use or the method of any of the preceding claims, wherein a CD19 CAR comprises any light chain variable region of a scFv listed in **Tables 2 or 3** and any heavy chain variable region of a scFv listed **Tables 2 or 3**.

14. The composition for use or the method of any of the preceding claims, wherein the CD19 CAR comprises a CD19 binding domain which comprises a sequence selected from a group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO: 4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11 and

SEQ ID NO:12, or a sequence with 95-99% identity thereof, or a polypeptide of SEQ ID NO:58.

15. The composition for use or the method any of the preceding claims, wherein the B-cell inhibitor comprises a CD20 CAR which comprises an antibody or antibody fragment which includes a CD20 binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said CD20 binding domain comprises one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD20 light chain binding domain amino acid sequence listed in **Table 13**, and one or more of heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD20 heavy chain binding domain amino acid sequence listed in **Table 12A or 12B**.

16. The composition for use or the method of any of the preceding claims, wherein the B-cell inhibitor comprises a CD22 CAR which comprises an antibody or antibody fragment which includes a CD22 binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said CD22 binding domain comprises one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD22 light chain binding domain amino acid sequence listed in **Table 8A or 8B**, and one or more of heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD22 heavy chain binding domain amino acid sequence listed in **Table 7A, 7B, or 7C**.

17. The composition for use or the method of claim 16, wherein the CD22 CAR comprises any light chain variable region listed in **Table 10A or 10B**, any heavy chain variable region listed in **Table 9A or 9B**, or any light chain variable region listed in **Table 10A or 10B** and any

heavy chain variable region listed **Table 9A or 9B**.

18. The composition for use or the method of any of the preceding claims, wherein the B-cell inhibitor comprises a CD123 CAR which comprises an antibody or antibody fragment which includes a CD123 binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said CD123 binding domain comprises one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD123 light chain binding domain amino acid sequence listed in **Table 18**, and one or more of heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD123 heavy chain binding domain amino acid sequence listed in **Table 17**.

19. The composition for use or the method of claim 18, wherein the CD123 CAR comprises any light chain variable region listed in **Table 16**, any heavy chain variable region listed in **Table 16**, or any light chain variable region listed in **Table 16** and any heavy chain variable region listed **Table 16**.

20. The composition for use or the method of any of the preceding claims, wherein the B-cell inhibitor comprises a CD123 CAR which comprises an antibody or antibody fragment which includes a CD123 binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said CD123 binding domain comprises one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD123 light chain binding domain amino acid sequence listed in **Table 27**, and one or more of heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD123 heavy chain binding domain amino acid sequence listed in **Table 26**.

21. The composition for use or the method of claim 20, wherein the CD123 CAR comprises any light chain variable region listed in **Table 25**, any heavy chain variable region listed in **Table 25**, or any light chain variable region listed in **Table 25** and any heavy chain variable region listed **Table 25**.

22. The composition for use or the method of any of the preceding claims, wherein the B-cell inhibitor comprises a CAR which comprises an antibody or antibody fragment which includes an antigen binding domain, a transmembrane domain, and an intracellular signaling domain comprising a stimulatory domain, and wherein said antigen binding domain comprises one or more of (e.g., all of) light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3), and one or more of (e.g., all of) heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3).

23. The composition for use or the method of any of the preceding claims, wherein the B-cell inhibitor comprises a CAR which comprises a scFv.

24. The composition for use or the method of any of the preceding claims, wherein the B-cell inhibitor comprises a CAR which comprises a transmembrane domain that comprises a transmembrane domain of a protein selected from the group consisting of the alpha, beta or zeta chain of the T-cell receptor, CD28, CD3 epsilon, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137 and CD154.

25. The composition for use or the method of claim 22, wherein the antigen binding domain is connected to the transmembrane domain by a hinge region, wherein, optionally, the hinge region comprises SEQ ID NO:14, or a sequence with 95-99% identity thereof.

26. The composition for use or the method of claim 22, wherein the costimulatory domain is a functional signaling domain obtained from a protein selected from the group consisting of OX40, CD2, CD27, CD28, CDS, ICAM-1, LFA-1 (CD11a/CD18), ICOS (CD278), and 4-1BB (CD137), wherein optionally the costimulatory domain comprises a sequence of SEQ ID NO:16 or SEQ ID NO:51.

27. The composition for use or the method of claim 22, wherein the intracellular signaling domain comprises a functional signaling domain of 4-1BB and/or a functional signaling domain of CD3 zeta; or

wherein the intracellular signaling domain comprises the sequence of SEQ ID NO: 16 and/or the sequence of SEQ ID NO:17 or SEQ ID NO:43.

28. The composition for use or the method of claim 22, wherein the CAR further comprises a leader sequence, wherein, optionally, the leader sequence comprises SEQ ID NO: 13.

29. The composition for use or the method of any of the preceding claims, wherein the cells comprise T cells or NK cells.

30. The composition for use or the method of any of the preceding claims, wherein the disease associated with CD19 expression is selected from a proliferative disease such as a cancer, tumor, or malignancy or a precancerous condition such as a myelodysplasia, a myelodysplastic syndrome or a preleukemia, or is a non-cancer related indication associated with expression of CD19, or

wherein the disease is one or more of a hematologic cancer, acute leukemia, B-cell acute lymphoid leukemia (BALL), T-cell acute lymphoid leukemia (TALL), small lymphocytic leukemia (SLL), acute lymphoid leukemia (ALL); chronic leukemia, chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma, or myeloma; or

wherein the disease is a CD19-negative cancer, e.g., a CD19-negative relapsed cancer.

31. The composition for use or the method of any of the preceding claims, further comprising administering:

an agent that increases the efficacy of a cell expressing a CAR molecule, or

an agent that ameliorates one or more side effects associated with administration of a cell expressing a CAR molecule, or

an agent that treats the disease associated with CD19; or

a checkpoint inhibitor.

32. The composition for use or the method of any of the preceding claims, wherein the subject receives a pre-treatment, concurrent treatment, or post-CART therapy treatment with an agent, e.g., a checkpoint inhibitor, prior to the initiation of a CART therapy.

33. The composition for use or the method of any of the preceding claims, wherein the patient comprises a CD19-negative cancer cell, and a cancer cell that is positive for one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

34. The composition for use or the method of any of the preceding claims, further comprises administering to the patient an inhibitor of one or more of the CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a for which the cancer cell is positive.

35. The composition for use or the method of any of the preceding claims, which further comprises a step of determining whether the patient comprises a CD19-negative cancer cell or whether the patient comprises a cancer cell that is positive for one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

36. A composition comprising, separately or admixed:

(i) one or more cells that express a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein, e.g., a CD19 CAR, and

(ii) one or more B-cell inhibitors chosen from inhibitors of one or more of CD10, CD20,CD22, CD34, CD123, FLT-3, or ROR1.

37. A composition comprising:

(i) a first nucleic acid encoding a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein, e.g., a CD19 CAR, and

(ii) a second nucleic acid encoding a CAR molecule that binds a B-cell antigen chosen from one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a, wherein the first nucleic acid and second nucleic acid are in the same or a separate nucleic acid molecule.

38. A composition comprising one or more immune effector cells and:

(i) a first nucleic acid encoding, or a first polypeptide comprising, a CAR molecule that binds CD19, e.g., a CAR molecule that binds CD19 described herein, e.g., a CD19 CAR, and

(ii) a second nucleic acid encoding, or a second polypeptide comprising, a CAR molecule that binds a B-cell antigen chosen from one or more of CD10, CD20, CD22, CD34, CD123, FLT-3, ROR1, CD79b, CD179b, or CD79a.

39. The composition of claim 38, wherein:

the first nucleic acid or first polypeptide and the second nucleic acid or second polypeptide are each contained within, e.g., expressed by, a first immune effector cell;

the composition comprises a first immune effector cell containing e.g., expressing the first nucleic acid or first polypeptide and a second immune effector cell containing e.g., expressing the second nucleic acid or second polypeptide;

or the composition does not comprise a cell containing, e.g., expressing both of the first nucleic acid or first polypeptide and the second nucleic acid or second polypeptide.

40. A cell comprising a nucleic acid composition of claim 37, wherein the cell is optionally a human T cell, a CD8+ T cell, or an NK cell.

41. A cell of claim 40, further expressing an inhibitory molecule that comprises a first polypeptide that comprises at least a portion of an inhibitory molecule, associated with a second polypeptide that comprises a positive signal from an intracellular signaling domain, wherein optionally the inhibitory molecule comprise first polypeptide that comprises at least a portion of PD1 and a second polypeptide comprising a costimulatory domain and primary signaling domain.

42. A method of making a cell, comprising introducing a nucleic acid composition of claim 37 into a T cell or NK cell, e.g., transducing a T cell or NK cell with a vector of any of claims 94-100, wherein the method optionally comprises:

assaying a gene signature that indicates whether a subject treated with the cell is likely to relapse, or has relapsed;

assaying the gene signature in the cell prior to infusion into the subject; or

decreasing the T_{REG} signature of a population of cells comprising the transduced cell, wherein decreasing the T_{REG} signature optionally comprises performing CD25-depletion on the population of cells.

43. The composition for use or the method of any of the preceding claims, wherein the CD19 inhibitor comprises a CD19 CAR and the B-cell inhibitor comprises a CD123 CAR, wherein optionally the CD19 CAR or CD123 CAR comprises a split intracellular signaling domain such that full activation of the cell, e.g., the population of immune effector cells, occurs when both the CD19 CAR and CD123 CAR bind to a target cell, e.g., a target CD19+CD123+ cell (e.g., a B-ALL blast cell), compared to activation when the CD19 CAR and CD123 CAR bind to a

target cell that expresses one of CD19 or CD123 (e.g., a hematopoietic stem cell).

44. The composition for use or the method of any of the preceding claims, further comprising transplanting a cell, e.g., a hematopoietic stem cell, or a bone marrow, into the mammal.

45. A method of treating a subject having DLBCL, e.g. primary DLBCL, comprising administering to the subject an effective number of one or more cells that express a CAR molecule that binds CD19, e.g., a CD19 CAR, in combination with a PD1 inhibitor, wherein optionally the subject has, or is identified as having, at least 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of DLBCL cells which are CD3+/PD1+.

46. A method of treating a subject having DLBCL, e.g. primary DLBCL, comprising administering to the subject an effective number of one or more cells that express a CAR molecule that binds CD19, e.g., a CD19 CAR, wherein optionally the subject has, or is identified as having, at least 5%, 6%, 7%, 8%, 9%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of DLBCL cells which are CD3+/PD1+.

47. A method of treating a subject having DLBCL, comprising administering to the subject an effective number of one or more cells that express a CAR molecule that binds CD19, e.g., a CD19 CAR, in combination with a PD-L1 inhibitor, wherein optionally the subject has, or is identified as having, less than 20%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% of cells in the cancer, e.g., cancer microenvironment, are double positive for CD19 and PD-L1.

48. An isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR), wherein the CAR comprises an antibody or antibody fragment which includes a CD20 binding domain, a transmembrane domain, and an intracellular signaling domain, and wherein said CD20 binding domain comprises one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD20 light chain binding domain amino acid sequence listed in **Table 13, 15A, or 15B**, and one or more of heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC

CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD20 heavy chain binding domain amino acid sequence listed in **Table 12A, 12B, 14A, or 14B**.

49. An isolated CAR molecule comprising a CD20 binding domain, a transmembrane domain, and an intracellular signaling domain, wherein the CD20 binding domain comprises one or more light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD20 binding domain listed in **Table 13, 15A, or 15B**, and one or more heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD20 binding domain listed in **Table 12A, 12B, 14A, or 14B**.

50. A method of treating a mammal having a disease associated with expression of CD20 comprising administering to the mammal an effective amount of the cell of comprising a CAR molecule of claim 49.

51. An isolated nucleic acid molecule encoding a chimeric antigen receptor (CAR), wherein the CAR comprises an antibody or antibody fragment which includes a CD22 binding domain, a transmembrane domain, and an intracellular signaling domain, and wherein said CD22 binding domain comprises one or more of light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD22 light chain binding domain amino acid sequence listed in **Table 8A, 8B, 10A or 10B**, and one or more of heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD22 heavy chain binding domain amino acid sequence listed in **Table 7A, 7B, 7C, 9A, or 9B**.

52. An isolated CAR molecule comprising a CD22 binding domain, a transmembrane domain, and an intracellular signaling domain, wherein the CD22 binding domain comprises one or

more light chain complementary determining region 1 (LC CDR1), light chain complementary determining region 2 (LC CDR2), and light chain complementary determining region 3 (LC CDR3) of any CD22 binding domain listed in **Table 8A, 8B, 10A or 10B**, and one or more heavy chain complementary determining region 1 (HC CDR1), heavy chain complementary determining region 2 (HC CDR2), and heavy chain complementary determining region 3 (HC CDR3) of any CD22 binding domain listed in **Table 7A, 7B, 7C, 9A, or 9B**.

53. A method of treating a mammal having a disease associated with expression of CD22 comprising administering to the mammal an effective amount of the cell of comprising a CAR molecule of claim 52.

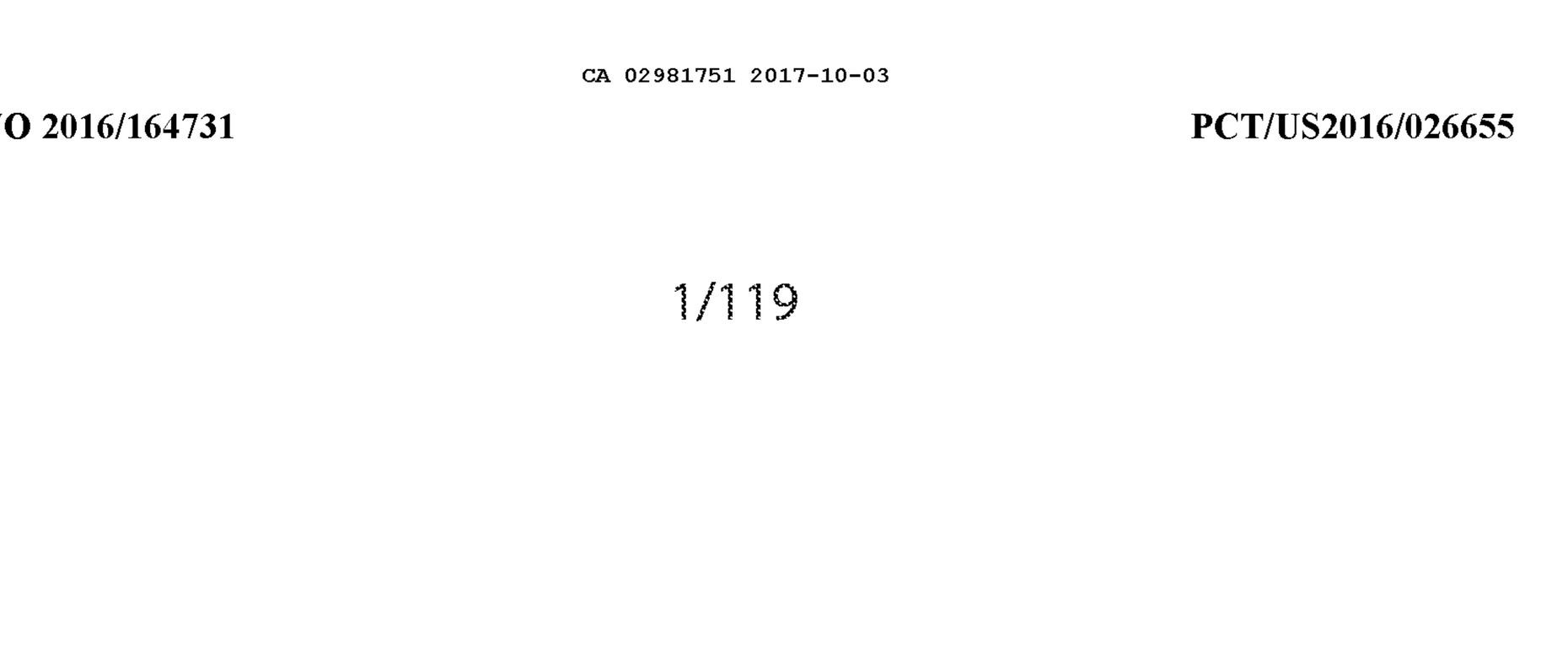

αCD19-Δζ
VH
VL
hCD8α hinge
αCD19-ζ
VH
VL
hCD8α hinge
TCR-ζ
αCD19-BB-ζ
VH
VL
hCD8α hinge
4-1BB
TCR-ζ
αCD19-28-ζ
VH
VL
hCD8α hinge
CD28
TCR-ζ
αCD19-28-BB-ζ
VH
VL
hCD8α hinge
CD28
4-1BB
TCR-ζ

FIG. 1A

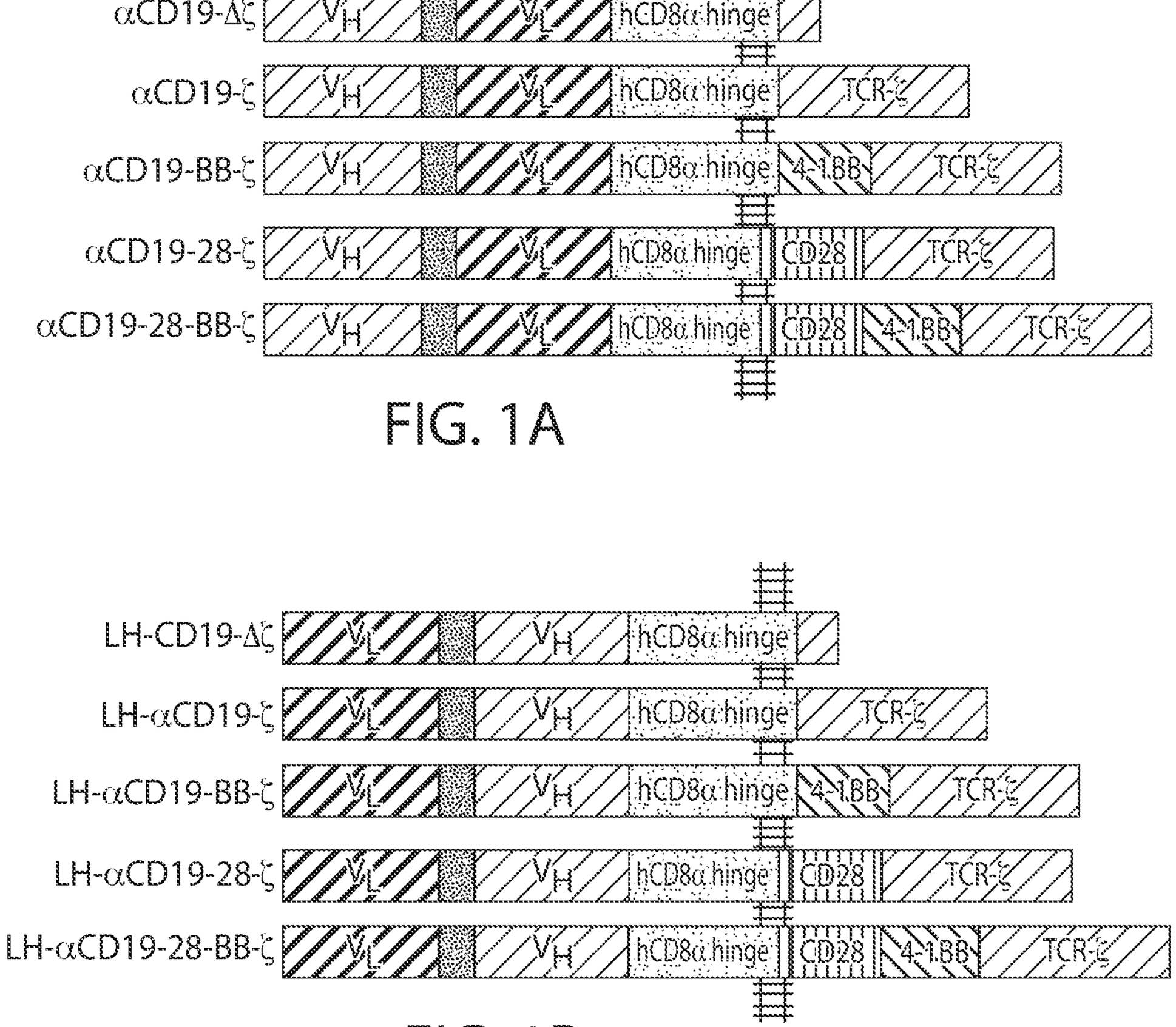

LH-CD19-Δζ
VL
VH
hCD8α hinge
LH-αCD19-ζ
VL
VH
hCD8α hinge
TCR-ζ
LH-αCD19-BB-ζ
VL
VH
hCD8α hinge
4-1BB
TCR-ζ
LH-αCD19-28-ζ
VL
VH
hCD8α hinge
CD28
TCR-ζ
LH-αCD19-28-BB-ζ
VL
VH
hCD8α hinge
CD28
4-1BB
TCR-ζ

FIG. 1B

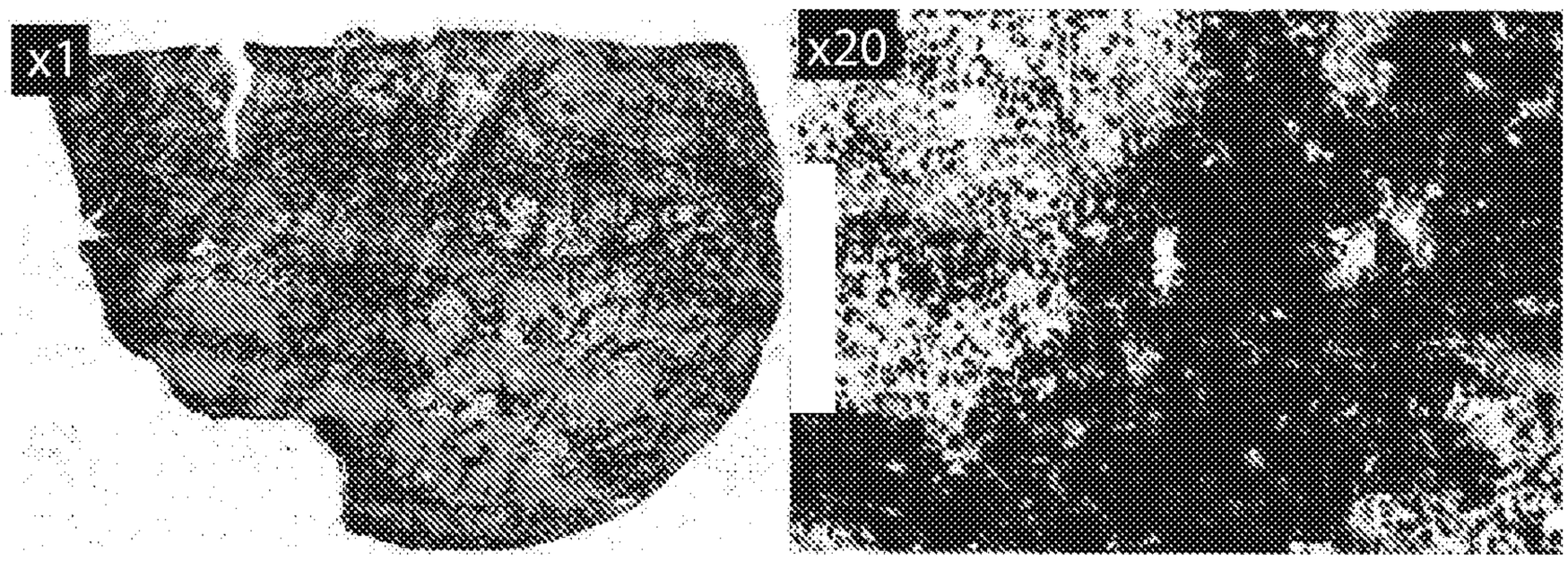


FIG. 2

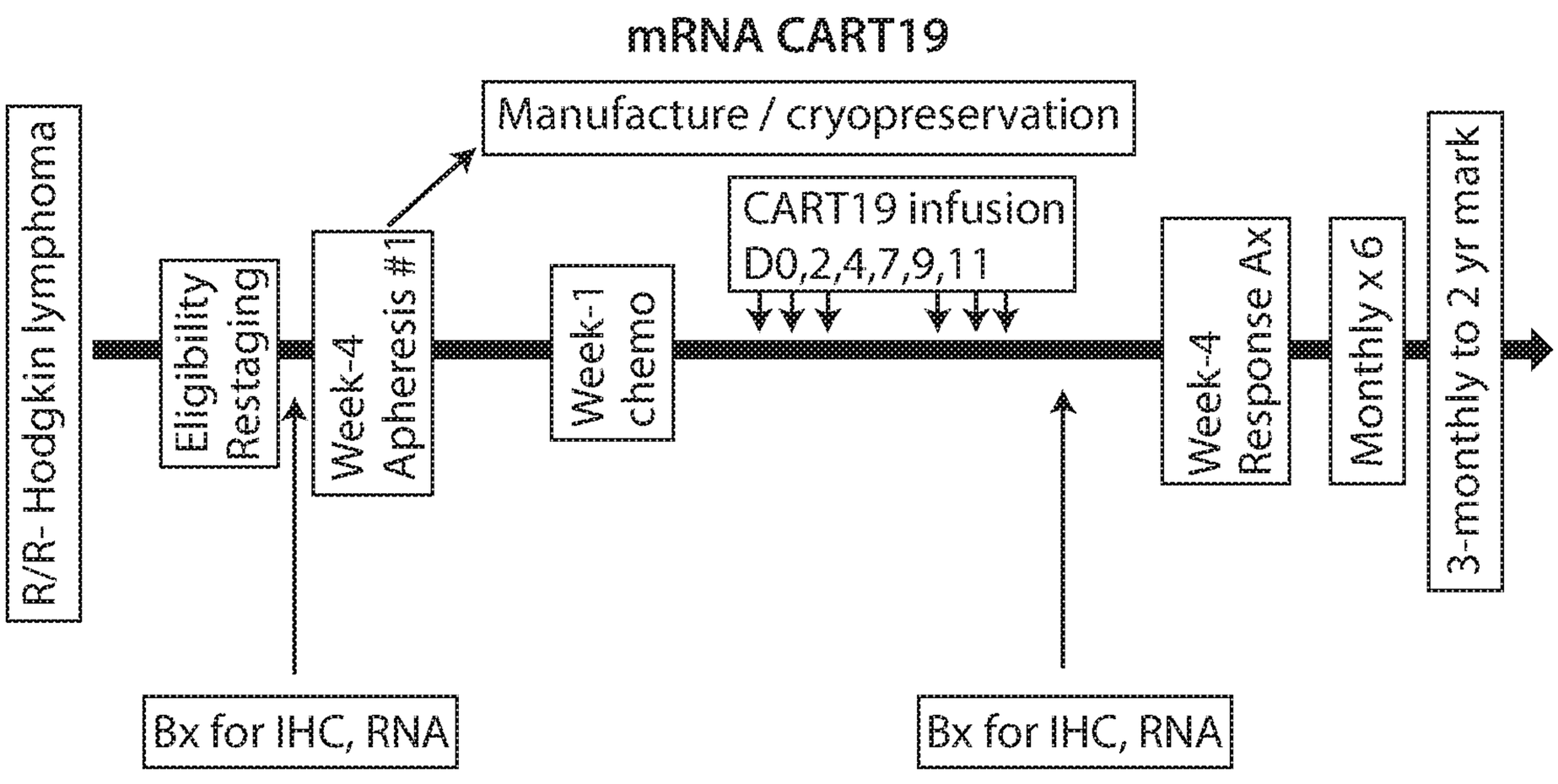

mRNA CART19
Manufacture / cryopreservation
R/R- Hodgkin lymphoma
Eligibility Restaging
Week-4 Apheresis #1
Week-1 chemo
CART19 infusion D0,2,4,7,9,11
Week-4 Response Ax
Monthly x 6
3-monthly to 2 yr mark
Bx for IHC, RNA
Bx for IHC, RNA

FIG. 3

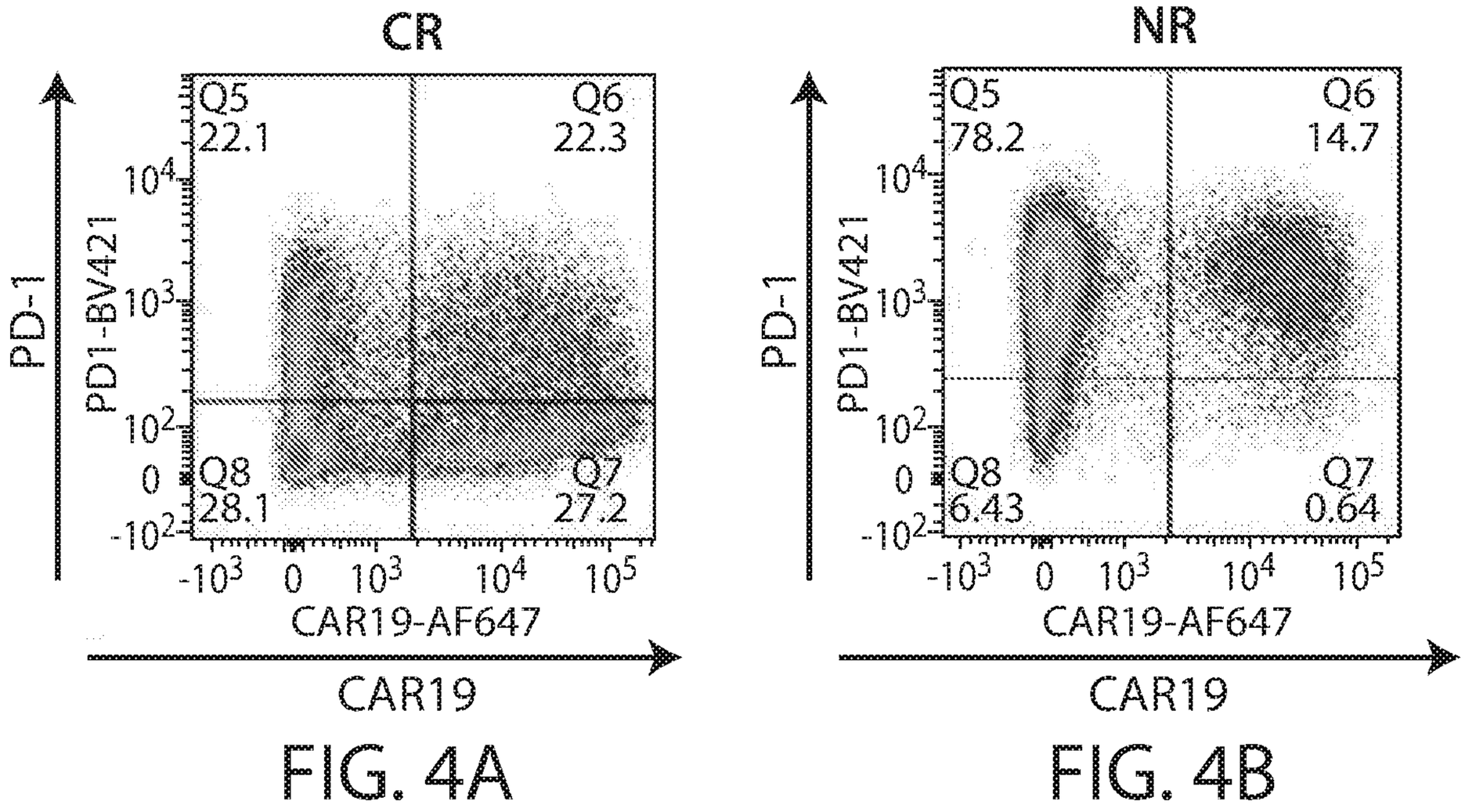

Gated on CD4+
CR
NR
PD-1
PD1-BV421
Q5
22.1
Q6
22.3
Q8
28.1
Q7
27.2
Q5
78.2
Q6
14.7
Q8
6.43
Q7
0.64
10^4
10^3
10^2
0
-10^2
-10^3
0
10^3
10^4
10^5
CAR19-AF647
CAR19
CAR19

FIG. 4A

FIG. 4B

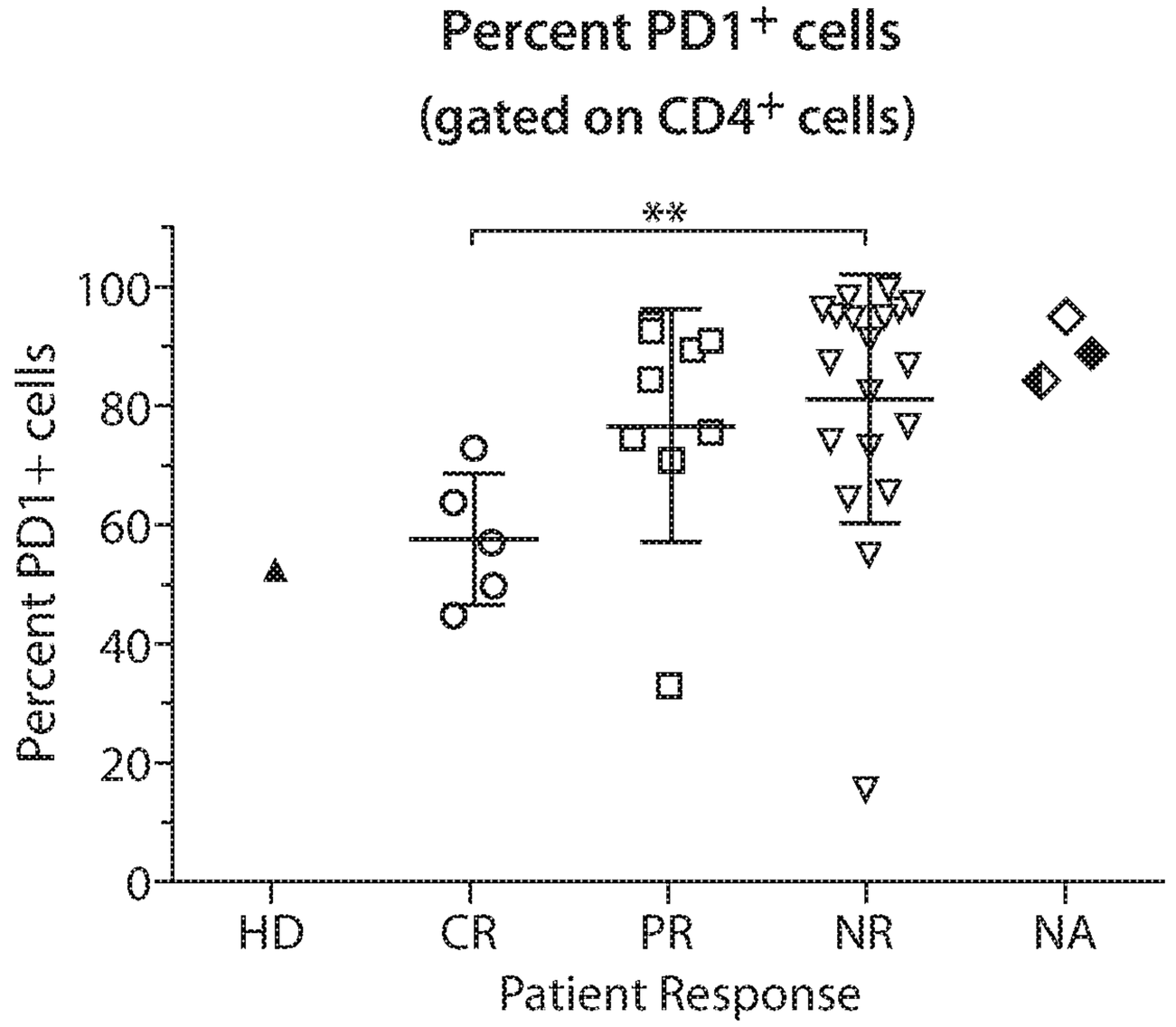

Percent PD1+ cells
(gated on CD4+ cells)
**
Percent PD1+ cells
100
80
60
40
20
0
HD
CR
PR
NR
NA
Patient Response

FIG. 4C

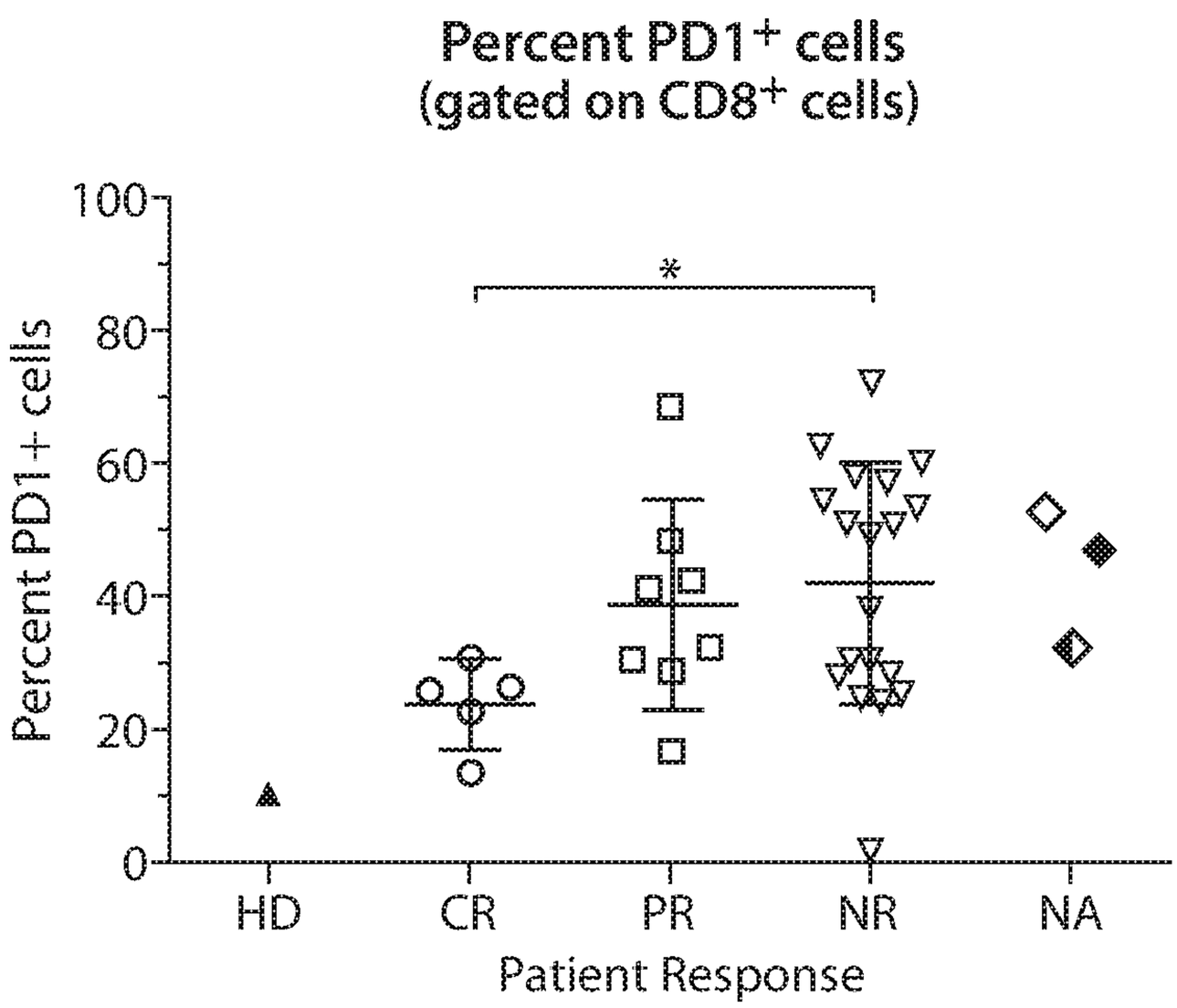

Percent PD1+ cells
(gated on CD8+ cells)
*
Percent PD1+ cells
100
80
60
40
20
0
HD
CR
PR
NR
NA
Patient Response

FIG. 4D

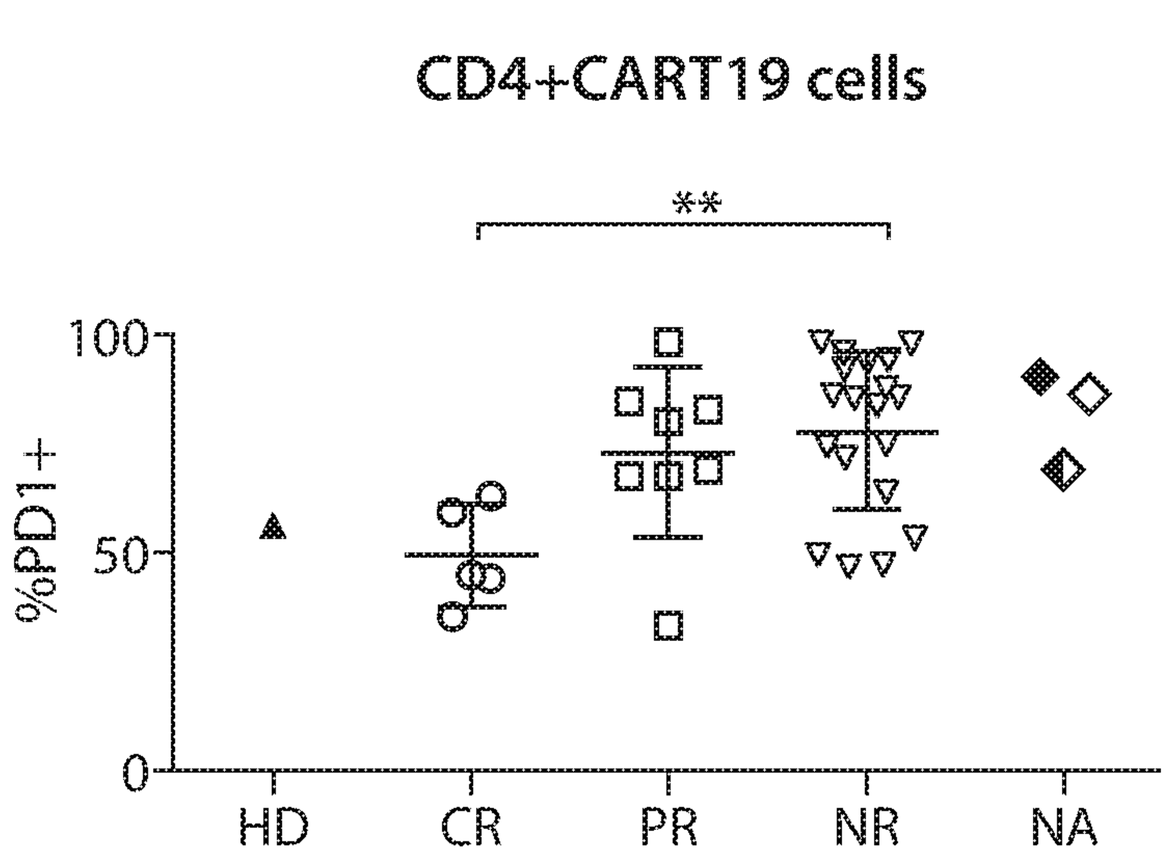

CD4+CART19 cells
**
100
50
0
%PD1+
HD
CR
PR
NR
NA

FIG. 5A

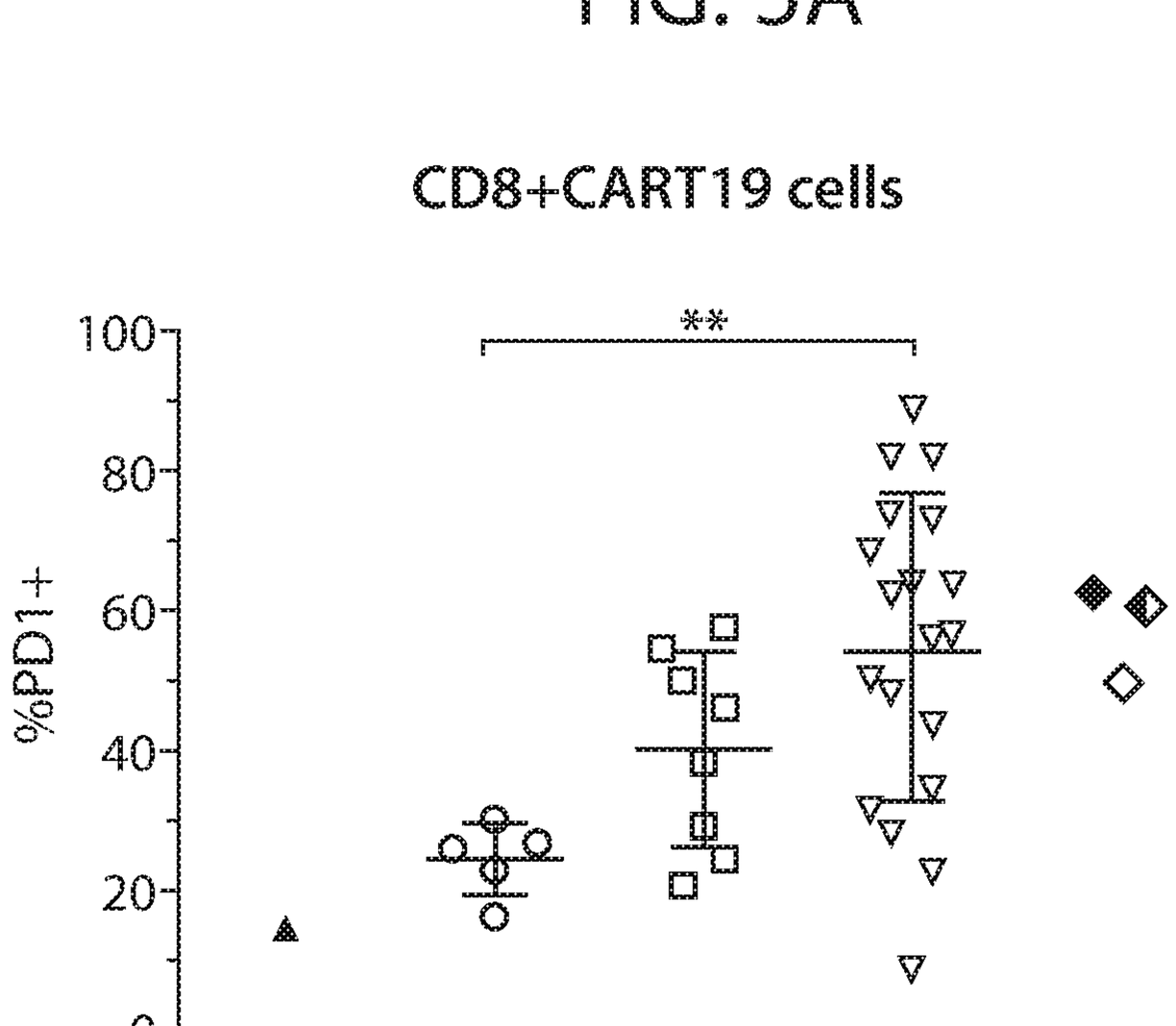

CD8+CART19 cells
**
100
80
60
40
20
0
%PD1+
HD
CR
PR
NR
NA

FIG. 5B

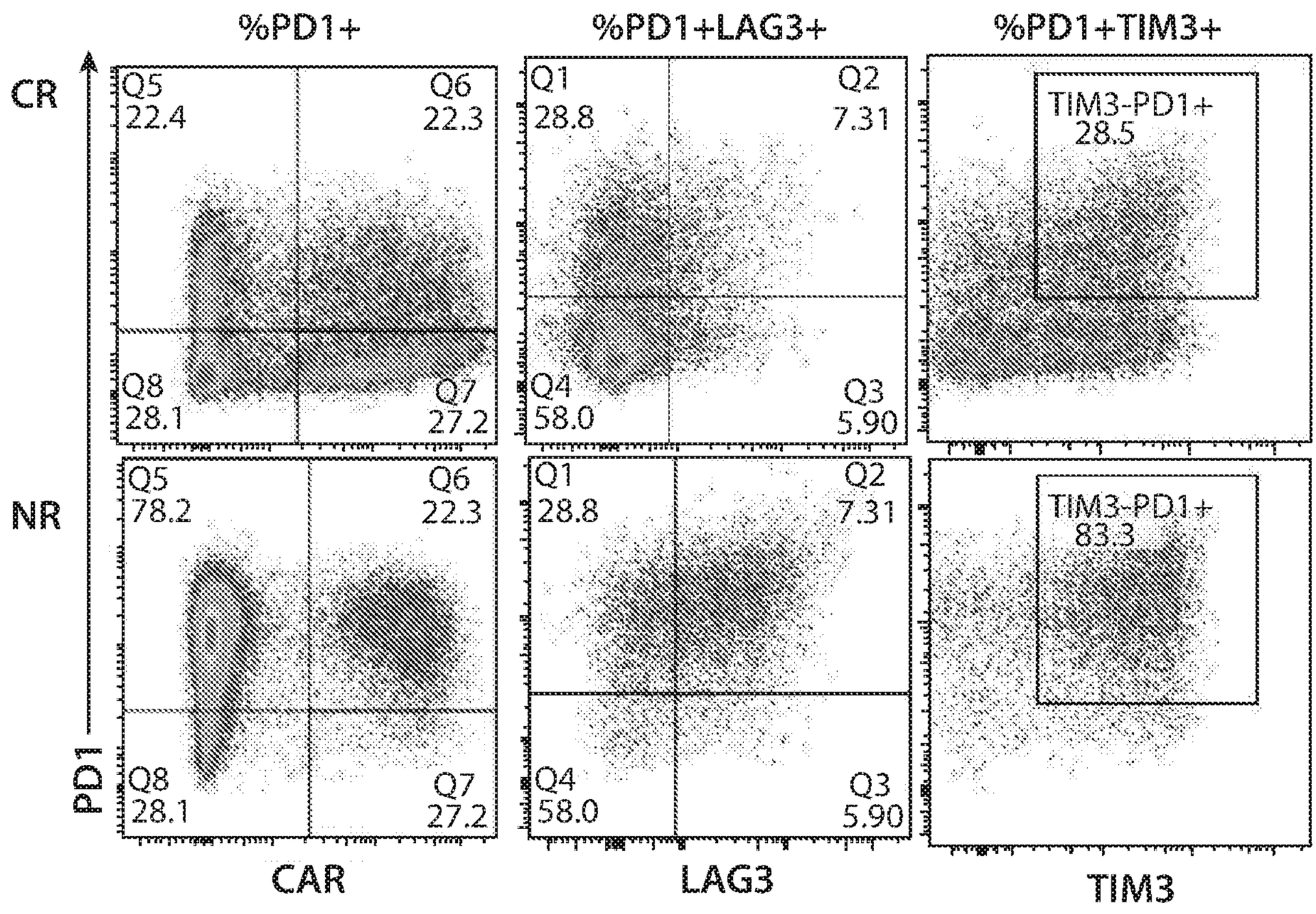

%PD1+
%PD1+LAG3+
%PD1+TIM3+
CR
NR
PD1
CAR
LAG3
TIM3
Q5
22.4
Q6
22.3
Q8
28.1
Q7
27.2
Q1
28.8
Q2
7.31
Q4
58.0
Q3
5.90
TIM3-PD1+
28.5
Q5
78.2
Q6
22.3
Q8
28.1
Q7
27.2
Q1
28.8
Q2
7.31
Q4
58.0
Q3
5.90
TIM3-PD1+
83.3

FIG. 6

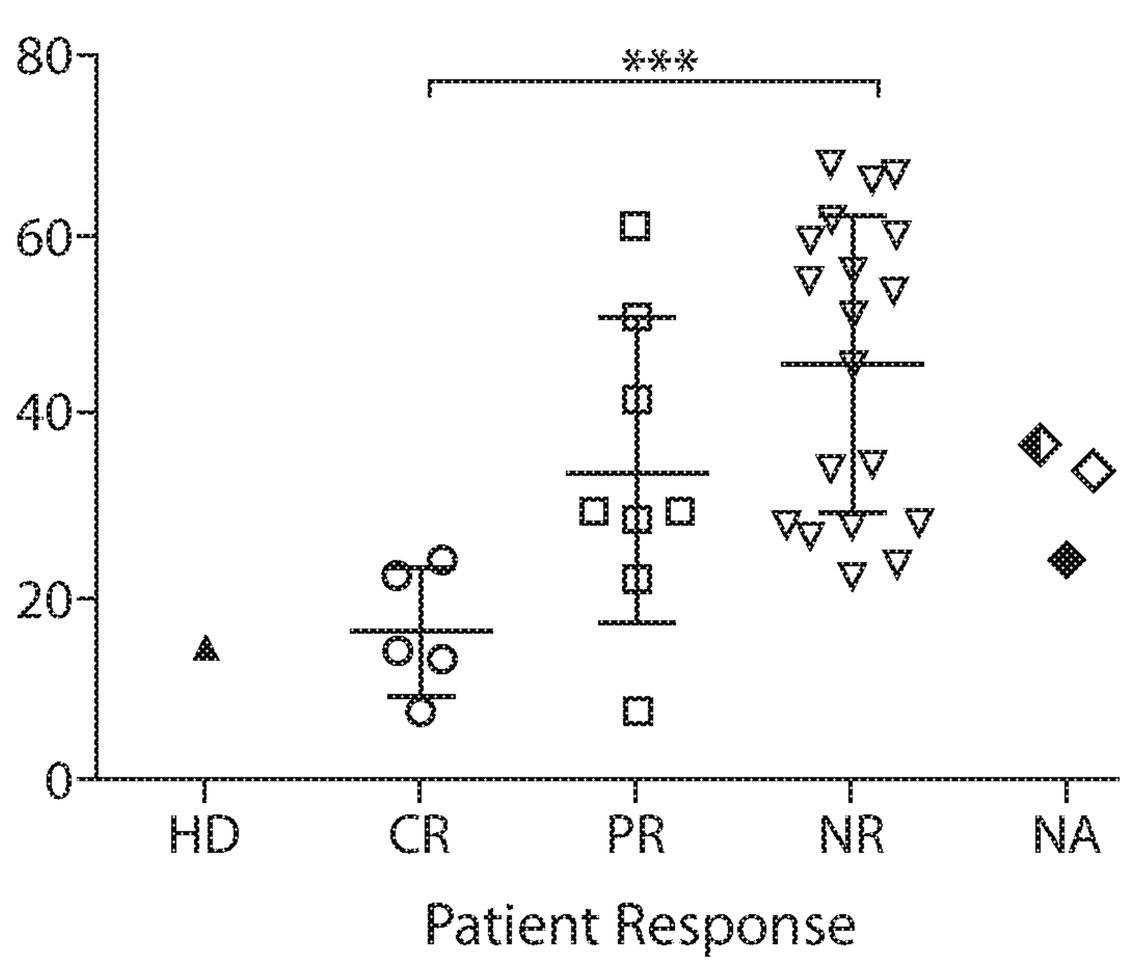

%PD1+LAG3+
***
80
60
40
20
0
HD
CR
PR
NR
NA
Patient Response

FIG. 7A

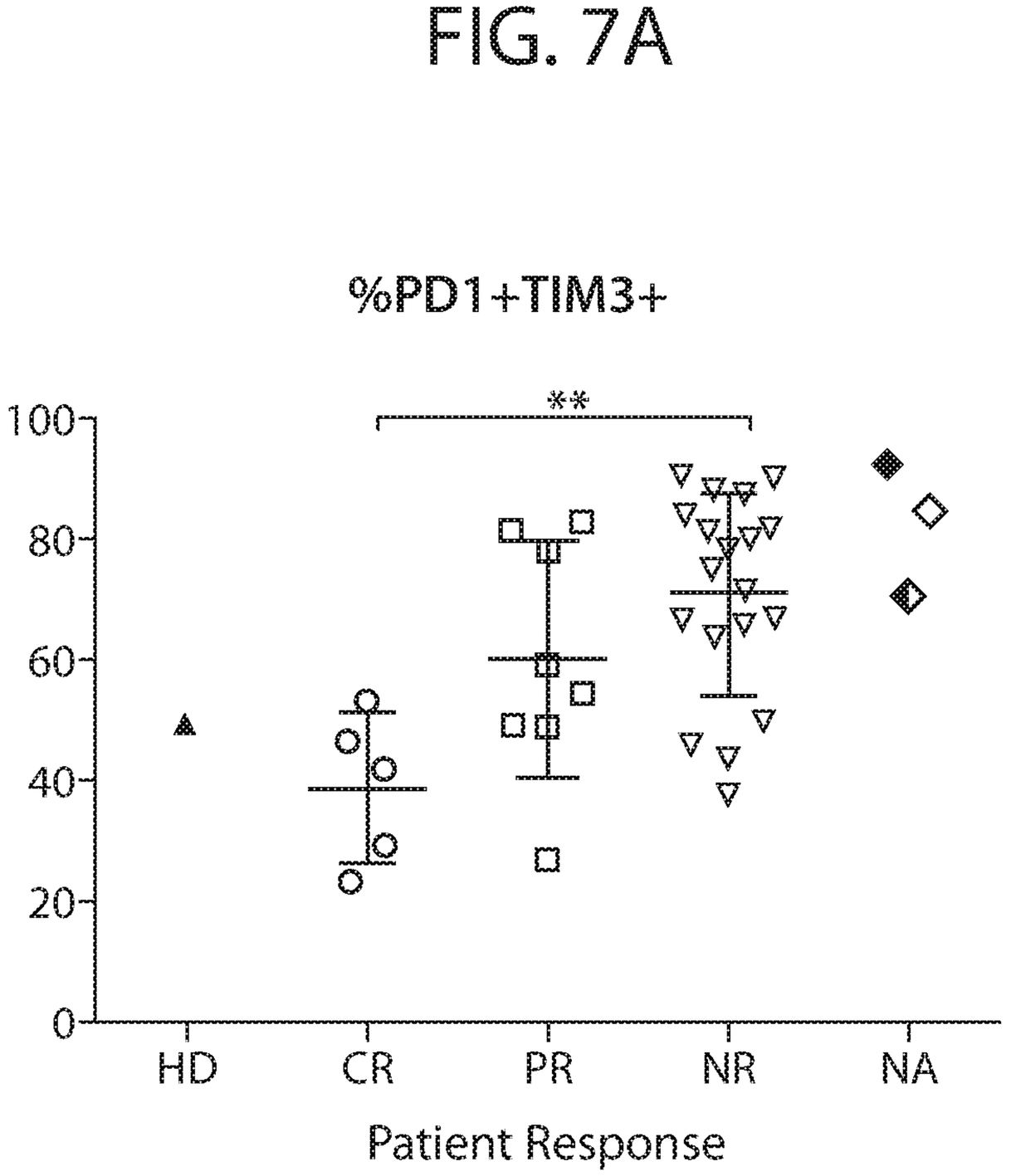

%PD1+TIM3+
**
100
80
60
40
20
0
HD
CR
PR
NR
NA
Patient Response

FIG. 7B

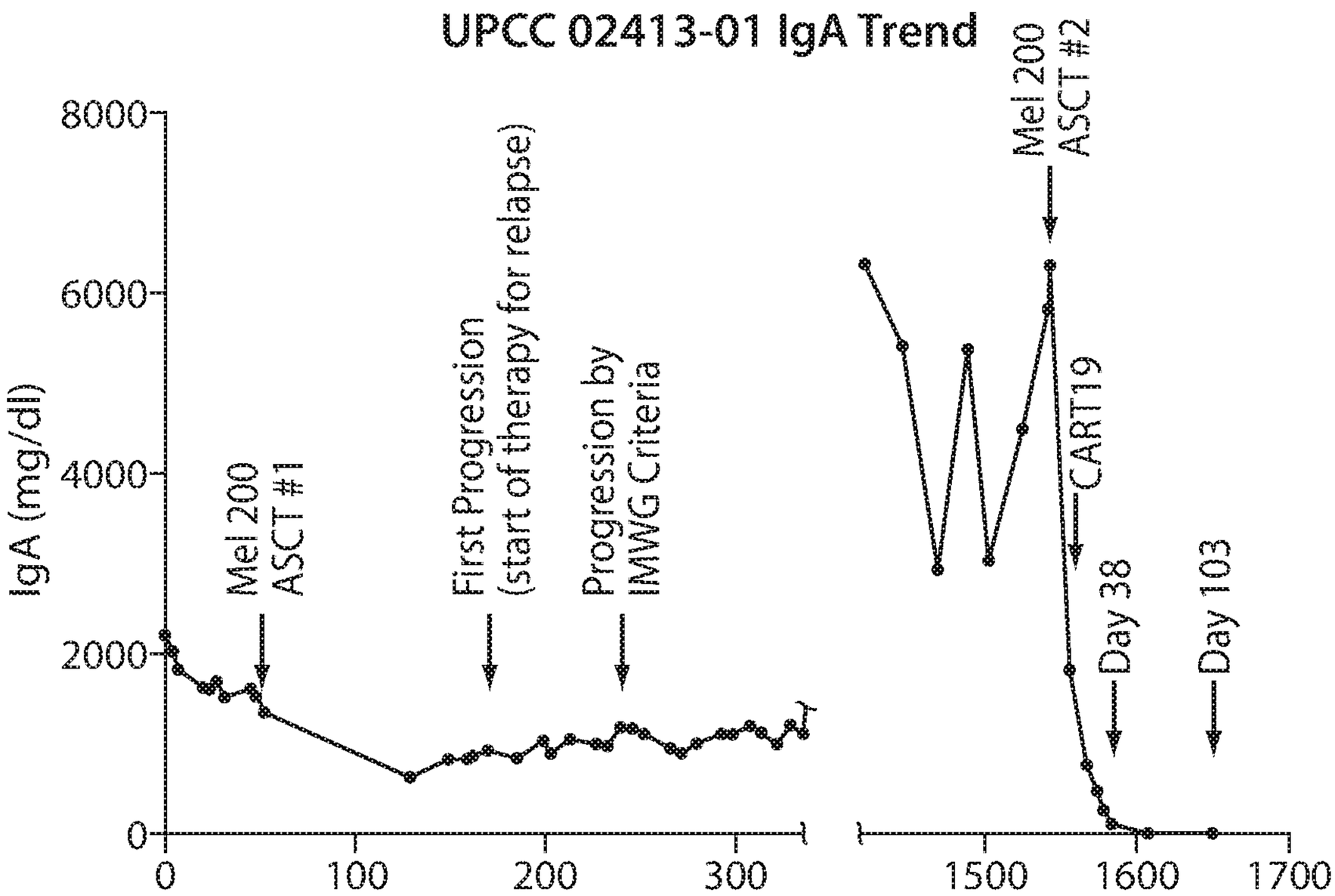

UPCC 02413-01 IgA Trend
IgA (mg/dl)
8000
6000
4000
2000
0
0
100
200
300
1500
1600
1700
Mel 200
ASCT #1
First Progression
(start of therapy for relapse)
Progression by
IMWG Criteria
Mel 200
ASCT #2
CART19
Day 38
Day 103

FIG. 8

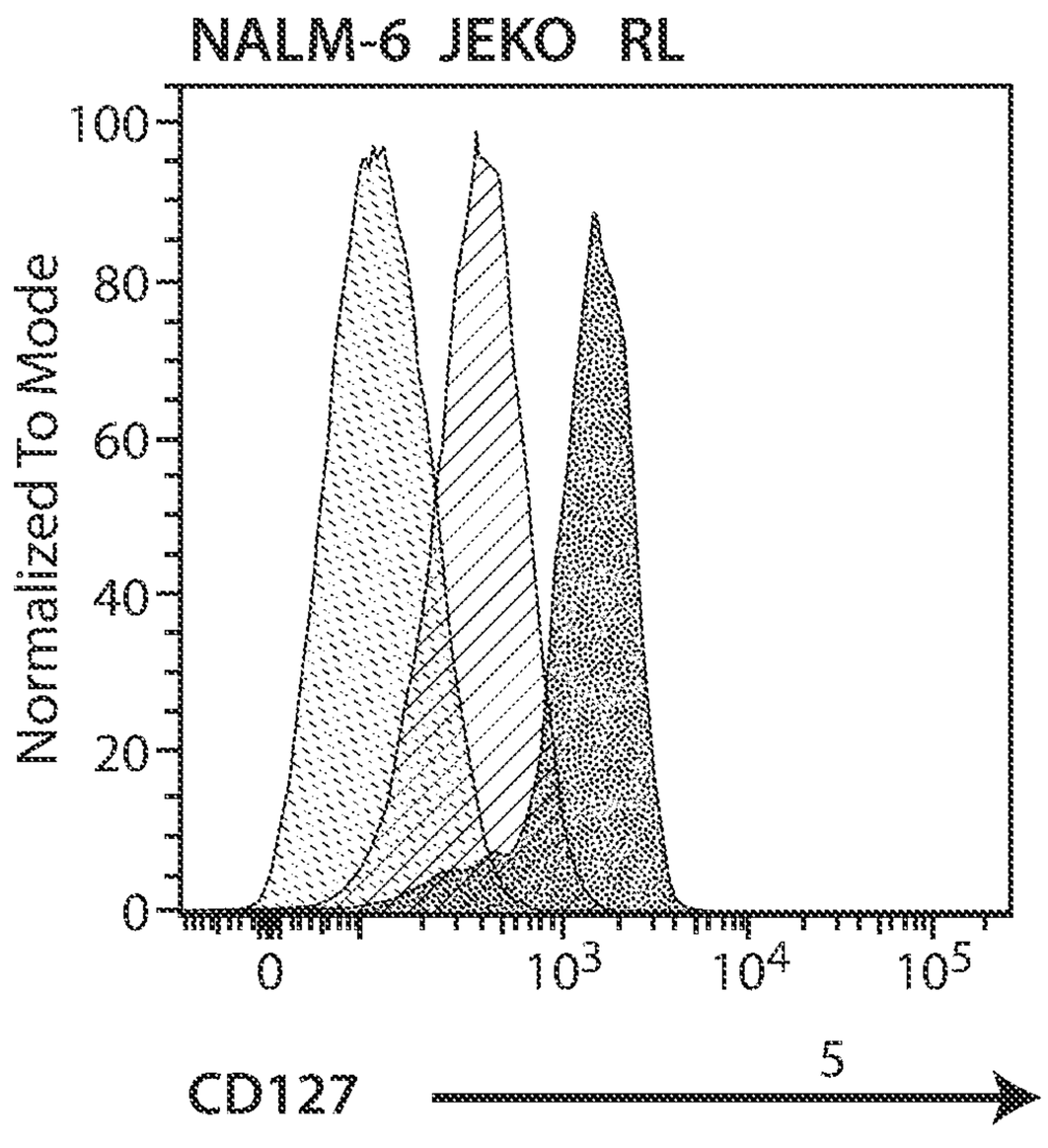

NALM-6 JEKO RL
100
80
60
40
20
0
Normalized To Mode
0
10^3
10^4
10^5
CD127
5

FIG. 9A

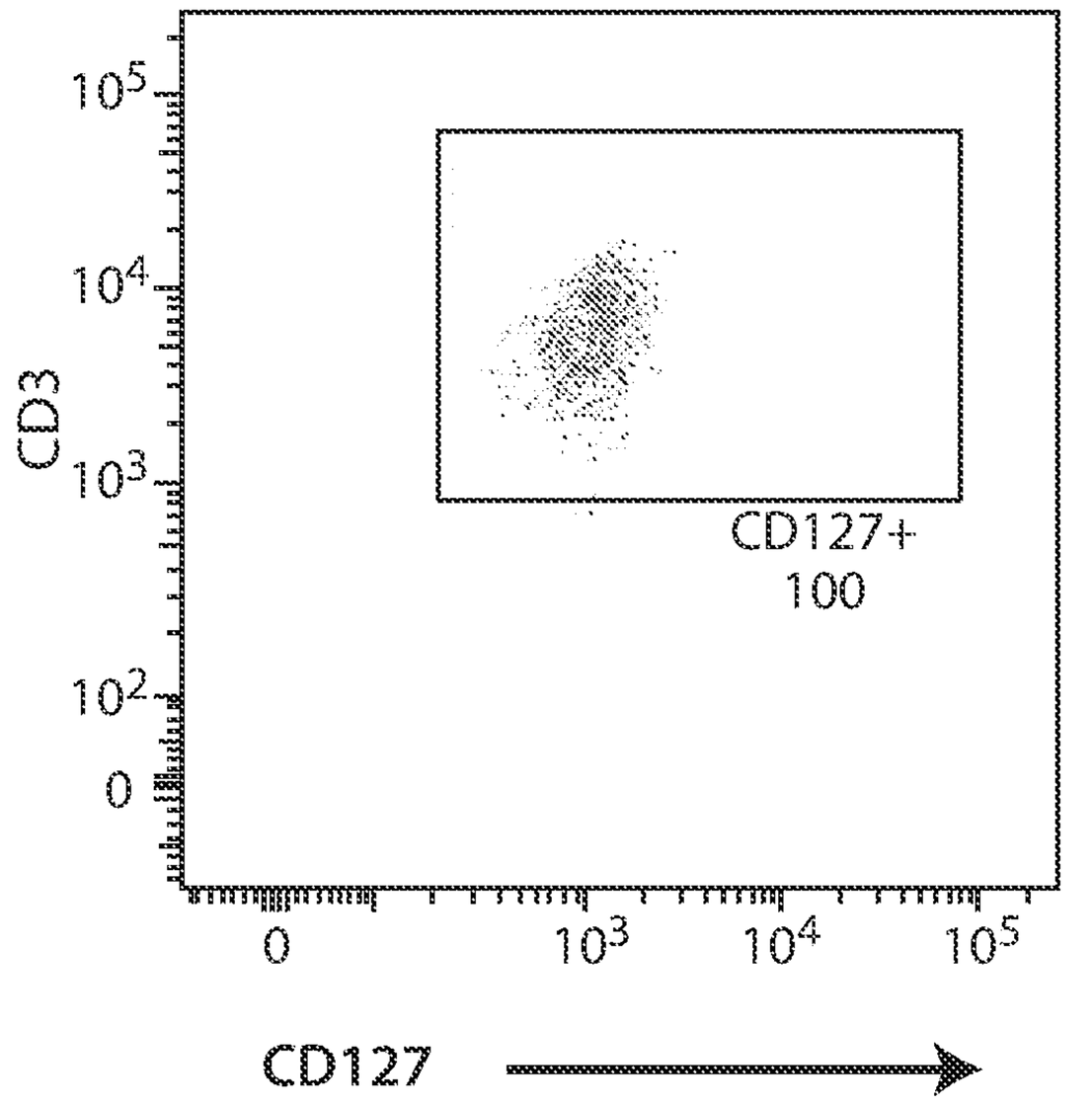

10^5
10^4
10^3
10^2
0
CD3
CD127+
100
0
10^3
10^4
10^5
CD127

FIG. 9B

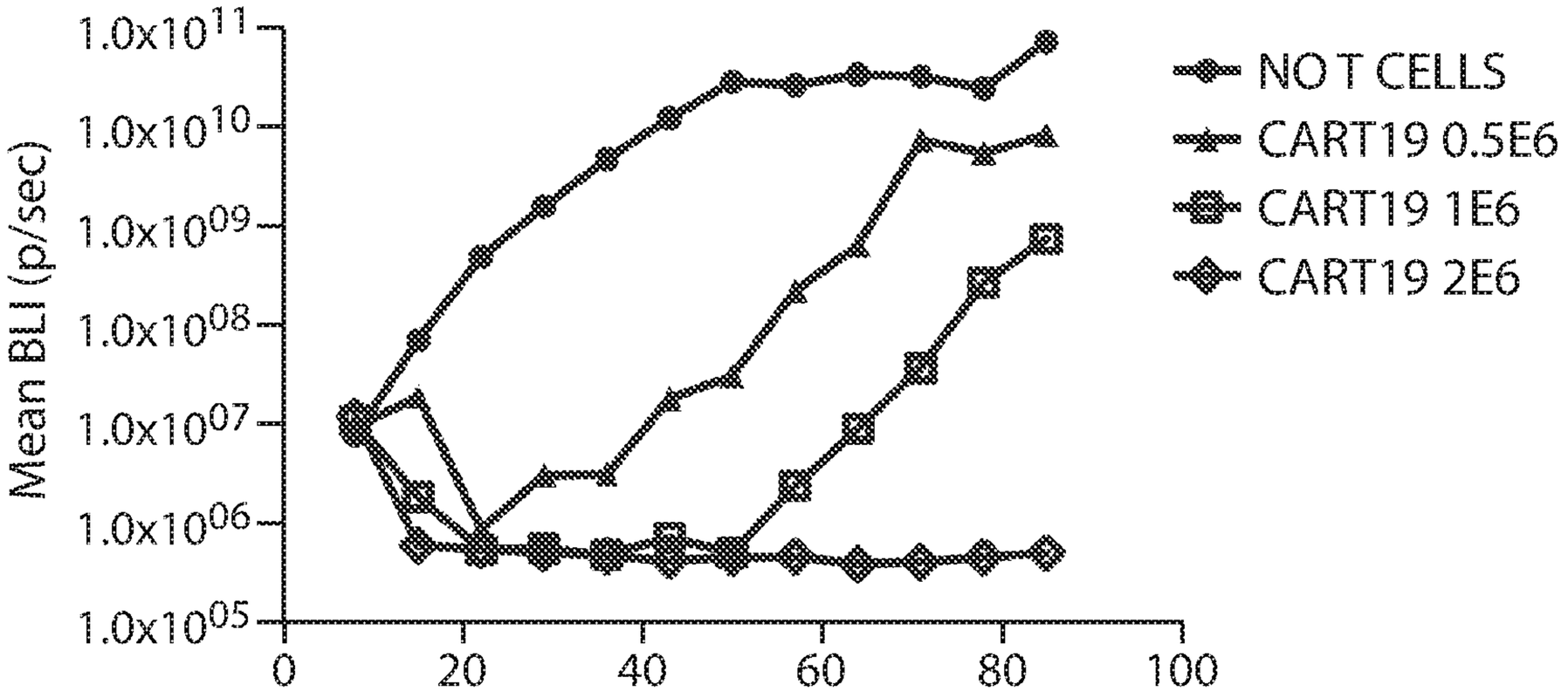

Mean BLI (p/sec)
1.0x10^11
1.0x10^10
1.0x10^09
1.0x10^08
1.0x10^07
1.0x10^06
1.0x10^05
0
20
40
60
80
100
NO T CELLS
CART19 0.5E6
CART19 1E6
CART19 2E6

FIG. 10A

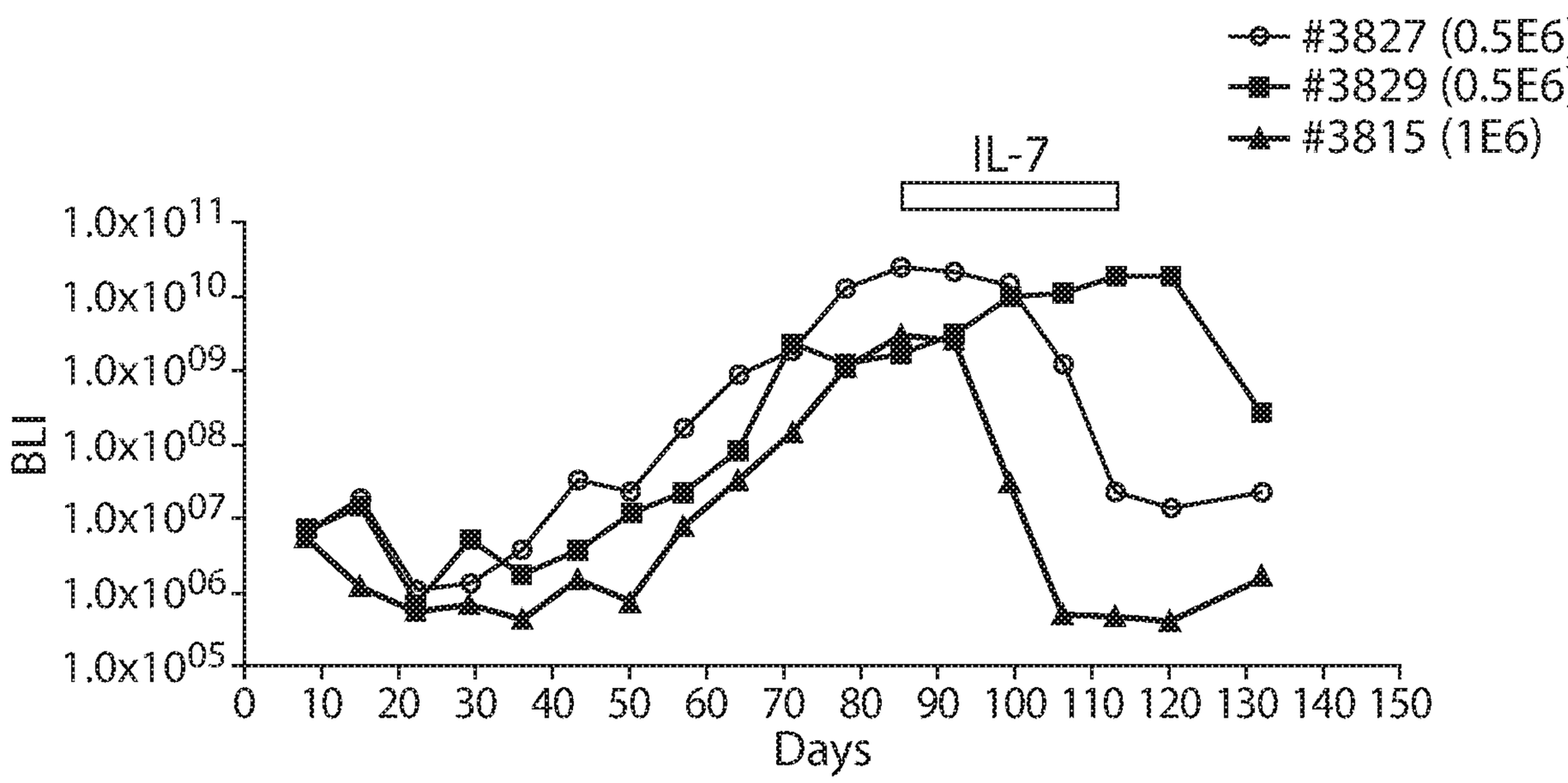


FIG. 10B

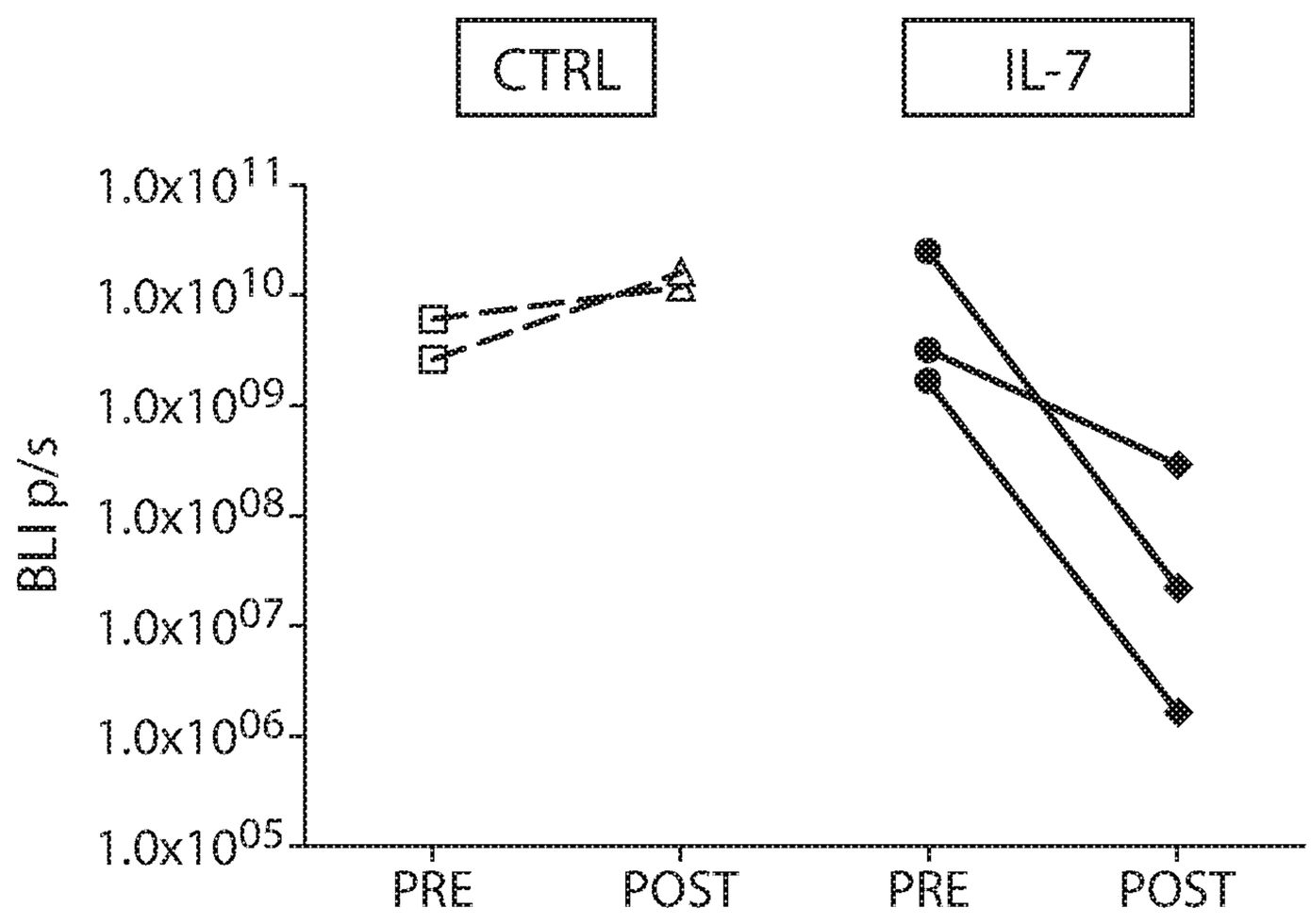


FIG. 10C

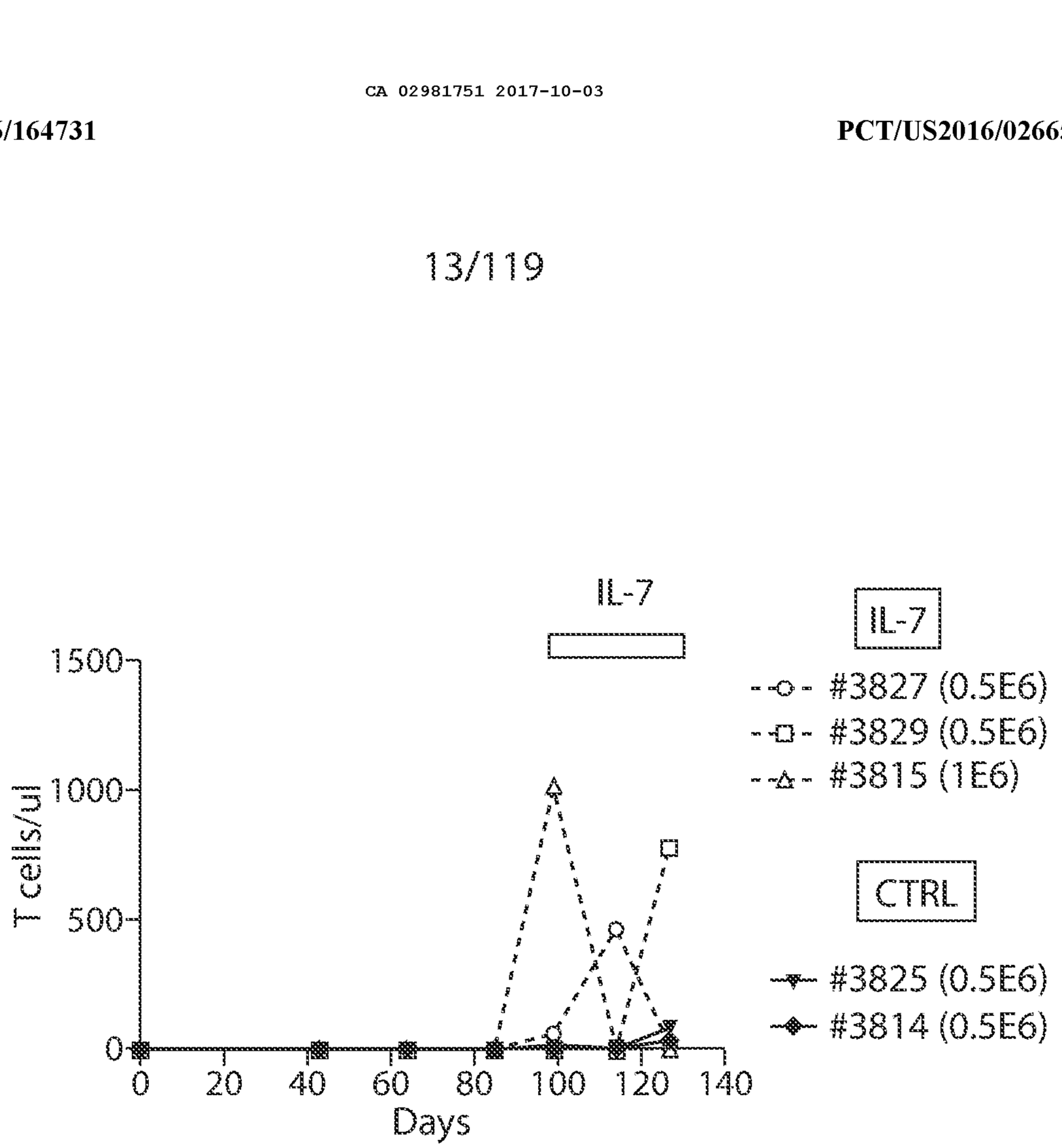


FIG. 11A

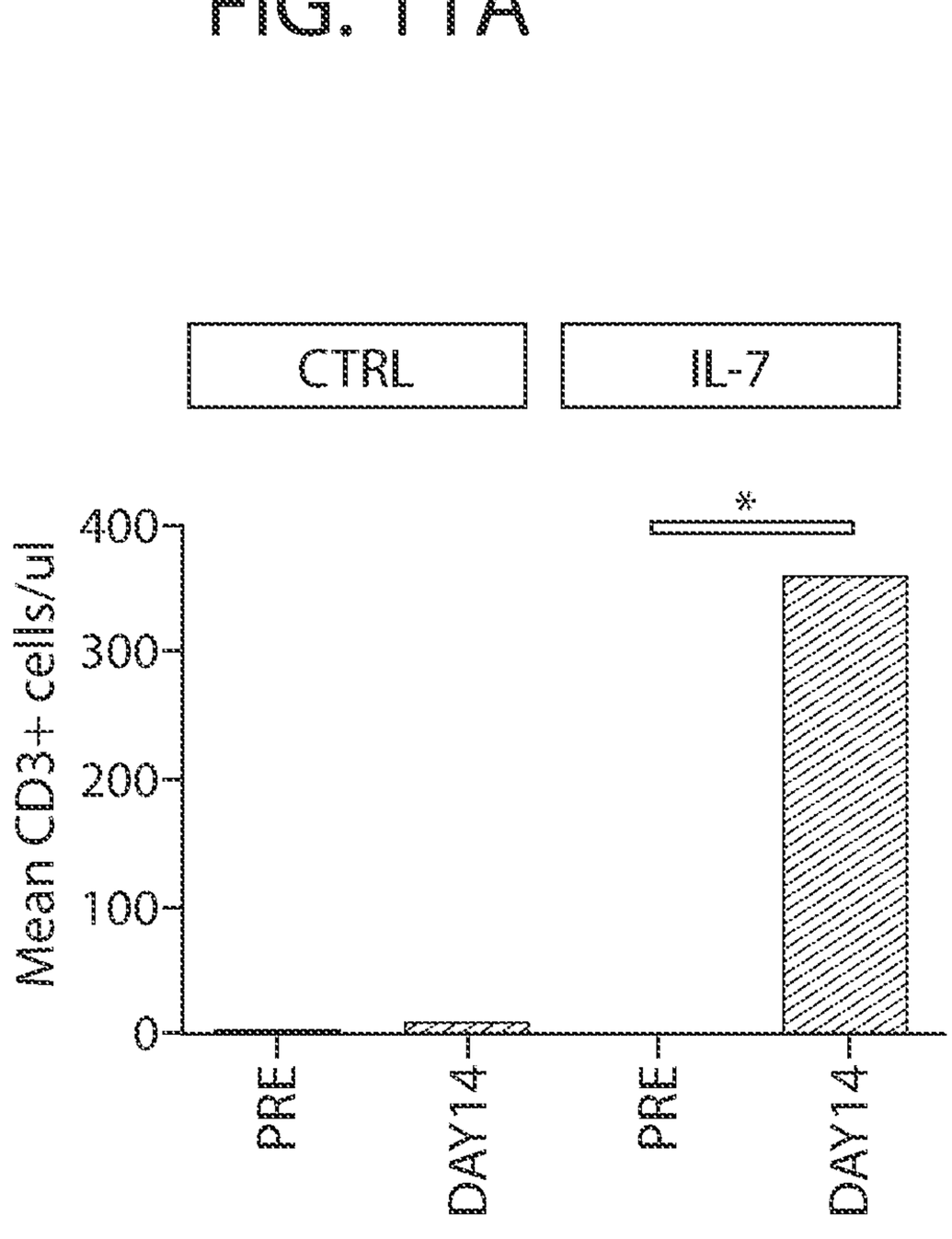


FIG. 11B

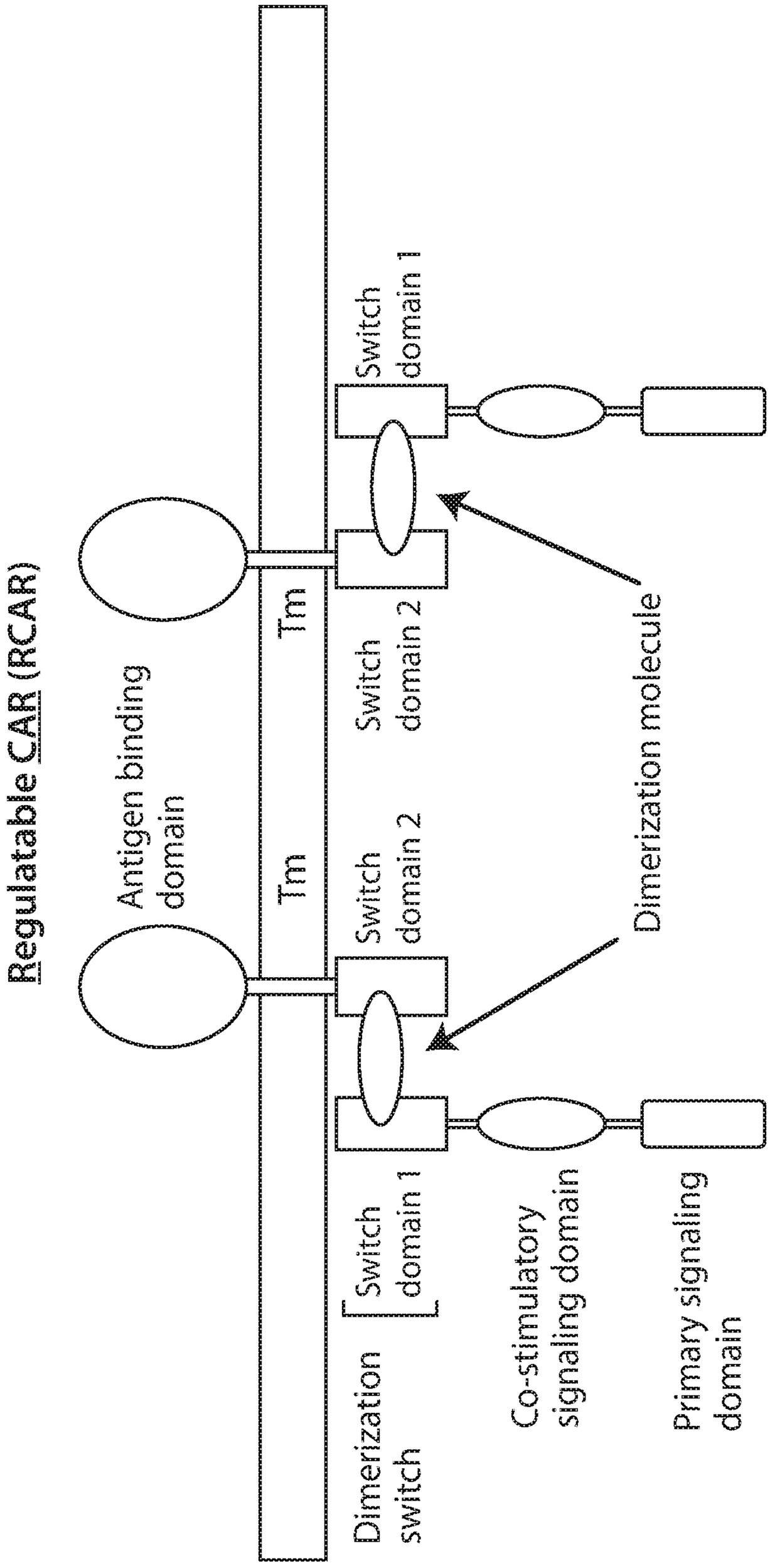

Regulatable CAR (RCAR)
Antigen binding domain
Tm
Switch domain 2
Dimerization molecule
Switch domain 1
Dimerization switch
Switch domain 1
Tm
Switch domain 2
Antigen binding domain
Co-stimulatory signaling domain
Primary signaling domain

Fig. 12

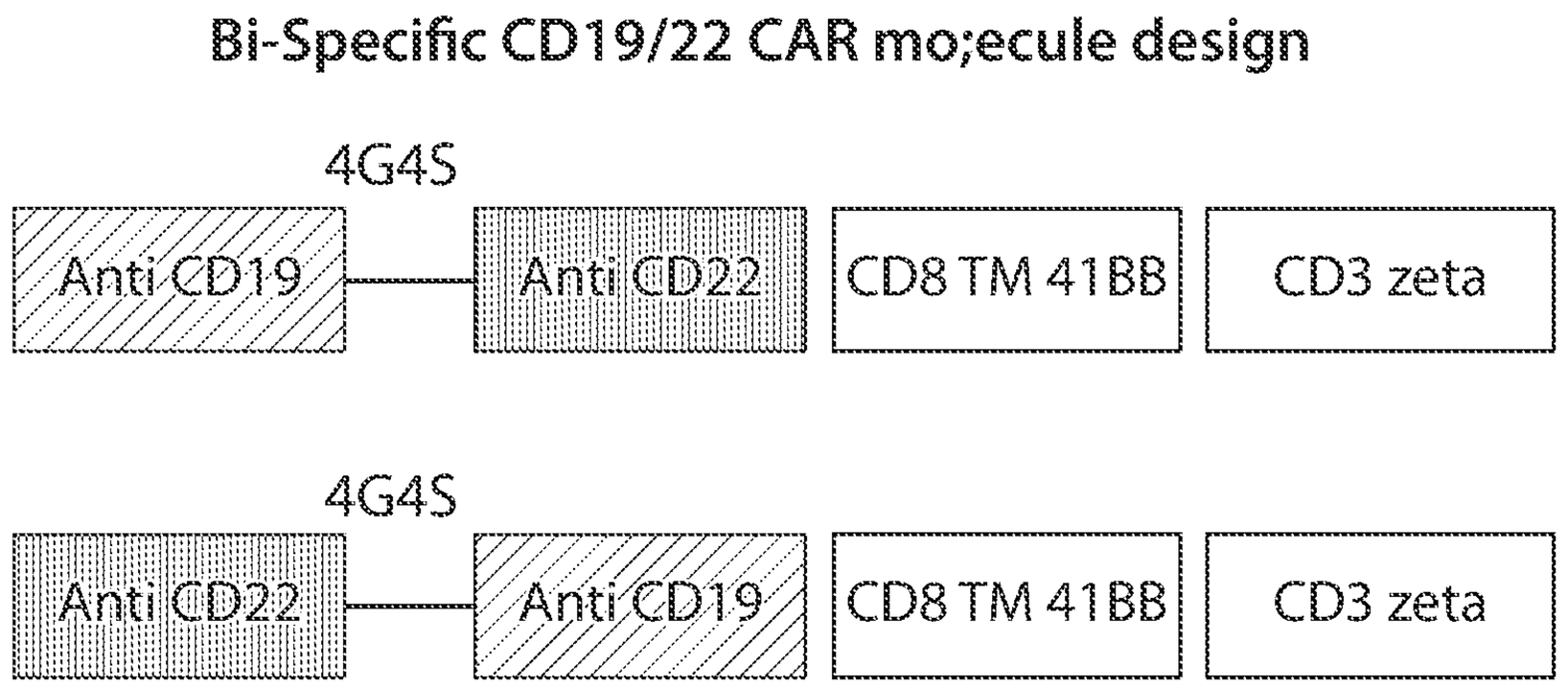

Bi-Specific CD19/22 CAR mo;ecule design
4G4S
Anti CD19
Anti CD22
CD8 TM 41BB
CD3 zeta
4G4S
Anti CD22
Anti CD19
CD8 TM 41BB
CD3 zeta

FIG. 13

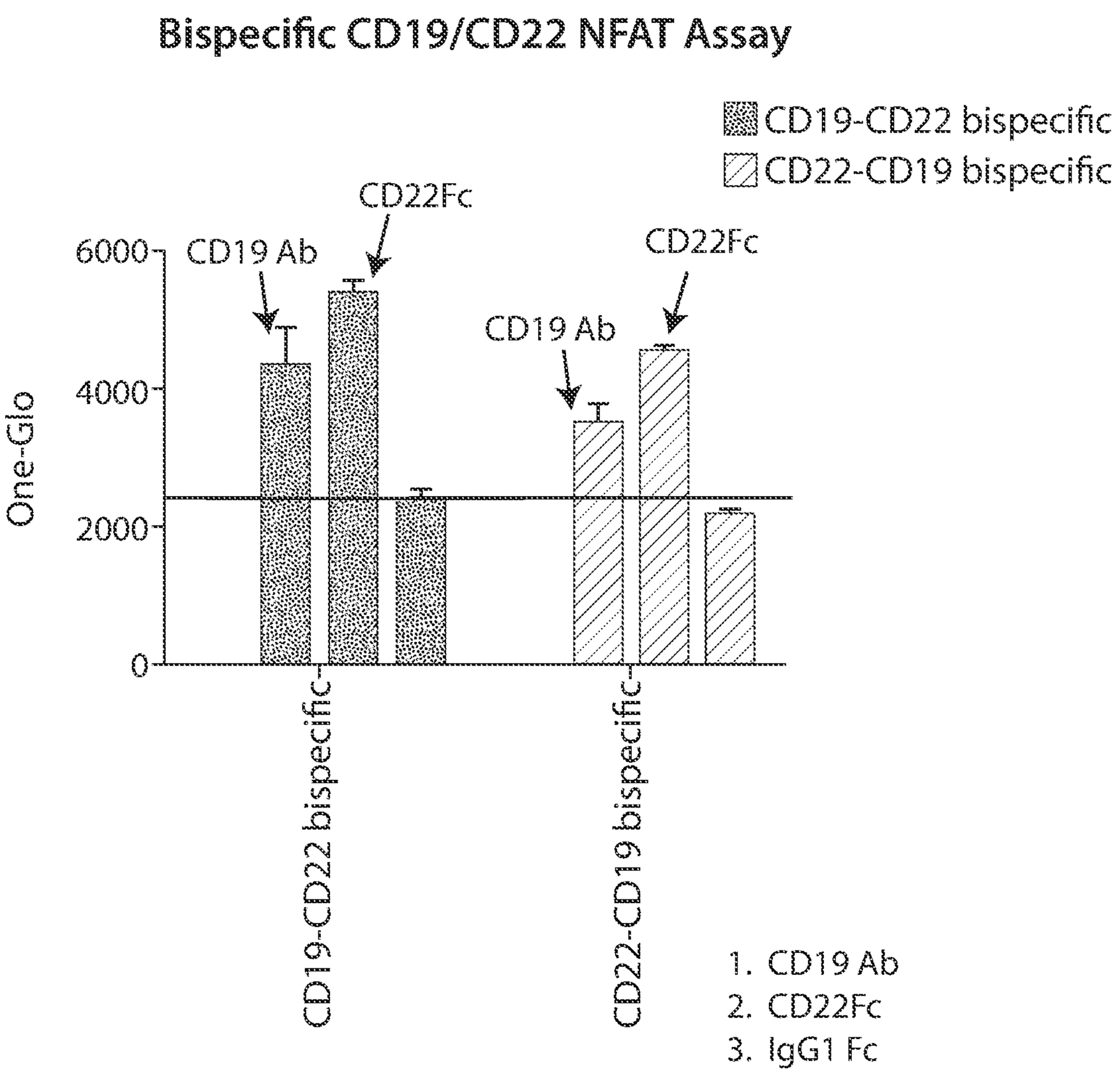

Bispecific CD19/CD22 NFAT Assay
CD19-CD22 bispecific
CD22-CD19 bispecific
One-Glo
6000
4000
2000
0
CD19 Ab
CD22Fc
CD19 Ab
CD22Fc
CD19-CD22 bispecific
CD22-CD19 bispecific
1. CD19 Ab
2. CD22Fc
3. IgG1 Fc

FIG. 14

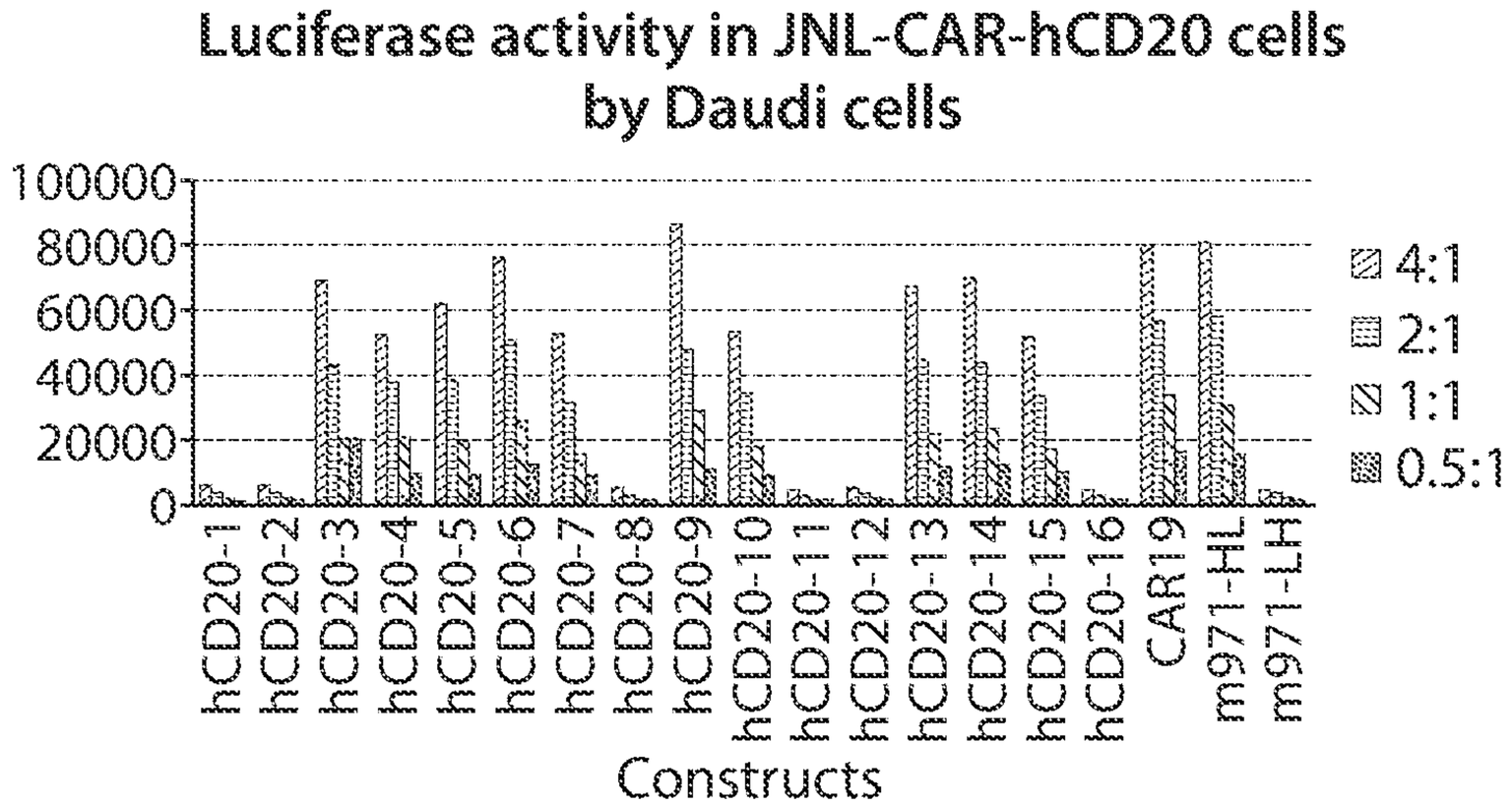


FIG. 15A

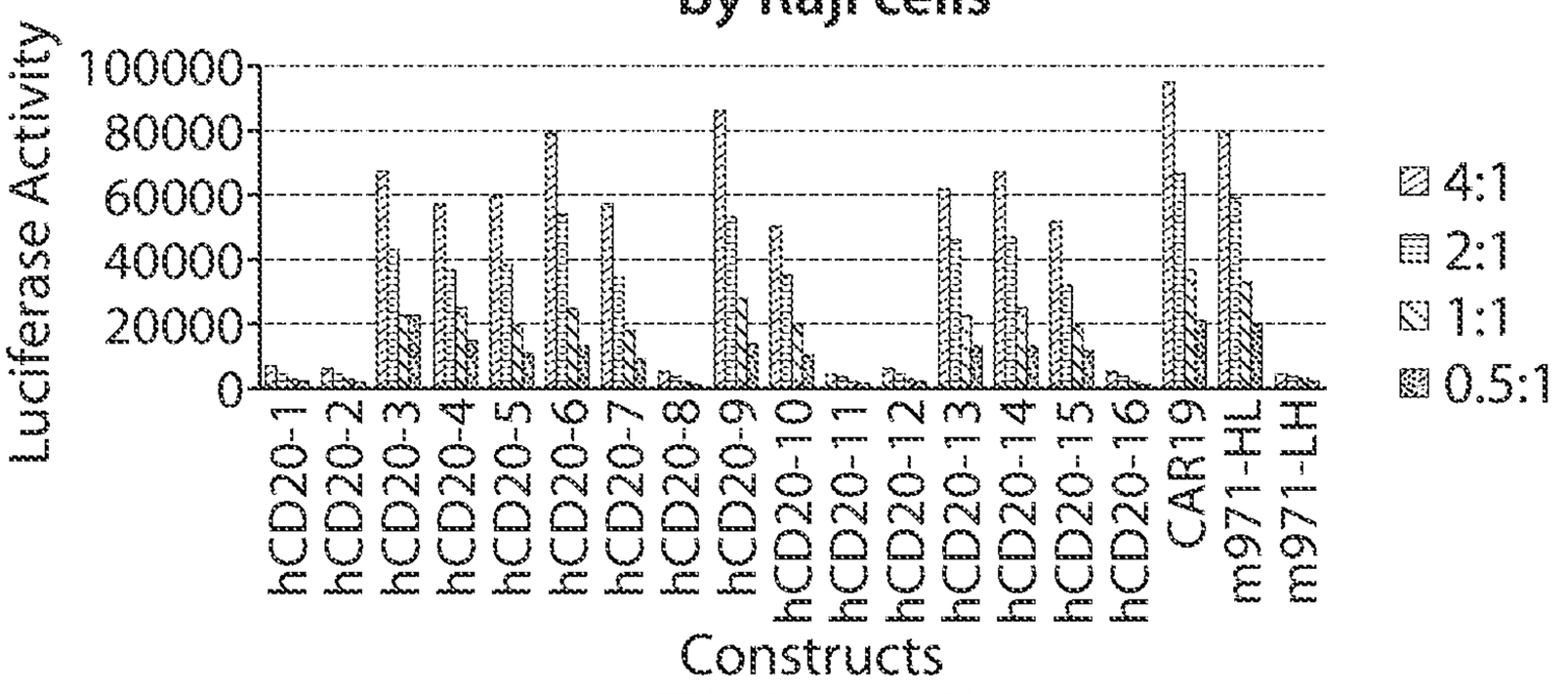


FIG. 15B

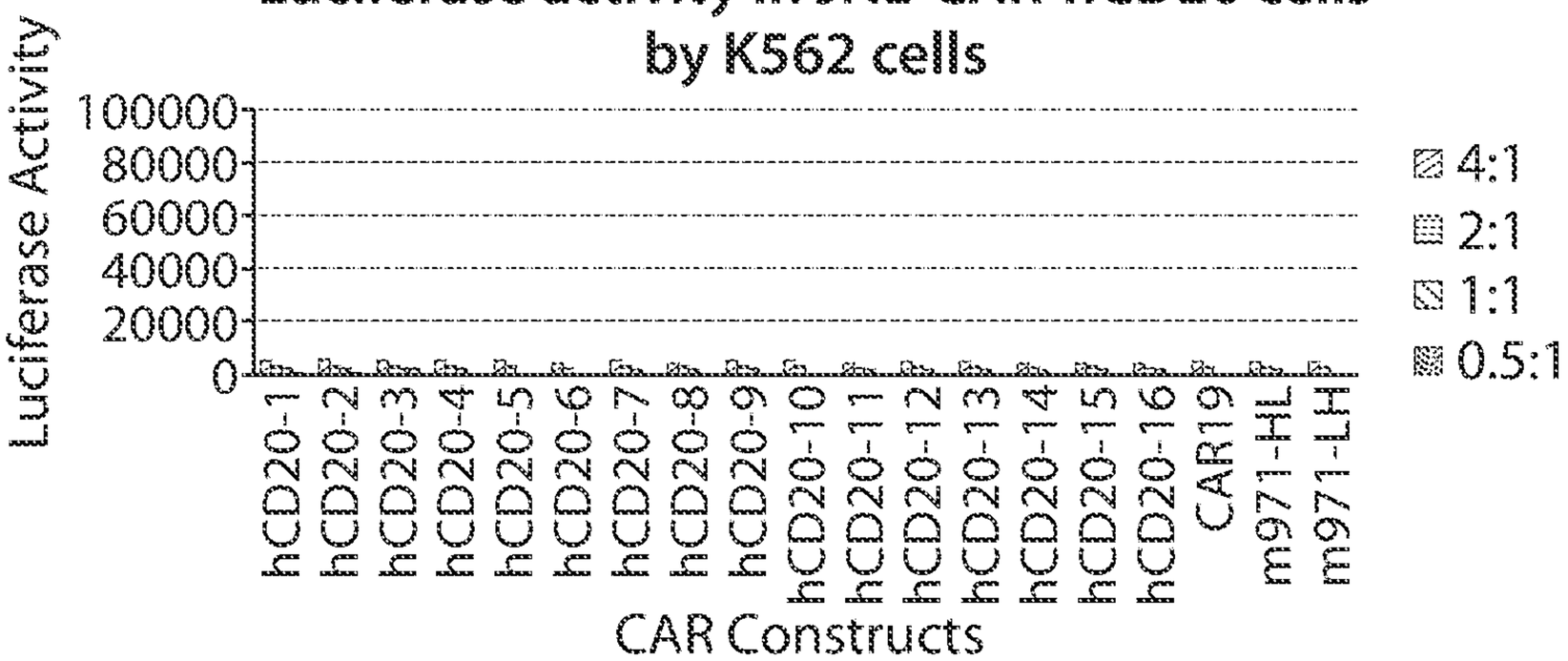


FIG. 15C

CA 02981751 2017-10-03

Activated Teff enriched in NRs vs. CRs

Genes upregulated in activated vs resting Teff are also upregulated in NRs

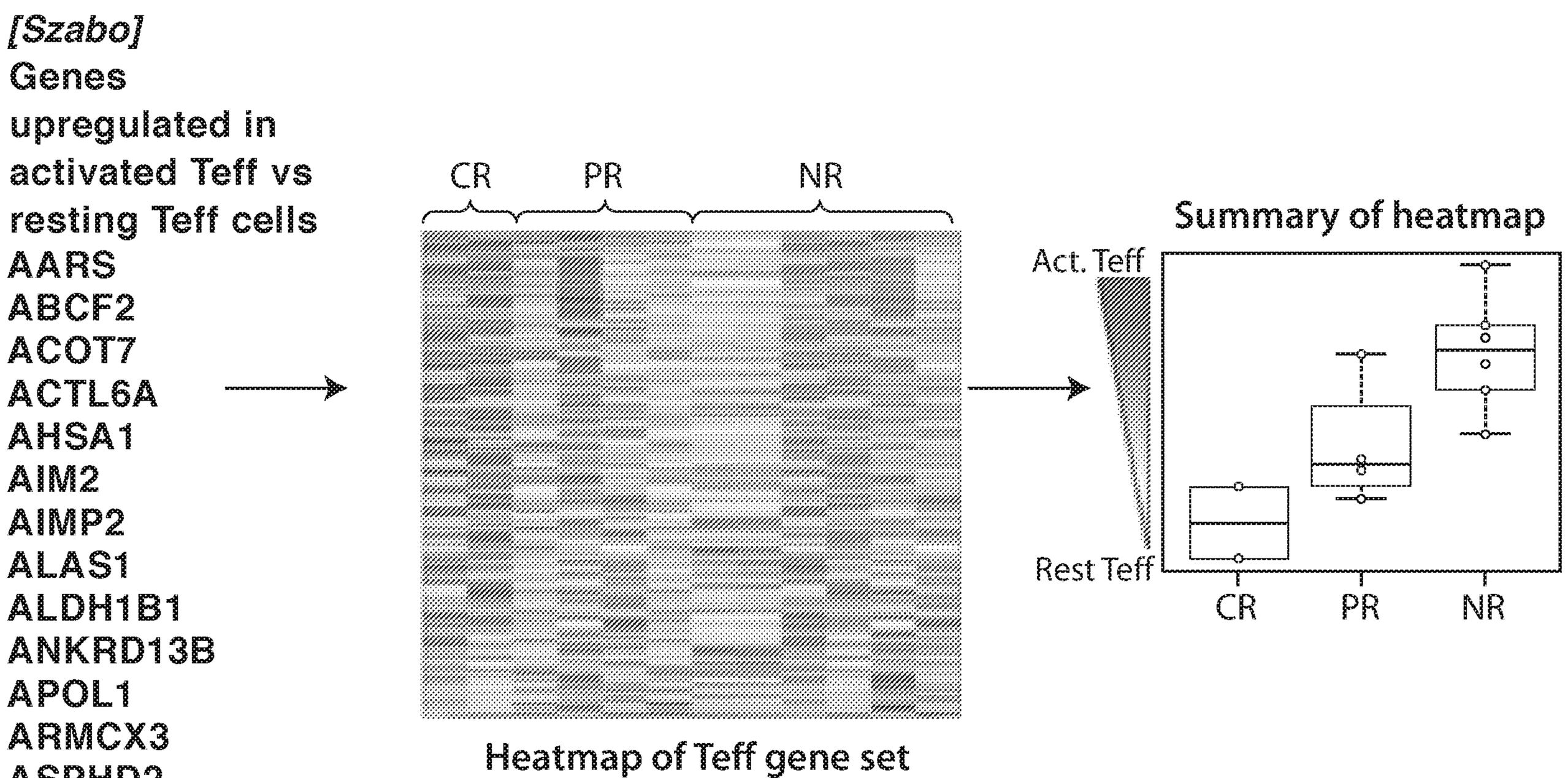


FIG. 16

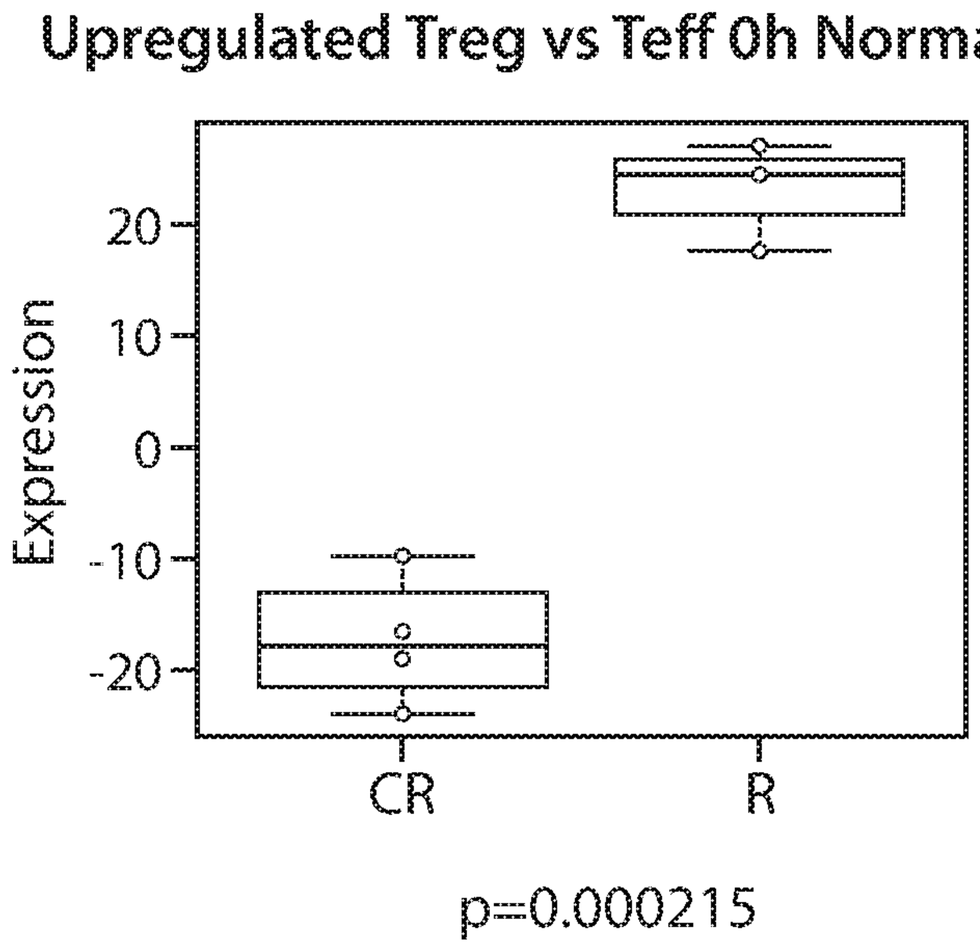

Upregulated Treg vs Teff 0h Normalized
Expression
20
10
0
-10
-20
CR
R
p=0.000215

FIG. 17

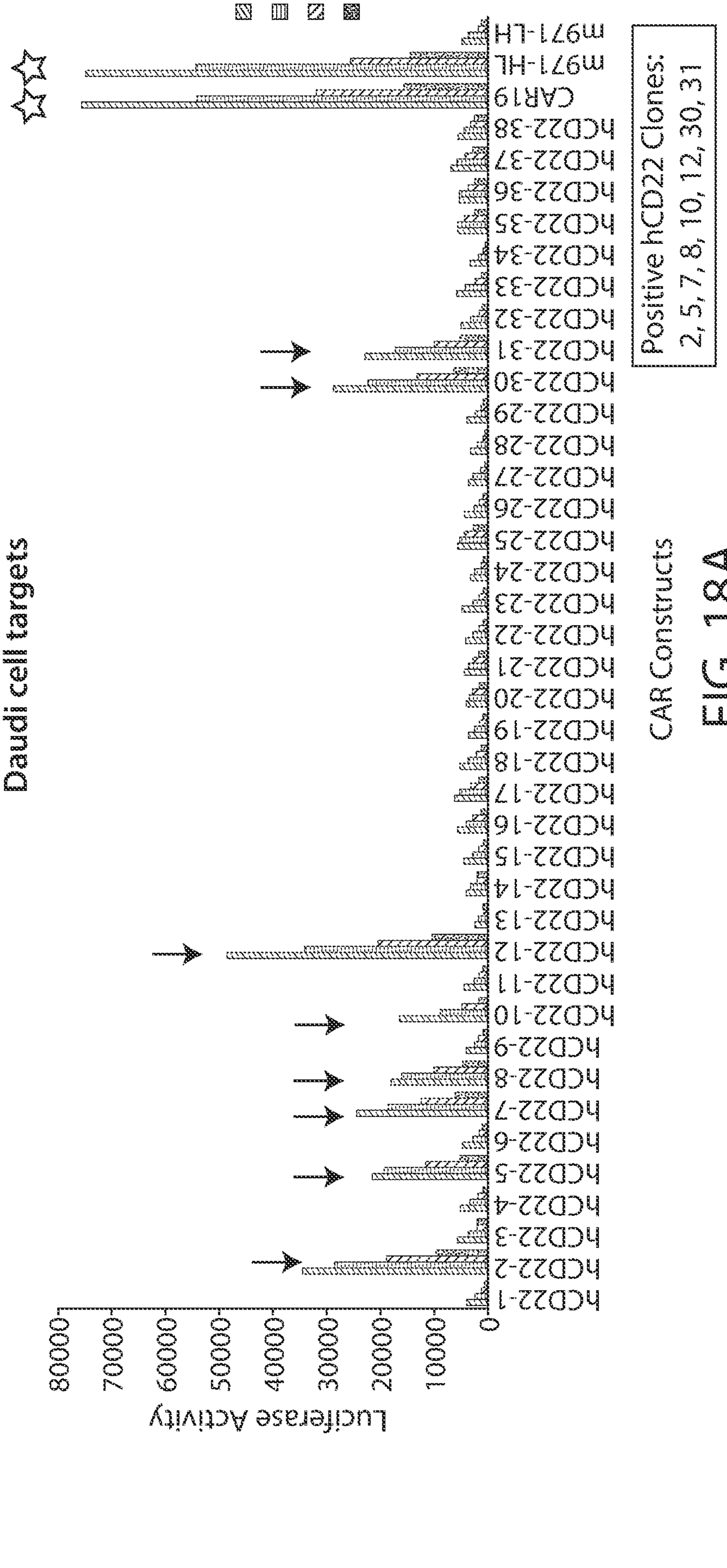

4:1
2:1
1:1
0.5:1
m971-LH
m971-HL
CAR19
hCD22-38
hCD22-37
hCD22-36
hCD22-35
hCD22-34
hCD22-33
hCD22-32
hCD22-31
hCD22-30
hCD22-29
hCD22-28
hCD22-27
hCD22-26
hCD22-25
hCD22-24
hCD22-23
hCD22-22
hCD22-21
hCD22-20
hCD22-19
hCD22-18
hCD22-17
hCD22-16
hCD22-15
hCD22-14
hCD22-13
hCD22-12
hCD22-11
hCD22-10
hCD22-9
hCD22-8
hCD22-7
hCD22-6
hCD22-5
hCD22-4
hCD22-3
hCD22-2
hCD22-1
Positive hCD22 Clones:
2, 5, 7, 8, 10, 12, 30, 31
CAR Constructs
Daudi cell targets
Luciferase Activity
80000
70000
60000
50000
40000
30000
20000
10000
0

FIG. 18A

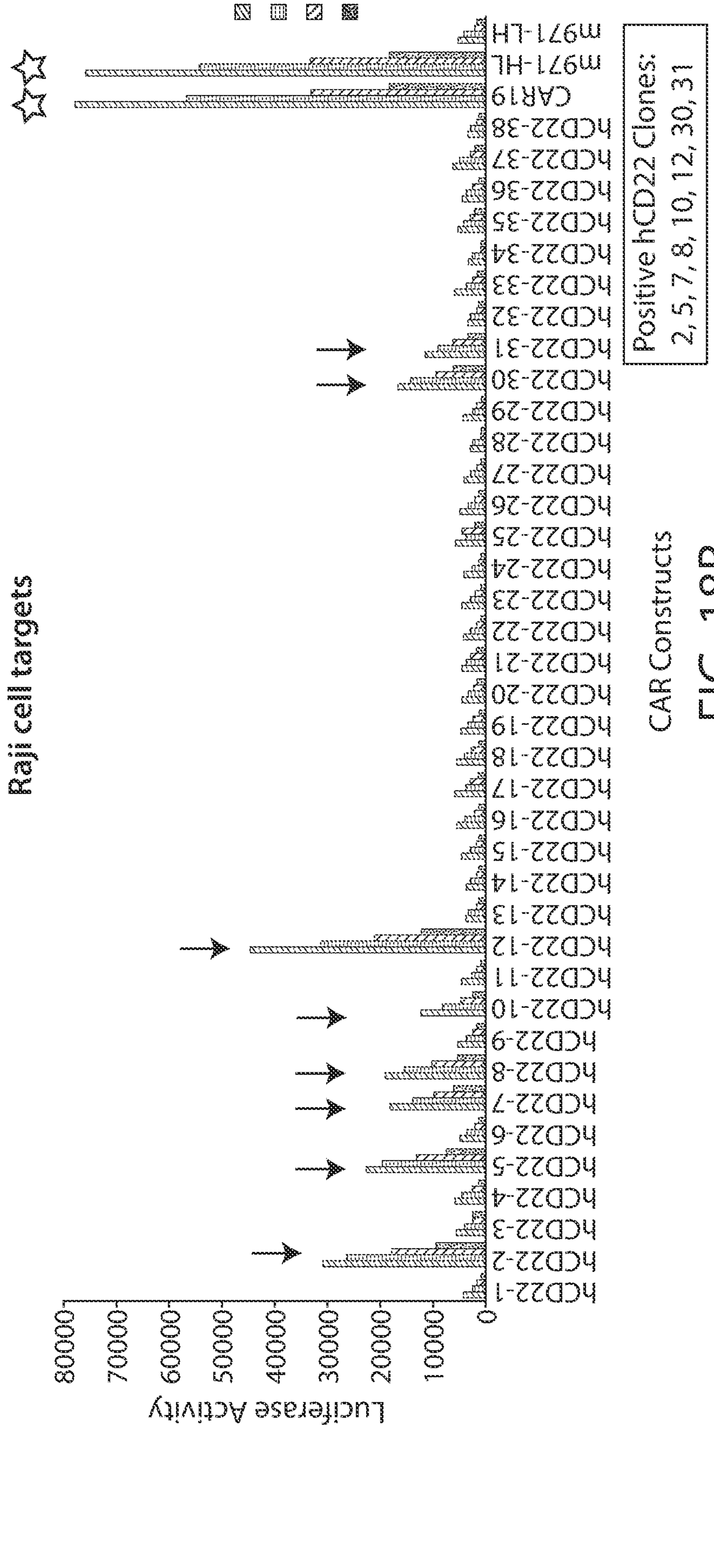

4:1
2:1
1:1
0.5:1
m971-LH
m971-HL
CAR19
hCD22-38
hCD22-37
hCD22-36
hCD22-35
hCD22-34
hCD22-33
hCD22-32
hCD22-31
hCD22-30
hCD22-29
hCD22-28
hCD22-27
hCD22-26
hCD22-25
hCD22-24
hCD22-23
hCD22-22
hCD22-21
hCD22-20
hCD22-19
hCD22-18
hCD22-17
hCD22-16
hCD22-15
hCD22-14
hCD22-13
hCD22-12
hCD22-11
hCD22-10
hCD22-9
hCD22-8
hCD22-7
hCD22-6
hCD22-5
hCD22-4
hCD22-3
hCD22-2
hCD22-1
Positive hCD22 Clones:
2, 5, 7, 8, 10, 12, 30, 31
CAR Constructs
Raji cell targets
Luciferase Activity
80000
70000
60000
50000
40000
30000
20000
10000
0

FIG. 18B

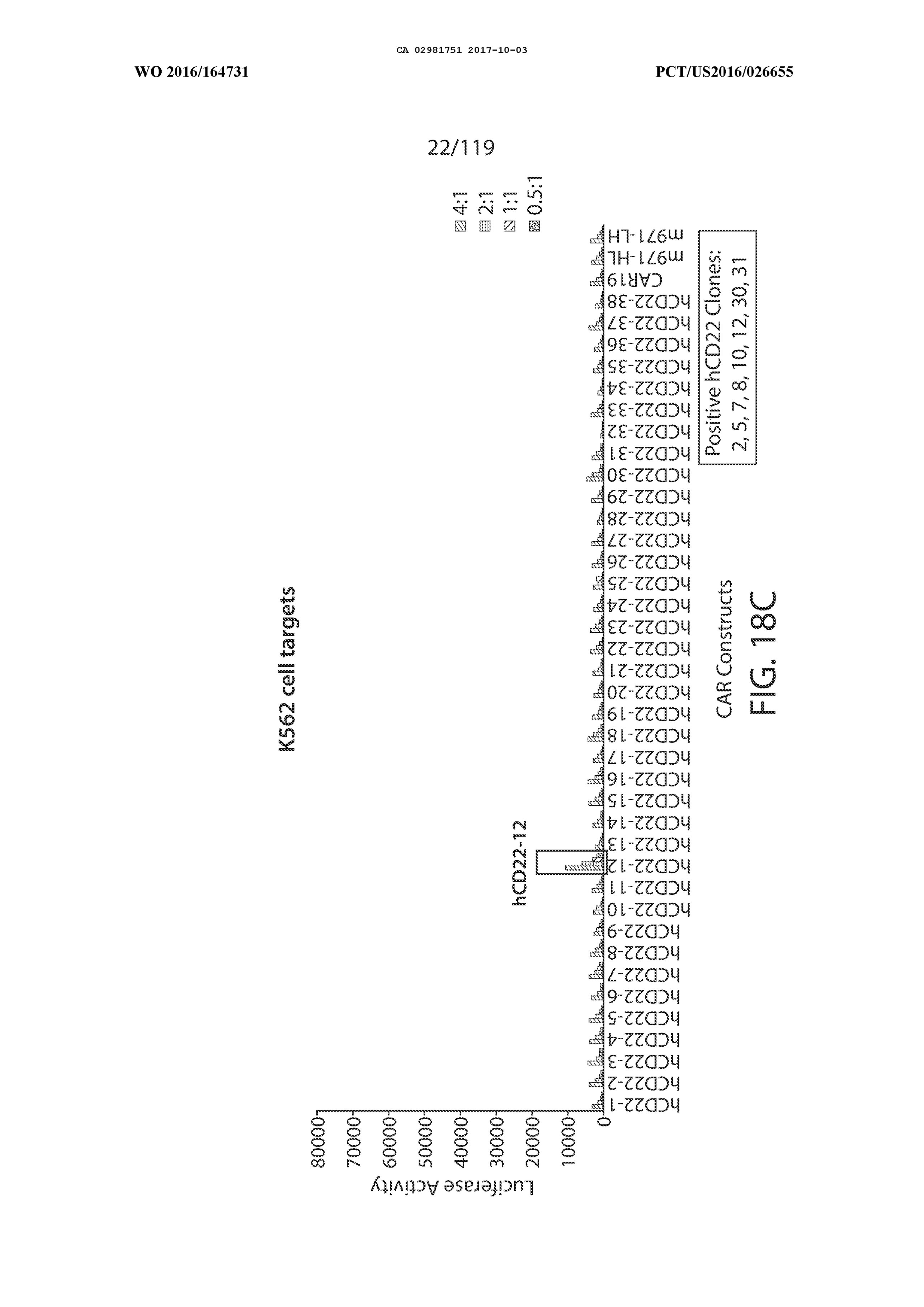

4:1
2:1
1:1
0.5:1
K562 cell targets
hCD22-12
Luciferase Activity
80000
70000
60000
50000
40000
30000
20000
10000
0
hCD22-1
hCD22-2
hCD22-3
hCD22-4
hCD22-5
hCD22-6
hCD22-7
hCD22-8
hCD22-9
hCD22-10
hCD22-11
hCD22-12
hCD22-13
hCD22-14
hCD22-15
hCD22-16
hCD22-17
hCD22-18
hCD22-19
hCD22-20
hCD22-21
hCD22-22
hCD22-23
hCD22-24
hCD22-25
hCD22-26
hCD22-27
hCD22-28
hCD22-29
hCD22-30
hCD22-31
hCD22-32
hCD22-33
hCD22-34
hCD22-35
hCD22-36
hCD22-37
hCD22-38
CAR19
m971-HL
m971-LH
CAR Constructs
Positive hCD22 clones:
2, 5, 7, 8, 10, 12, 30, 31

FIG. 18C

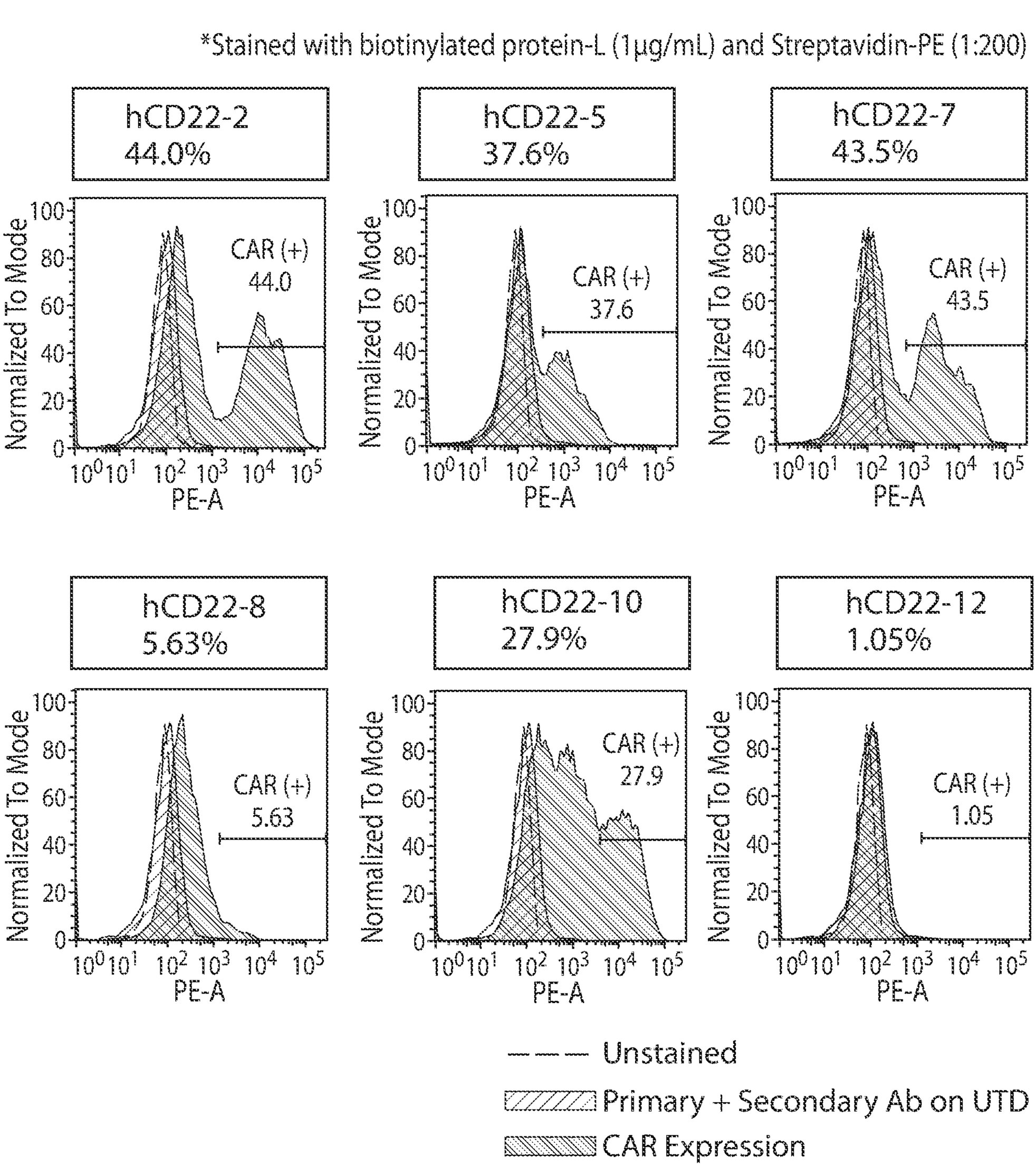

Protein L
*Stained with biotinylated protein-L (1µg/mL) and Streptavidin-PE (1:200)
hCD22-2
44.0%
hCD22-5
37.6%
hCD22-7
43.5%
hCD22-8
5.63%
hCD22-10
27.9%
hCD22-12
1.05%
Normalized To Mode
PE-A
CAR (+)
44.0
CAR (+)
37.6
CAR (+)
43.5
CAR (+)
5.63
CAR (+)
27.9
CAR (+)
1.05
Unstained
Primary + Secondary Ab on UTD
CAR Expression

FIG. 19-1

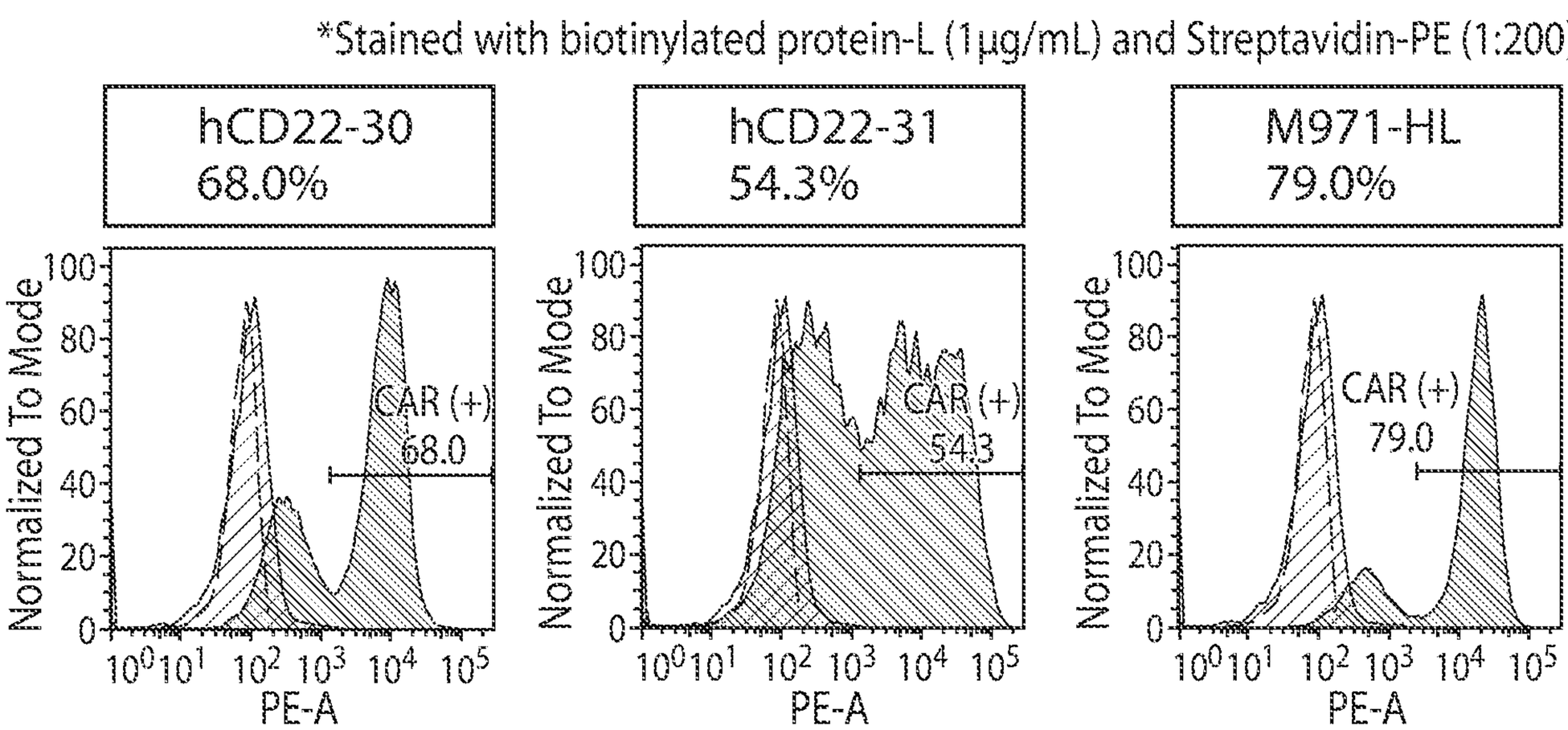

Protein L
*Stained with biotinylated protein-L (1µg/mL) and Streptavidin-PE (1:200)
hCD22-30
68.0%
hCD22-31
54.3%
M971-HL
79.0%
Normalized To Mode
CAR (+)
68.0
CAR (+)
54.3
CAR (+)
79.0
PE-A
M971-HL
69.8%
CAE19
69.9%
CAR (+)
69.8
CAR (+)
69.9
Unstained
Primary + Secondary Ab on UTD
CAR Expression

FIG. 19-2

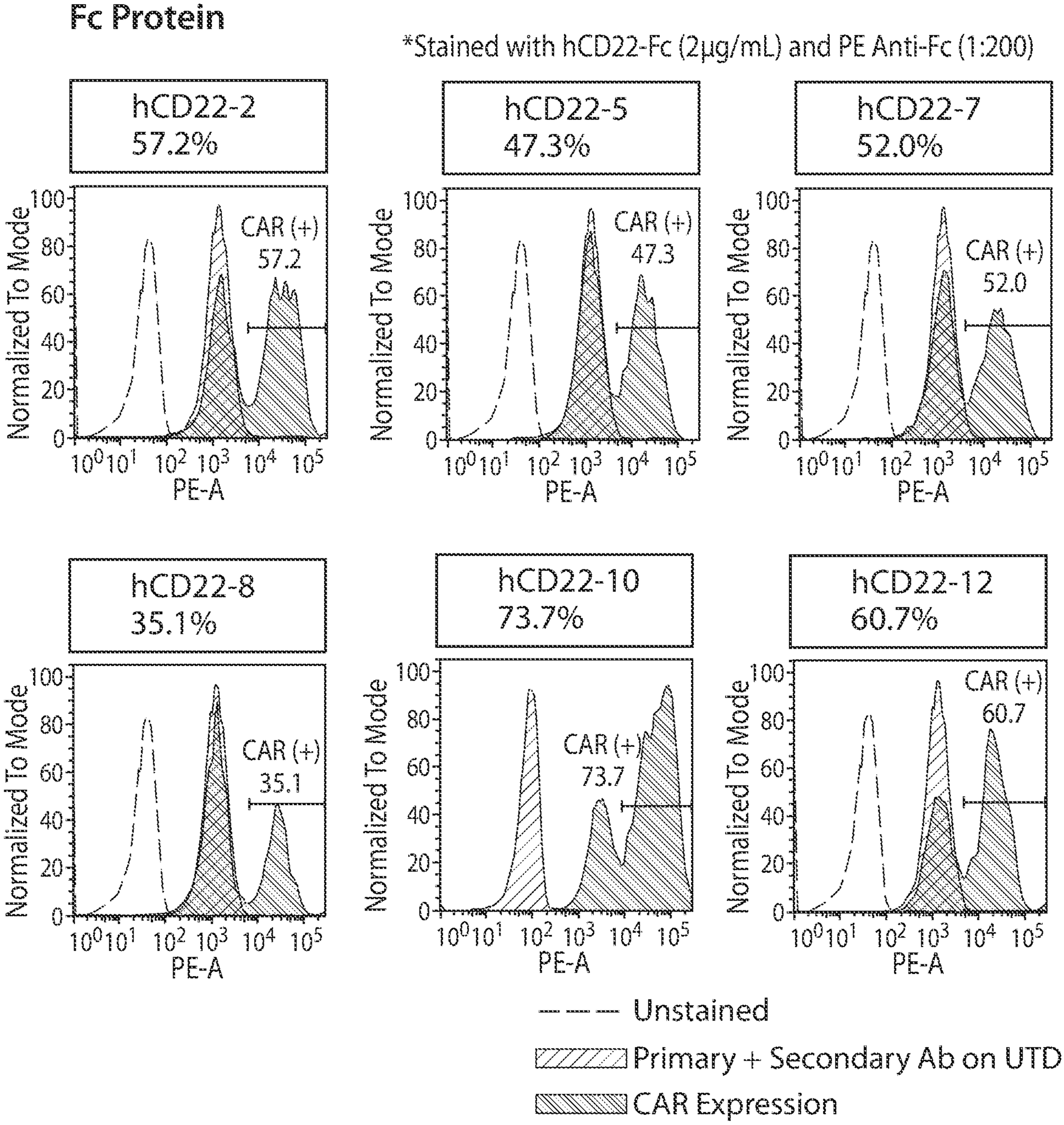

Fc Protein
*Stained with hCD22-Fc (2µg/mL) and PE Anti-Fc (1:200)
hCD22-2
57.2%
hCD22-5
47.3%
hCD22-7
52.0%
hCD22-8
35.1%
hCD22-10
73.7%
hCD22-12
60.7%
Normalized To Mode
PE-A
CAR (+)
57.2
CAR (+)
47.3
CAR (+)
52.0
CAR (+)
35.1
CAR (+)
73.7
CAR (+)
60.7
Unstained
Primary + Secondary Ab on UTD
CAR Expression

FIG. 19-3

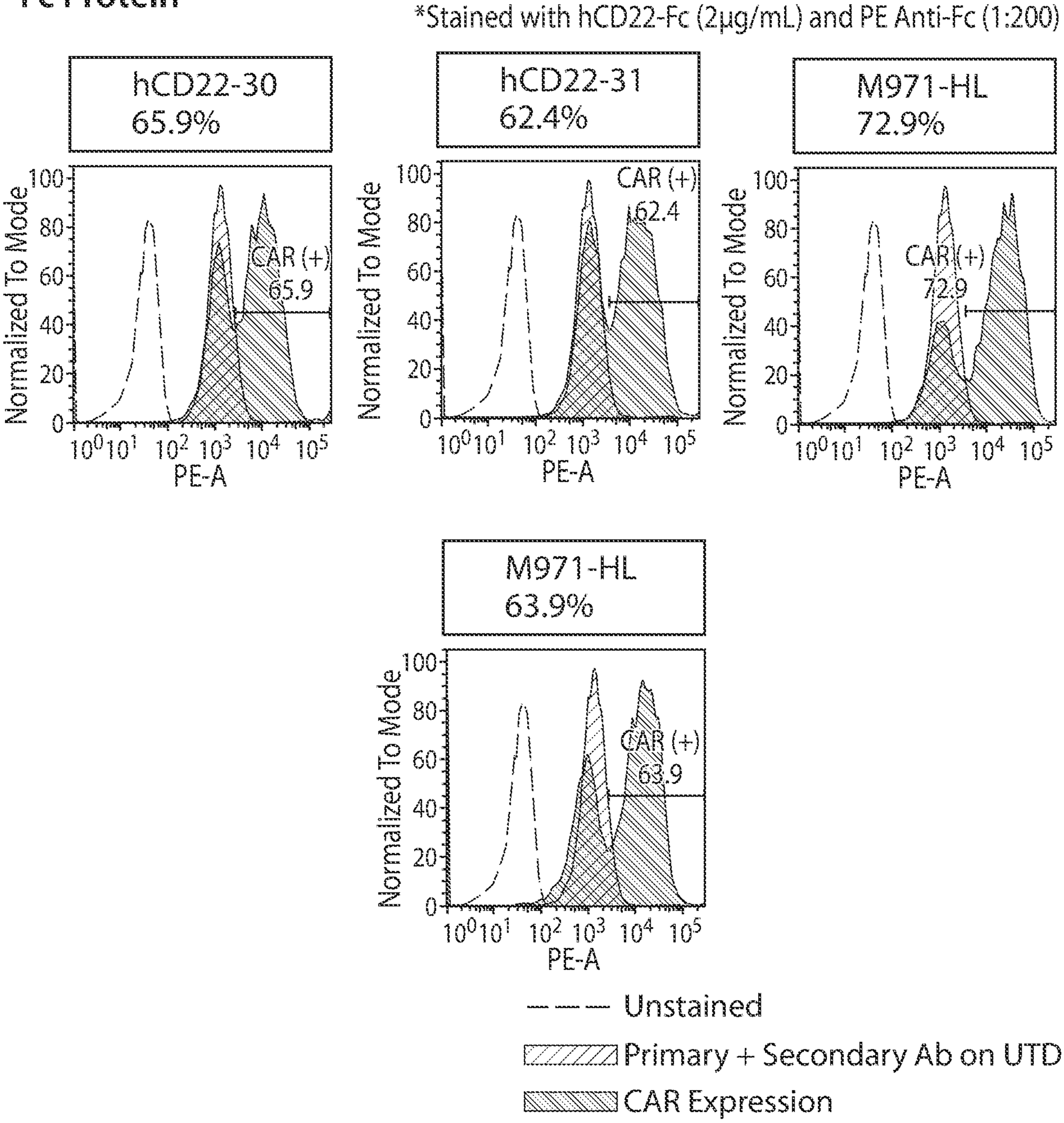

Fc Protein
*Stained with hCD22-Fc (2µg/mL) and PE Anti-Fc (1:200)
hCD22-30
65.9%
hCD22-31
62.4%
M971-HL
72.9%
M971-HL
63.9%
Normalized To Mode
PE-A
CAR (+)
65.9
CAR (+)
62.4
CAR (+)
72.9
CAR (+)
63.9
Unstained
Primary + Secondary Ab on UTD
CAR Expression

FIG. 19-4

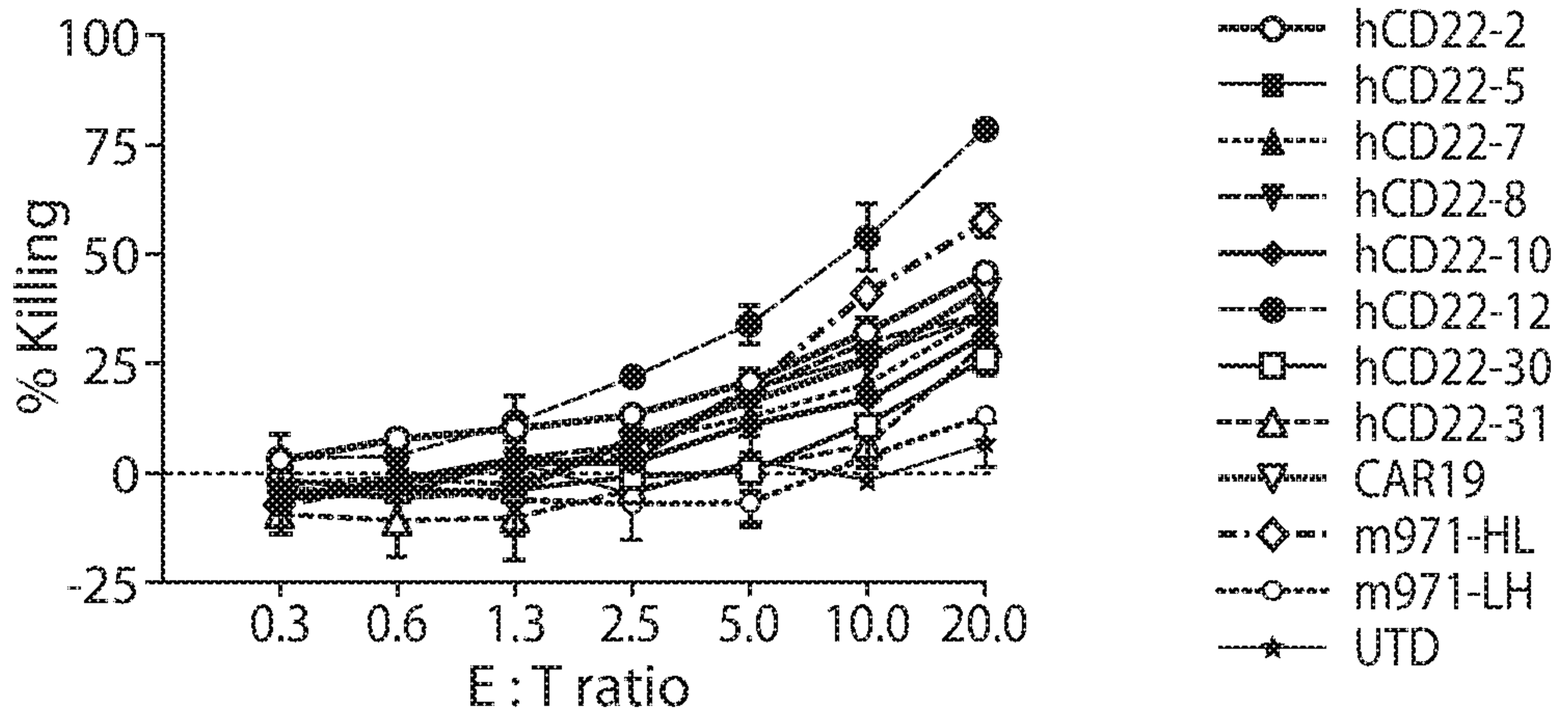


FIG. 20A

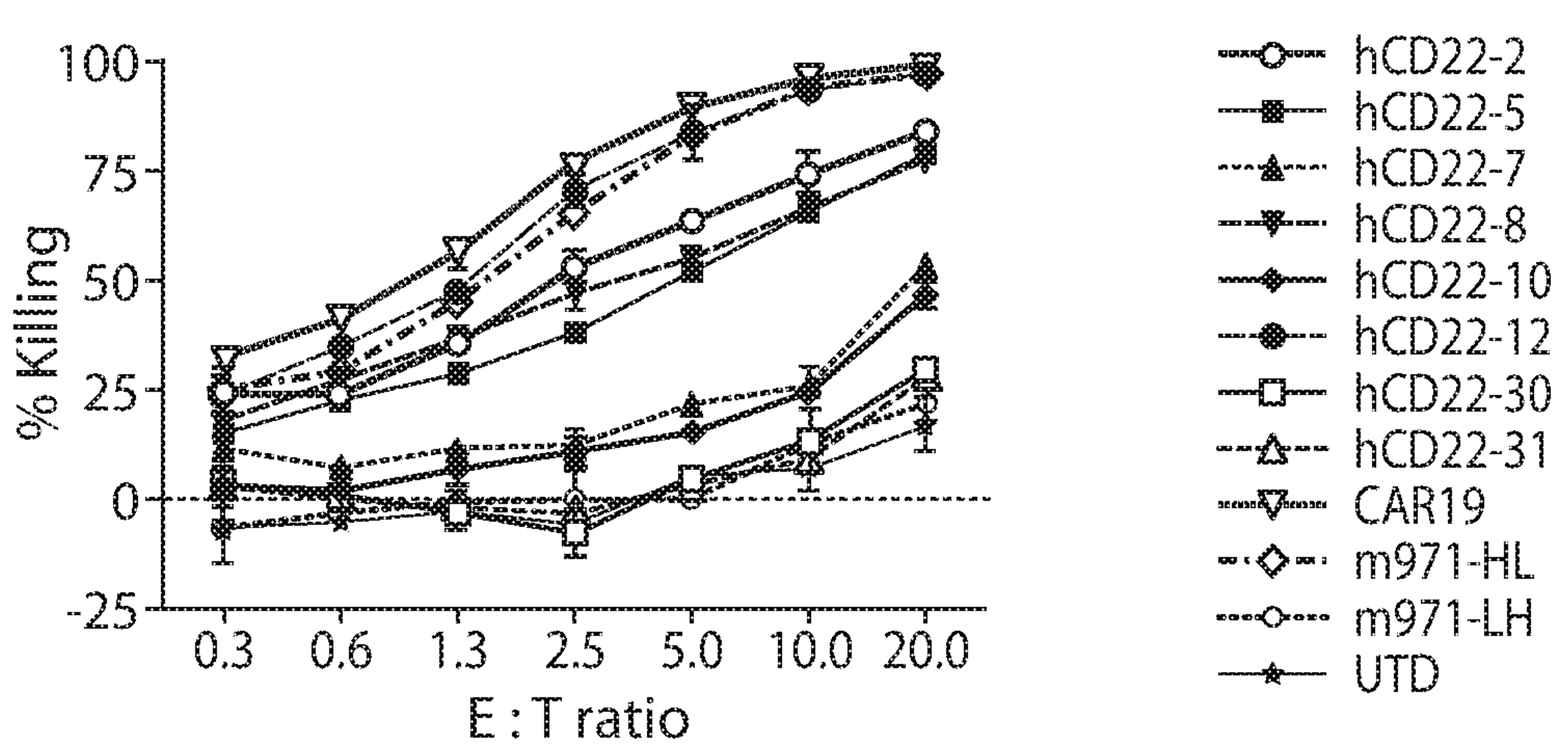


FIG. 20B

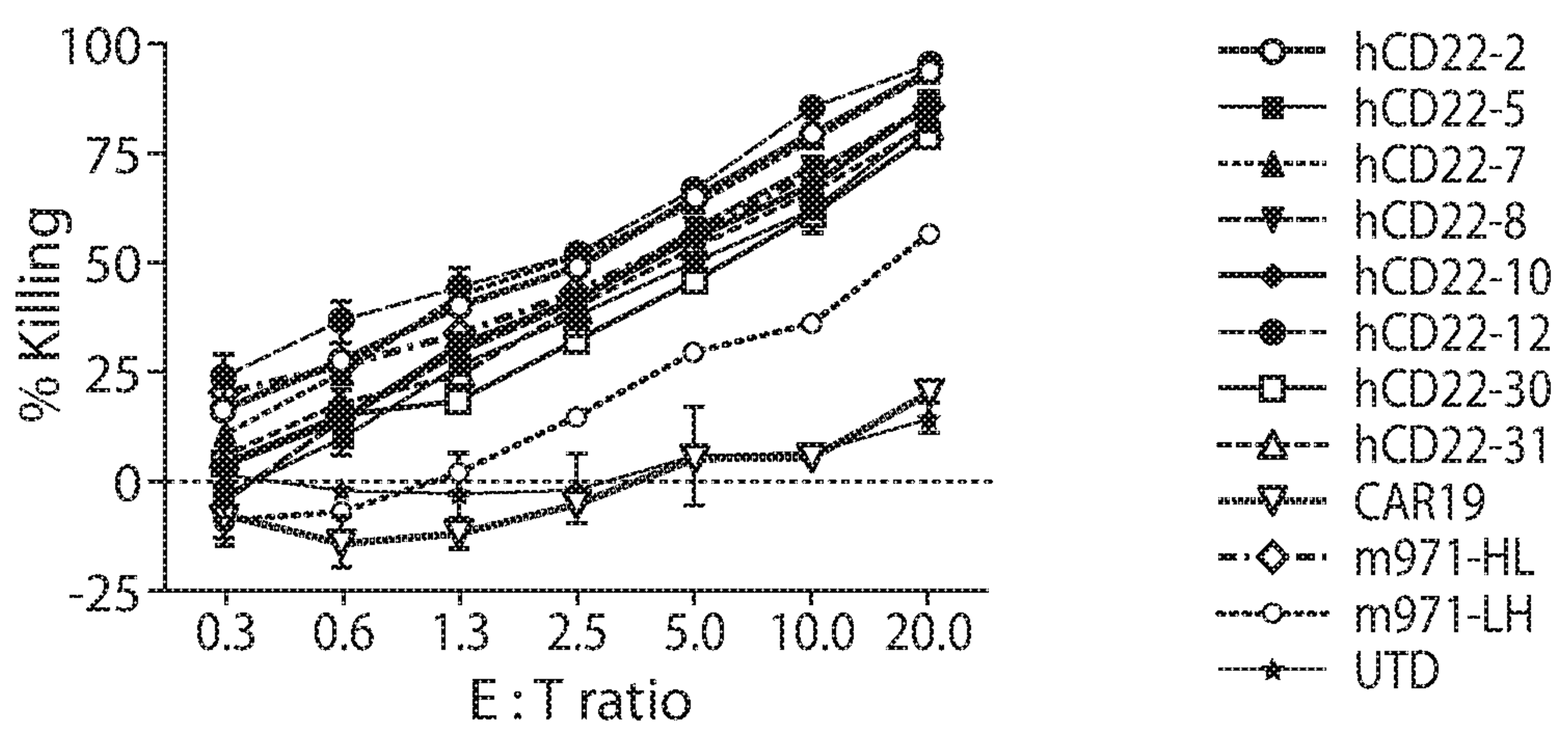


FIG. 20C

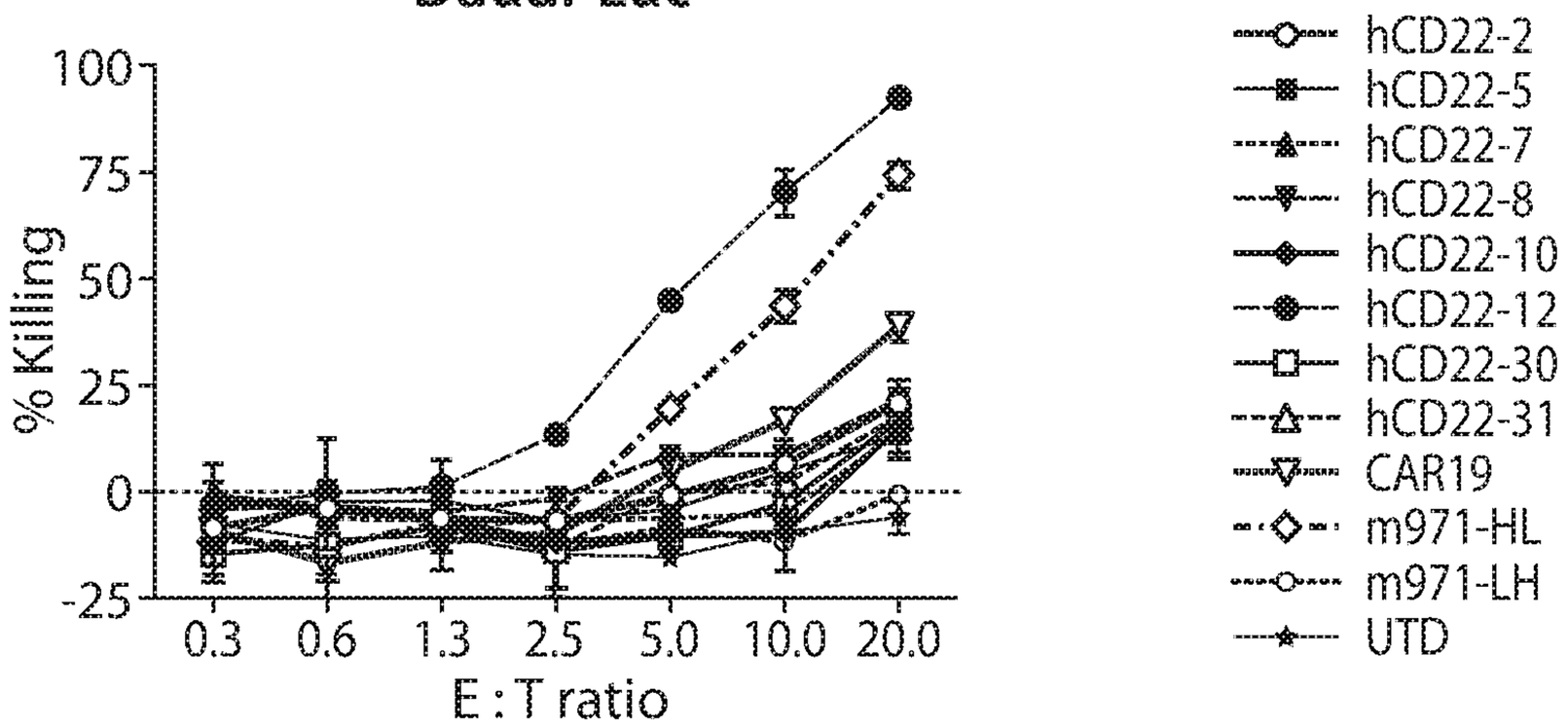


FIG. 20D

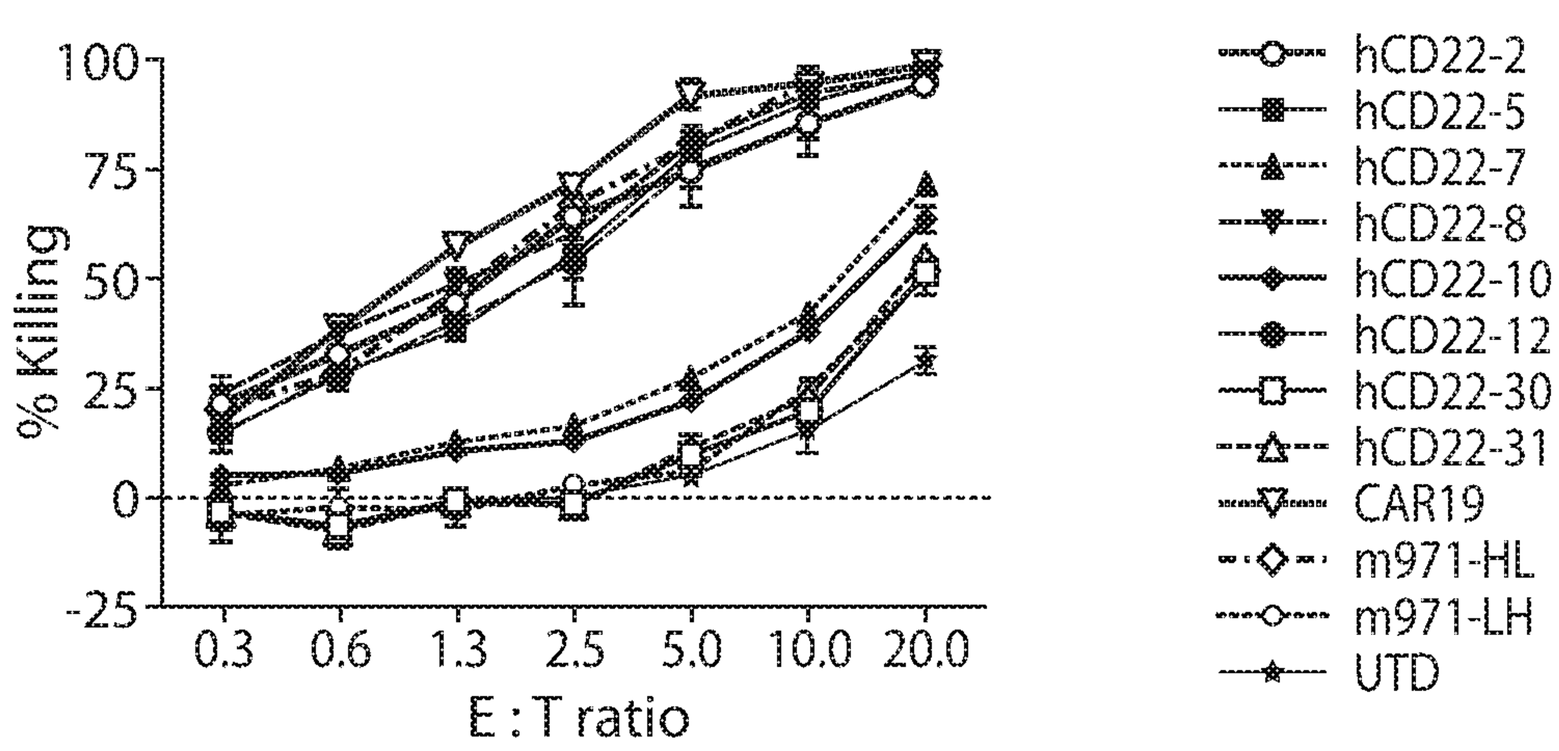


FIG. 20E

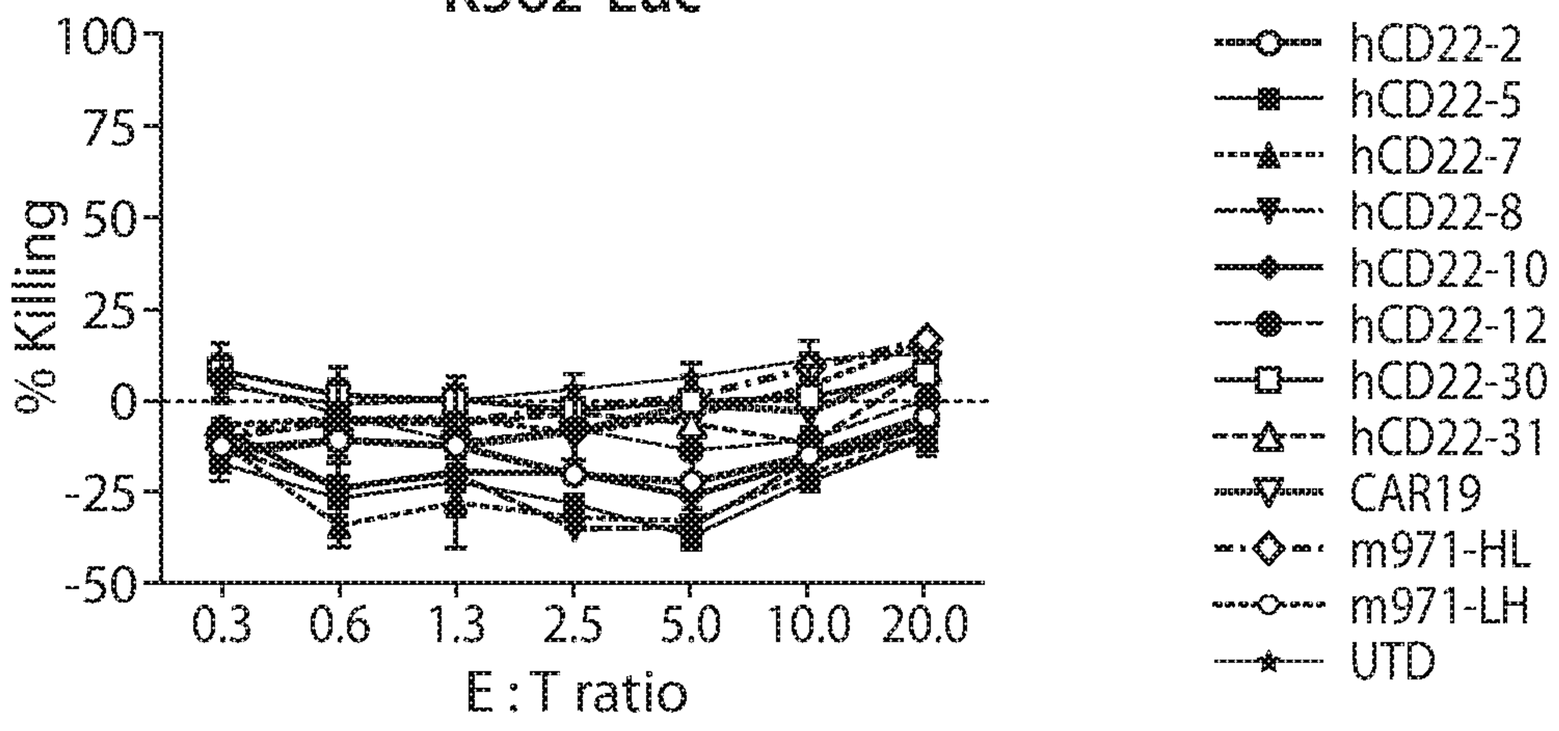


FIG. 20F

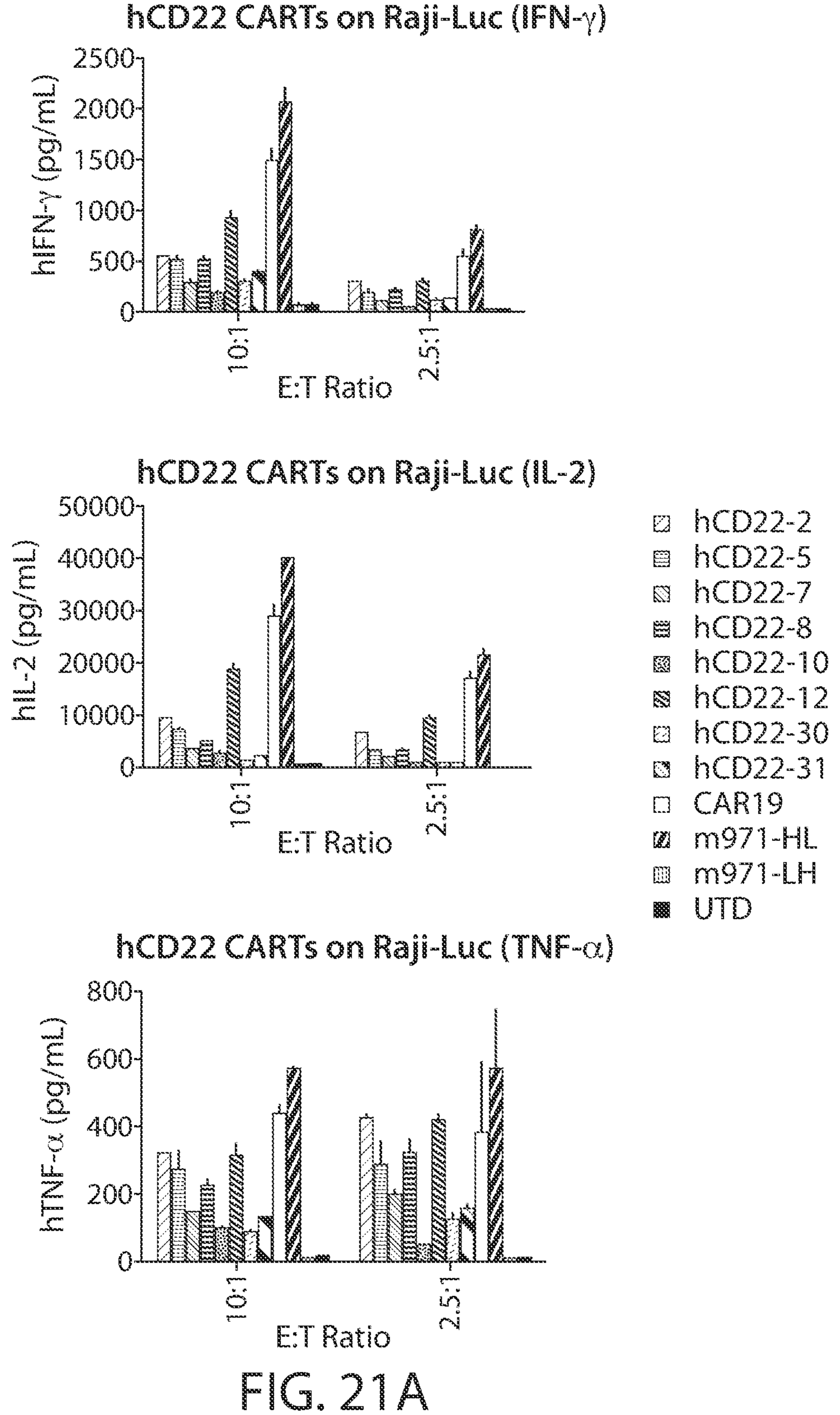

hCD22 CARTs on Raji-Luc (IFN-γ)
hIFN-γ (pg/mL)
2500
2000
1500
1000
500
0
10:1
2.5:1
E:T Ratio
hCD22 CARTs on Raji-Luc (IL-2)
hIL-2 (pg/mL)
50000
40000
30000
20000
10000
0
10:1
2.5:1
E:T Ratio
hCD22-2
hCD22-5
hCD22-7
hCD22-8
hCD22-10
hCD22-12
hCD22-30
hCD22-31
CAR19
m971-HL
m971-LH
UTD
hCD22 CARTs on Raji-Luc (TNF-α)
hTNF-α (pg/mL)
800
600
400
200
0
10:1
2.5:1
E:T Ratio

FIG. 21A

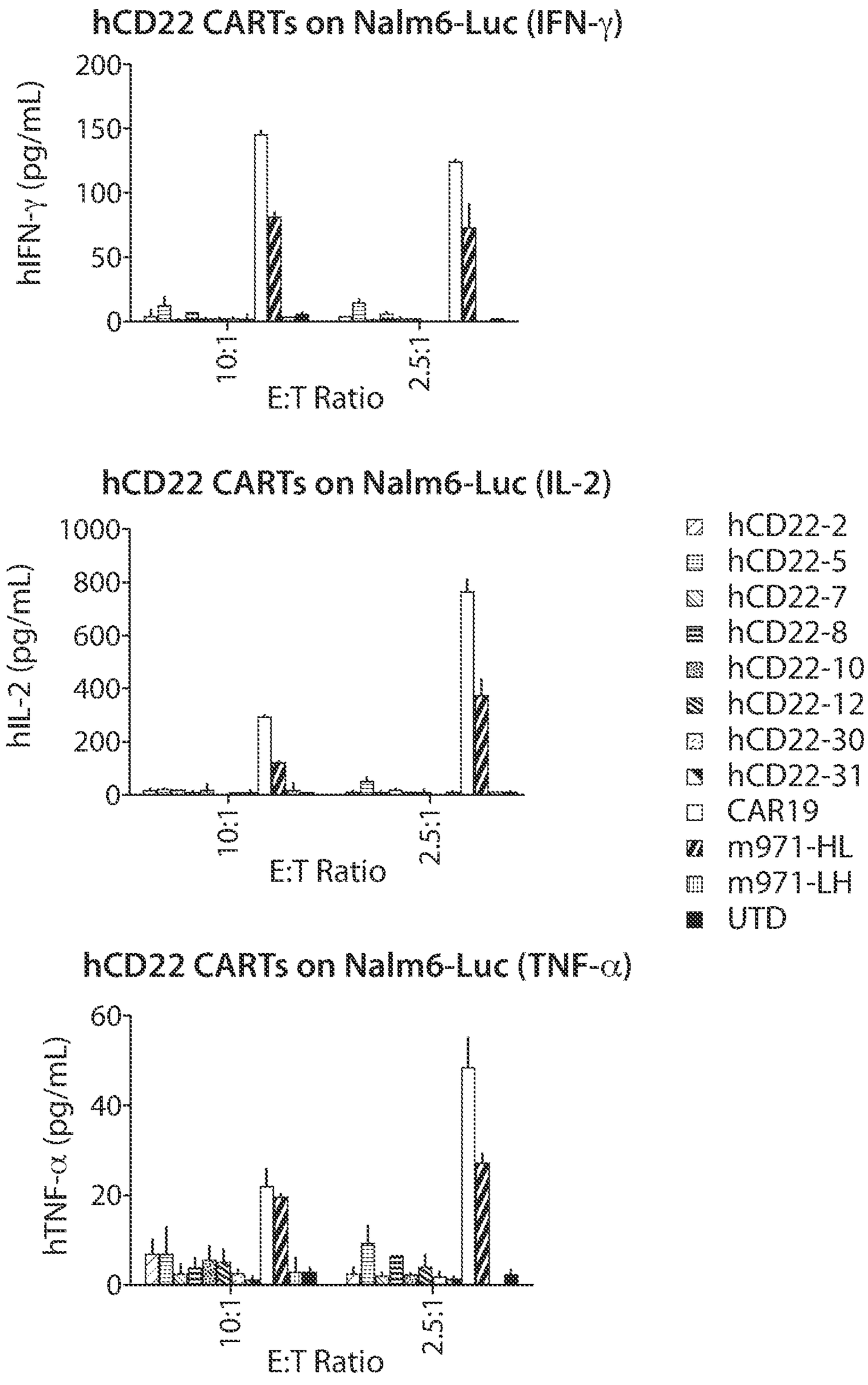
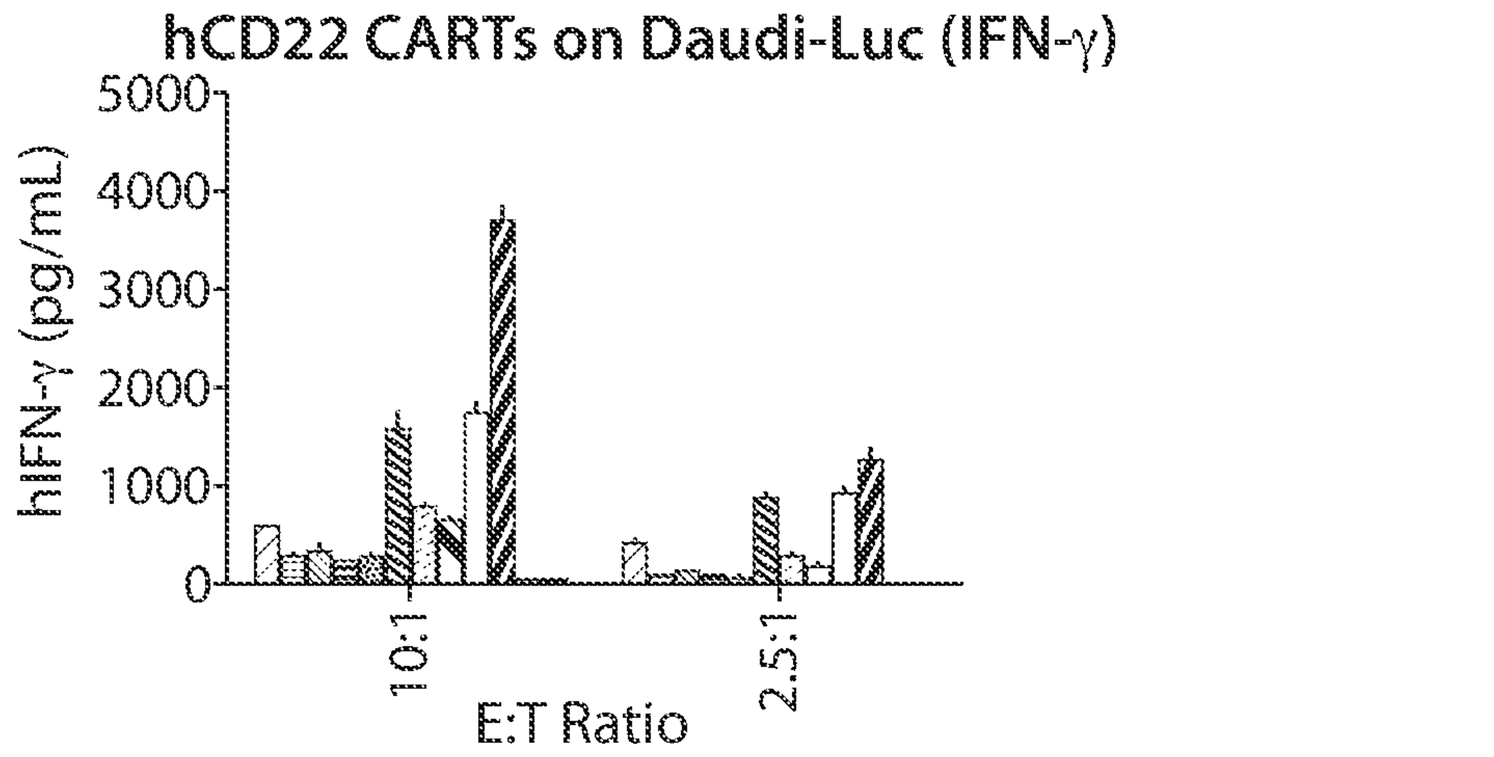
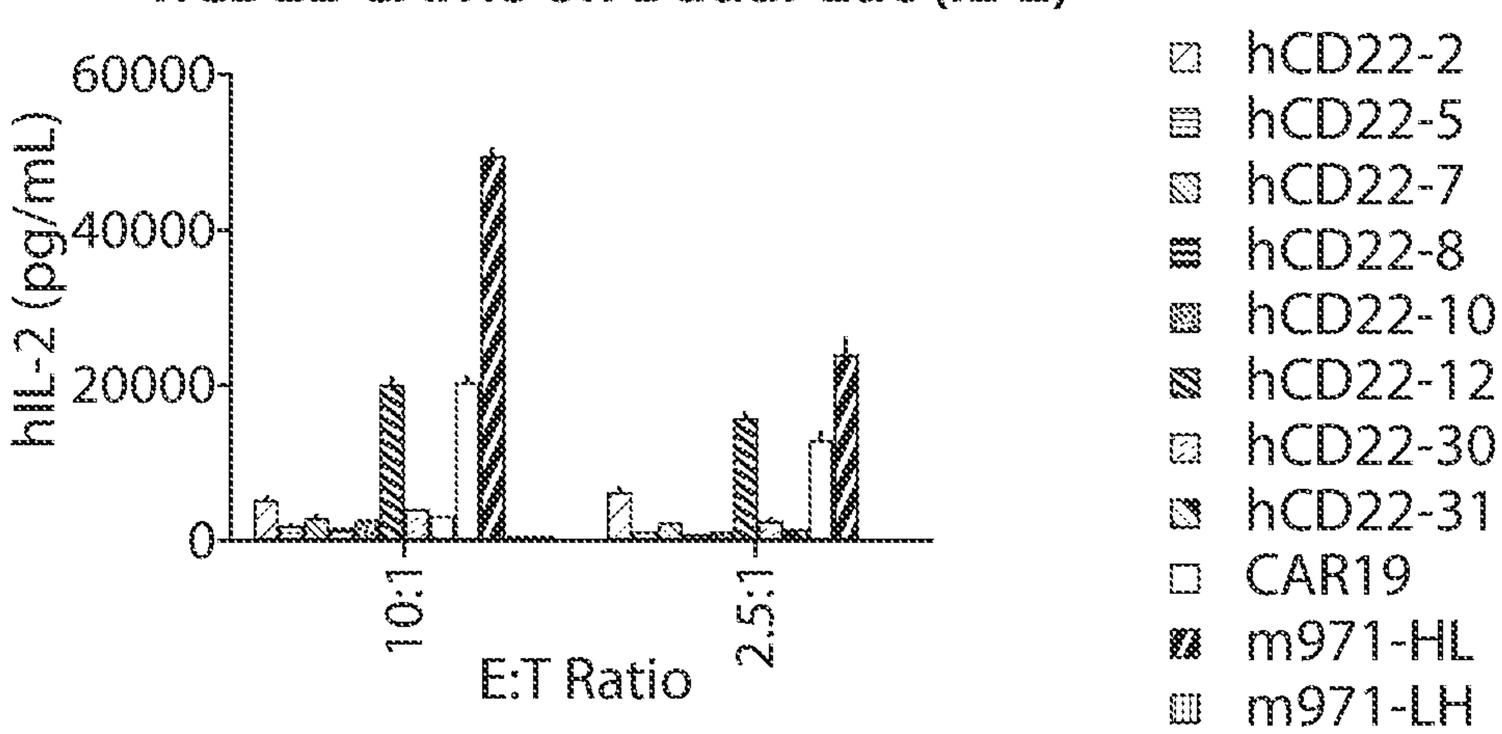

hCD22 CARTs on Nalm6-Luc (IFN-γ)
hIFN-γ (pg/mL)
200
150
100
50
0
10:1
2.5:1
E:T Ratio
hCD22 CARTs on Nalm6-Luc (IL-2)
hIL-2 (pg/mL)
1000
800
600
400
200
0
10:1
2.5:1
E:T Ratio
hCD22-2
hCD22-5
hCD22-7
hCD22-8
hCD22-10
hCD22-12
hCD22-30
hCD22-31
CAR19
m971-HL
m971-LH
UTD
hCD22 CARTs on Nalm6-Luc (TNF-α)
hTNF-α (pg/mL)
60
40
20
0
10:1
2.5:1
E:T Ratio

FIG. 21B


hCD22 CARTs on Daudi-Luc (IFN-γ)
hIFN-γ (pg/mL)
5000
4000
3000
2000
1000
0
10:1
2.5:1
E:T Ratio


hCD22 CARTs on Daudi-Luc (IL-2)
hIL-2 (pg/mL)
60000
40000
20000
0
10:1
2.5:1
E:T Ratio
hCD22-2
hCD22-5
hCD22-7
hCD22-8
hCD22-10
hCD22-12
hCD22-30
hCD22-31
CAR19
m971-HL
m971-LH
UTD

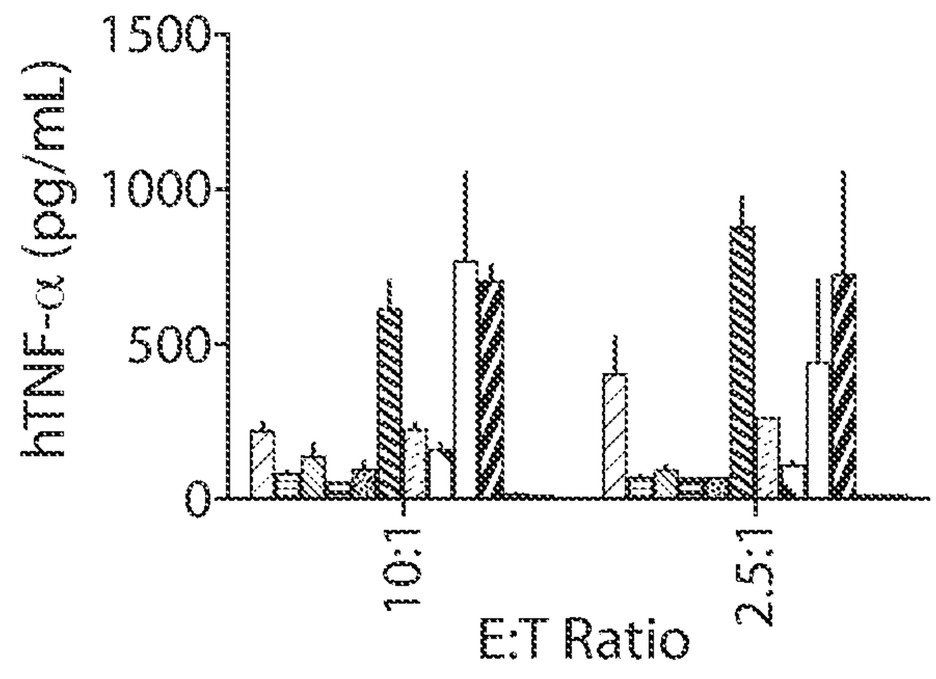
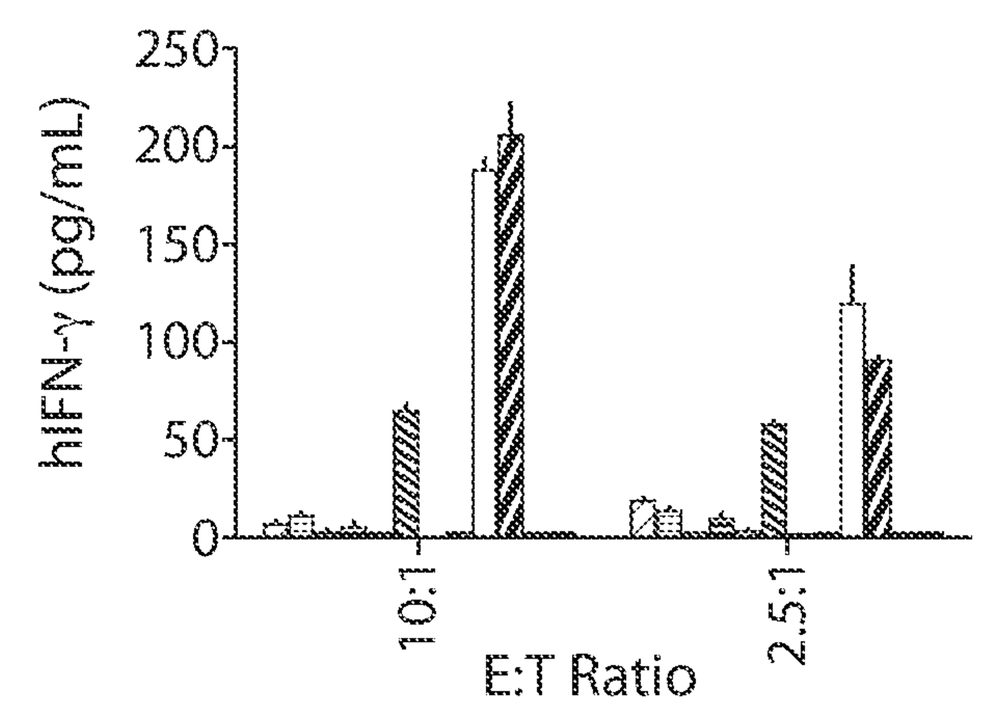
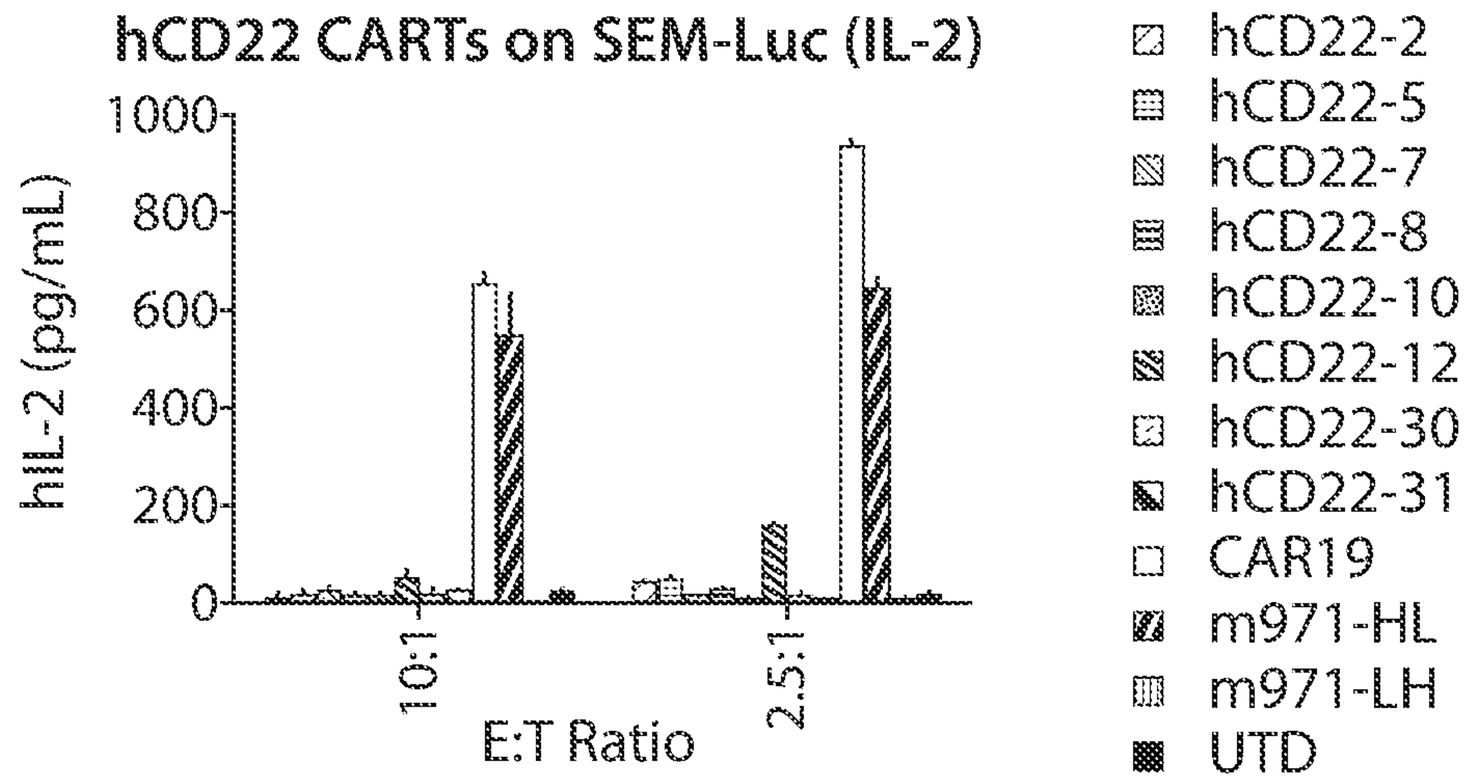

hCD22 CARTs on Daudi-Luc (TNF-α)
hTNF-α (pg/mL)
1500
1000
500
0
10:1
2.5:1
E:T Ratio

FIG. 21C


hCD22 CARTs on SEM-Luc (IFN-γ)
hIFN-γ (pg/mL)
250
200
150
100
50
0
10:1
2.5:1
E:T Ratio


hCD22 CARTs on SEM-Luc (IL-2)
hIL-2 (pg/mL)
1000
800
600
400
200
0
10:1
2.5:1
E:T Ratio
hCD22-2
hCD22-5
hCD22-7
hCD22-8
hCD22-10
hCD22-12
hCD22-30
hCD22-31
CAR19
m971-HL
m971-LH
UTD

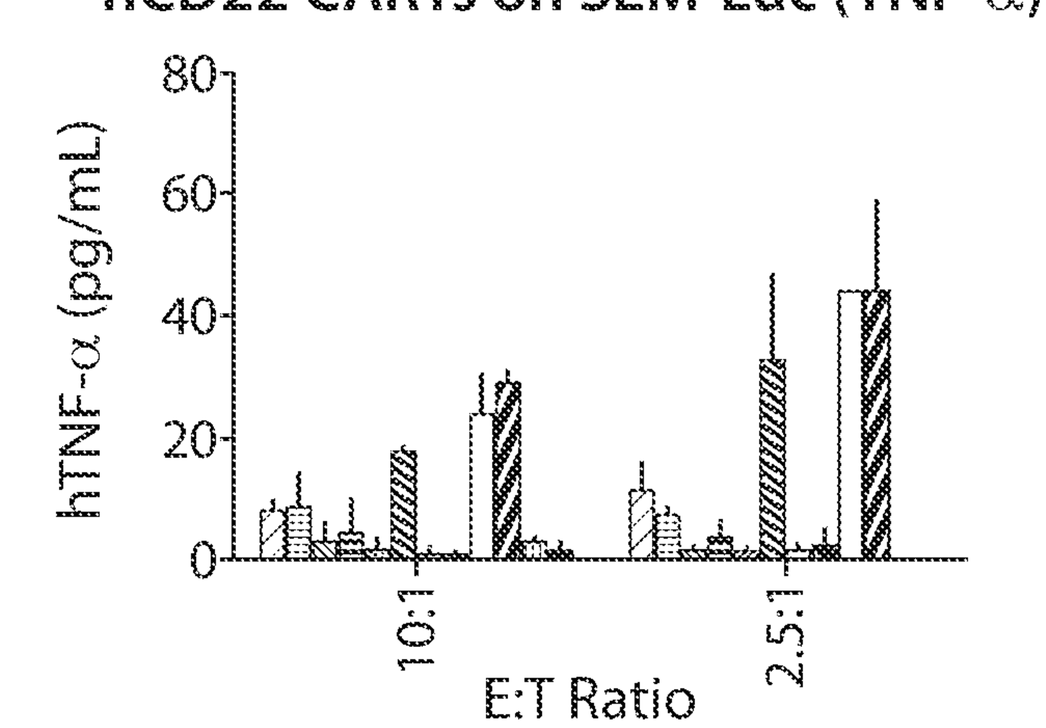

hCD22 CARTs on SEM-Luc (TNF-α)
hTNF-α (pg/mL)
80
60
40
20
0
10:1
2.5:1
E:T Ratio

FIG. 21D

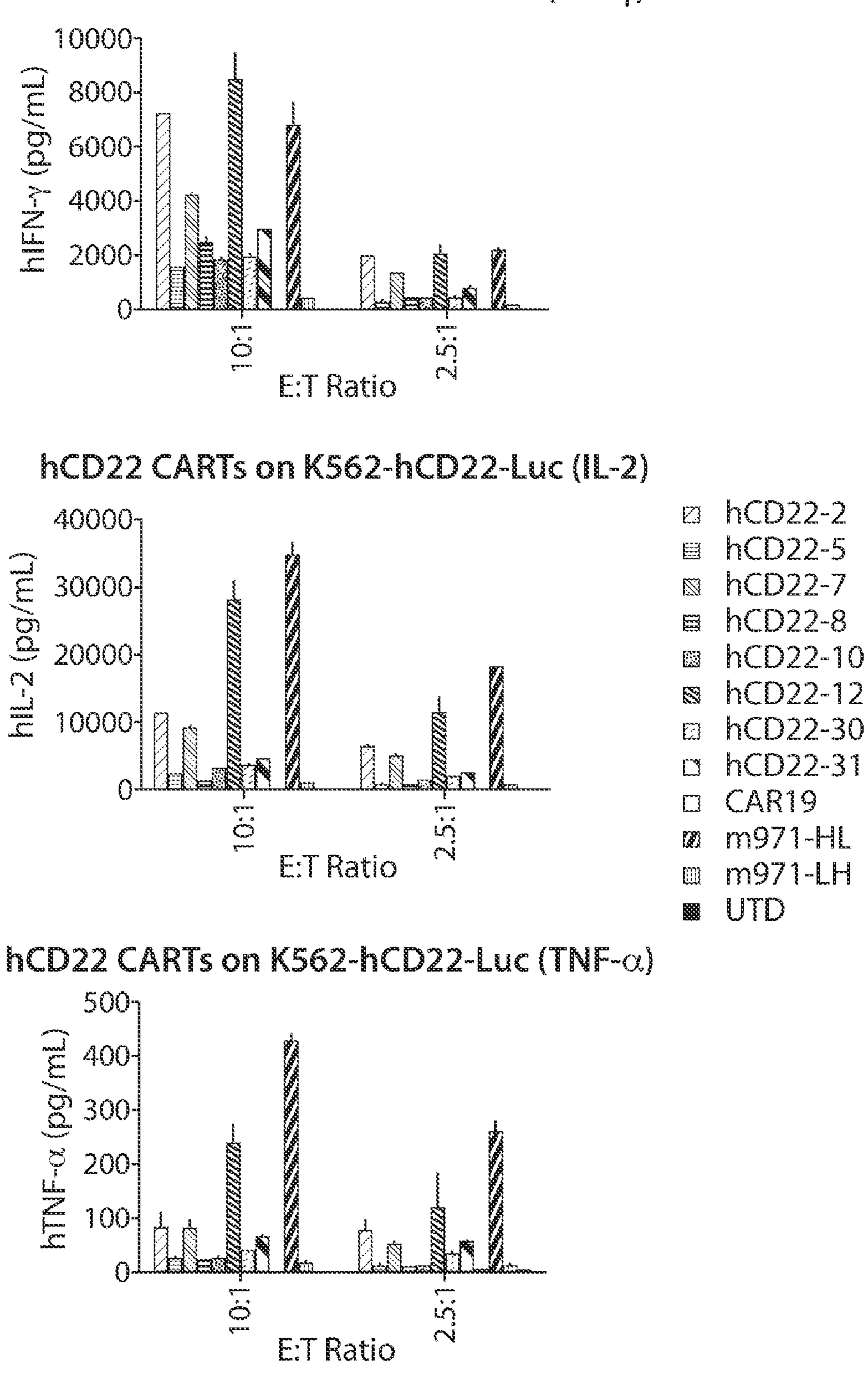

hCD22 CARTs on K562-hCD22-Luc (IFN-γ)
hIFN-γ (pg/mL)
10000
8000
6000
4000
2000
0
10:1
2.5:1
E:T Ratio
hCD22 CARTs on K562-hCD22-Luc (IL-2)
hIL-2 (pg/mL)
40000
30000
20000
10000
0
10:1
2.5:1
E:T Ratio
hCD22-2
hCD22-5
hCD22-7
hCD22-8
hCD22-10
hCD22-12
hCD22-30
hCD22-31
CAR19
m971-HL
m971-LH
UTD
hCD22 CARTs on K562-hCD22-Luc (TNF-α)
hTNF-α (pg/mL)
500
400
300
200
100
0
10:1
2.5:1
E:T Ratio

FIG. 21E

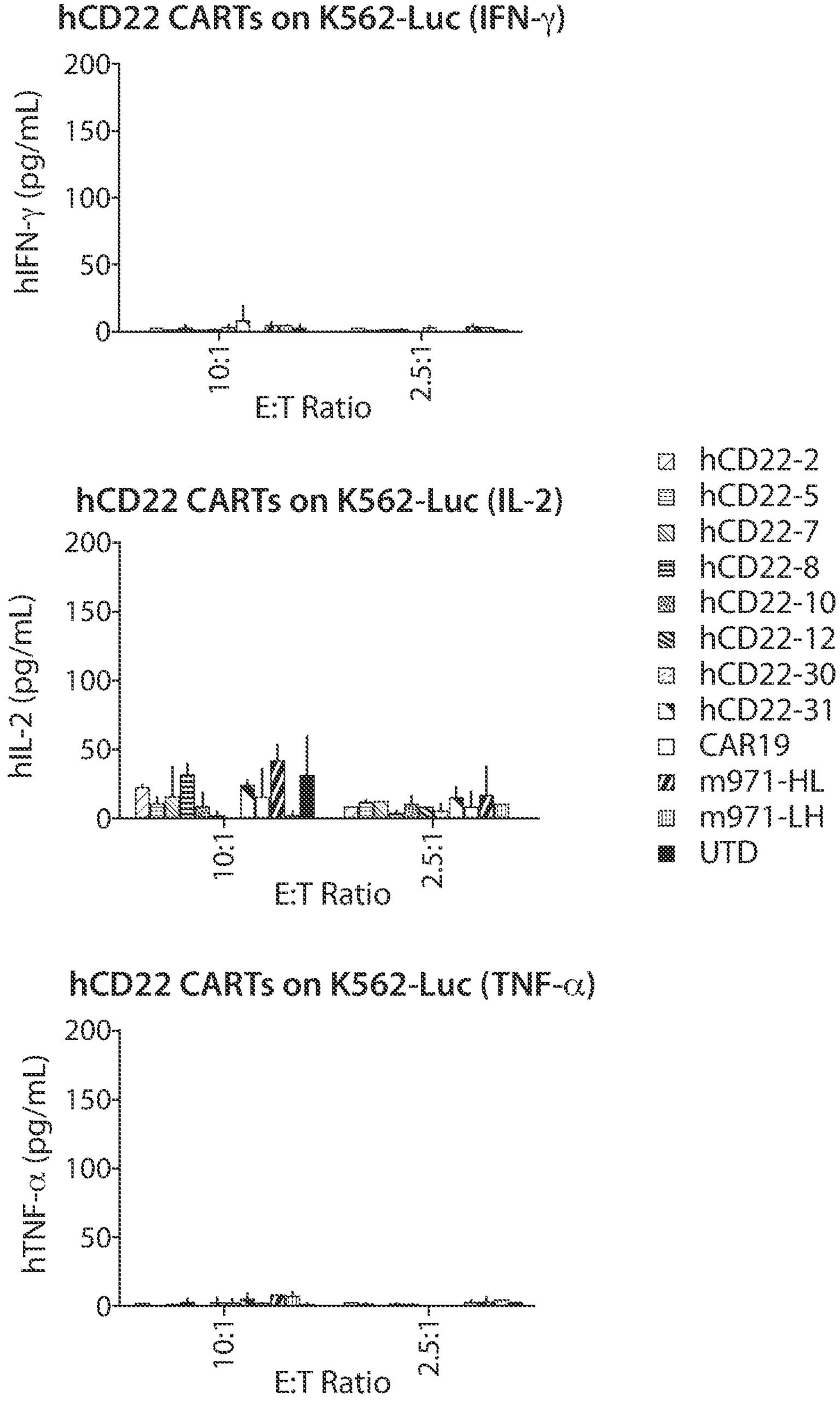

hCD22 CARTs on K562-Luc (IFN-γ)
hIFN-γ (pg/mL)
200
150
100
50
0
10:1
2.5:1
E:T Ratio
hCD22 CARTs on K562-Luc (IL-2)
hIL-2 (pg/mL)
200
150
100
50
0
10:1
2.5:1
E:T Ratio
hCD22-2
hCD22-5
hCD22-7
hCD22-8
hCD22-10
hCD22-12
hCD22-30
hCD22-31
CAR19
m971-HL
m971-LH
UTD
hCD22 CARTs on K562-Luc (TNF-α)
hTNF-α (pg/mL)
200
150
100
50
0
10:1
2.5:1
E:T Ratio

FIG. 21F

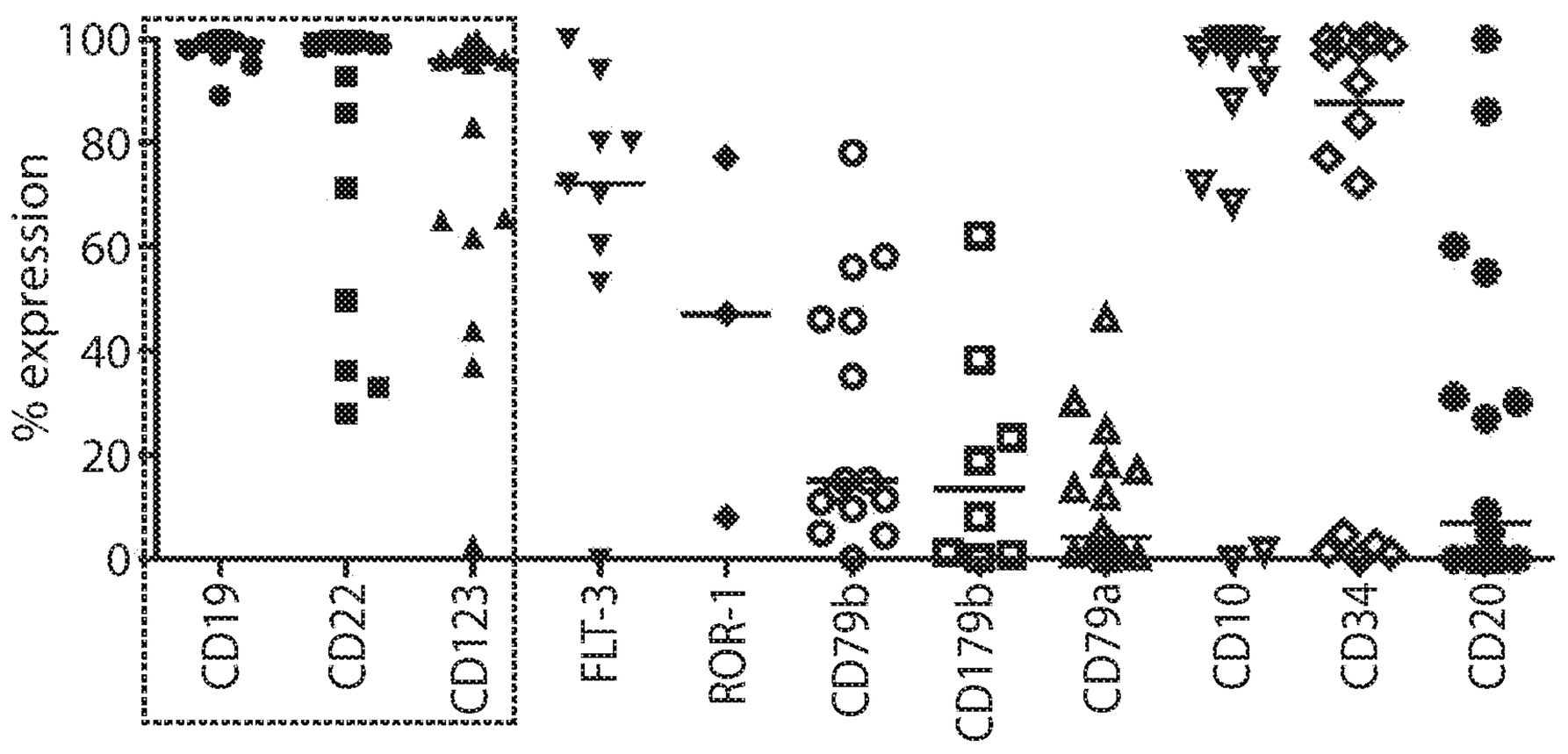

% expression
100
80
60
40
20
0
CD19
CD22
CD123
FLT-3
ROR-1
CD79b
CD179b
CD79a
CD10
CD34
CD20

FIG. 22

CA 02981751 2017-10-03

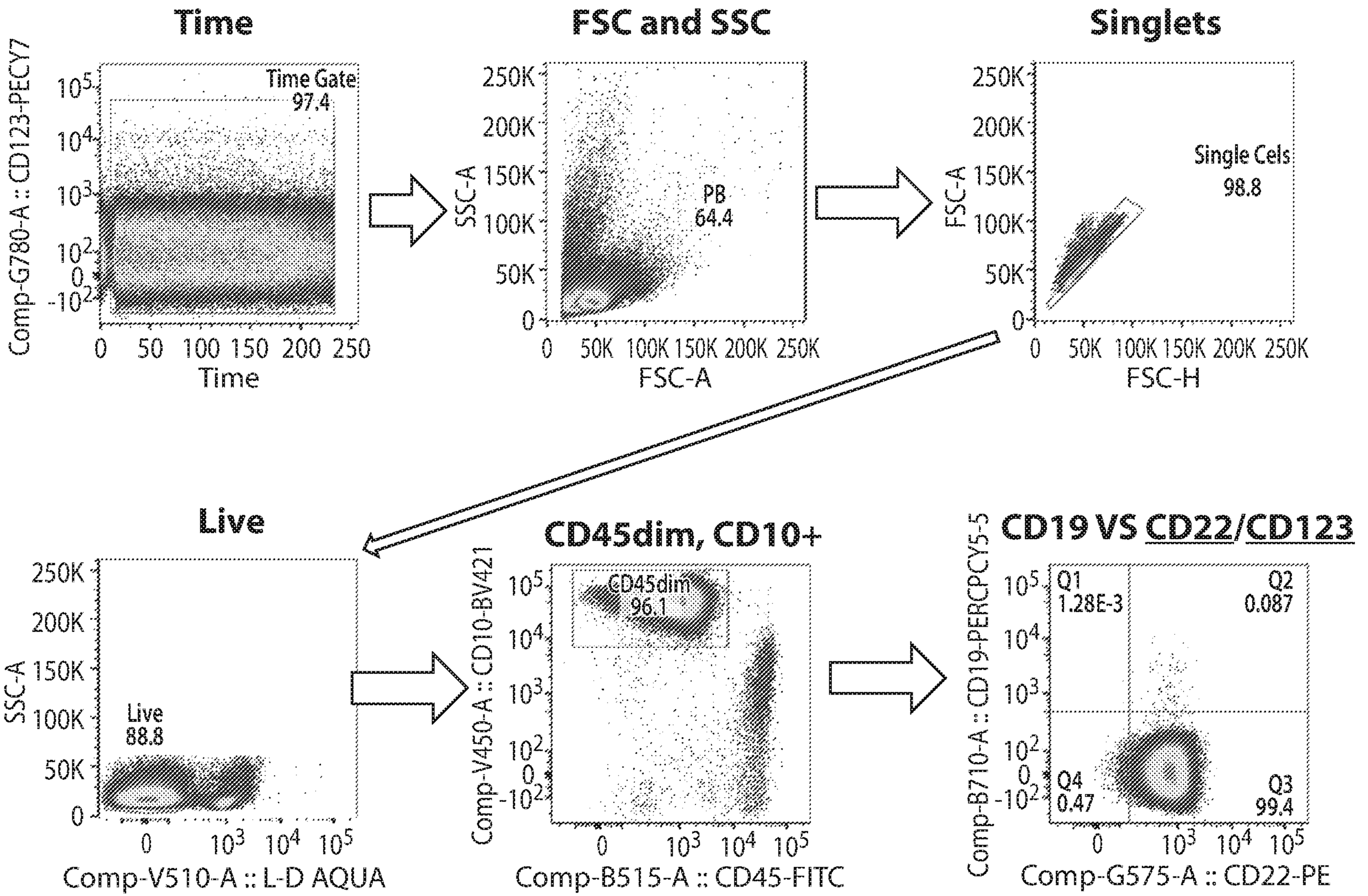


FIG. 23

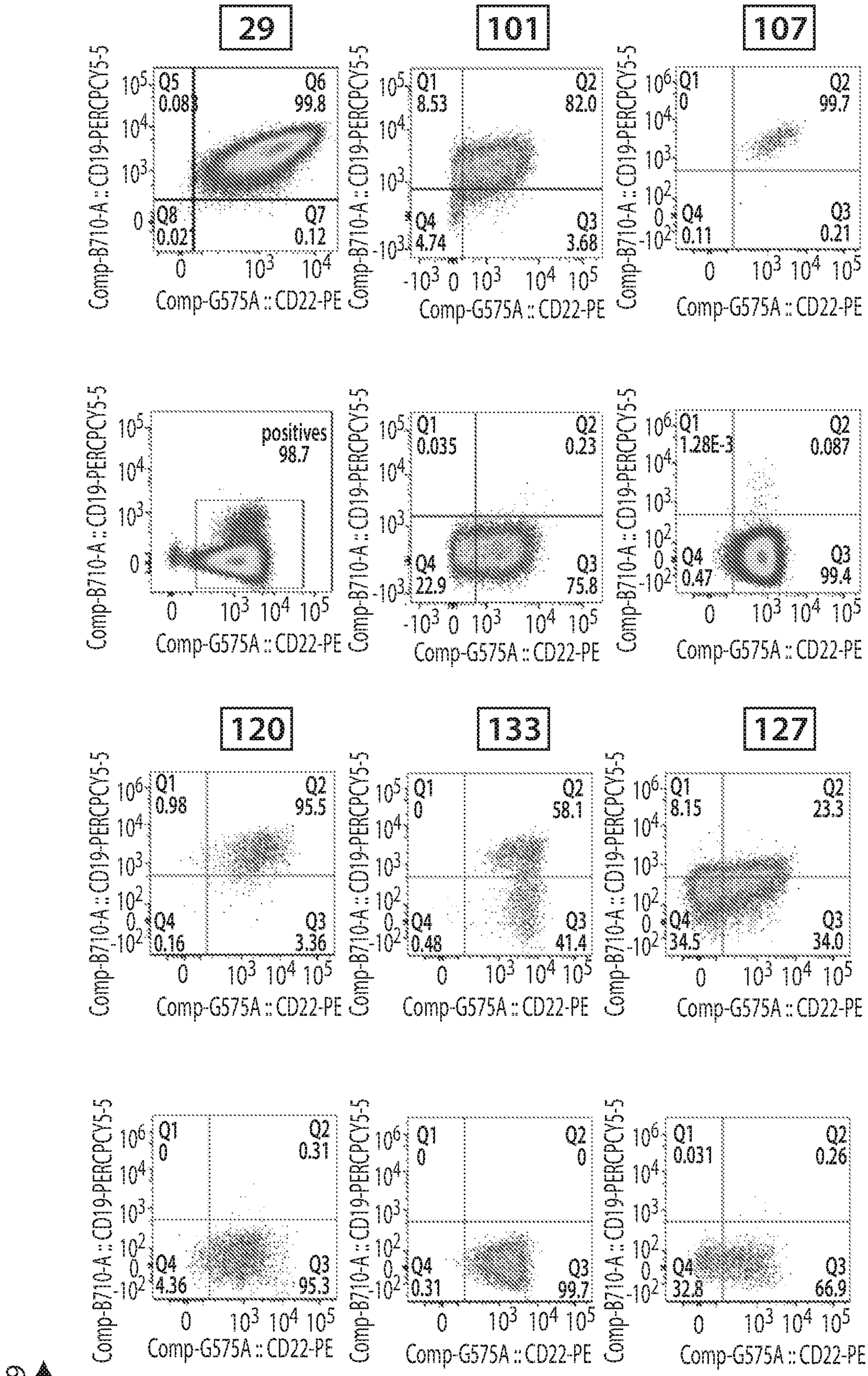

29
101
107
positives
98.7
120
133
127
Comp-B710-A :: CD19-PERCPCY5-5
Comp-G575A :: CD22-PE
CD19
CD22

FIG. 24

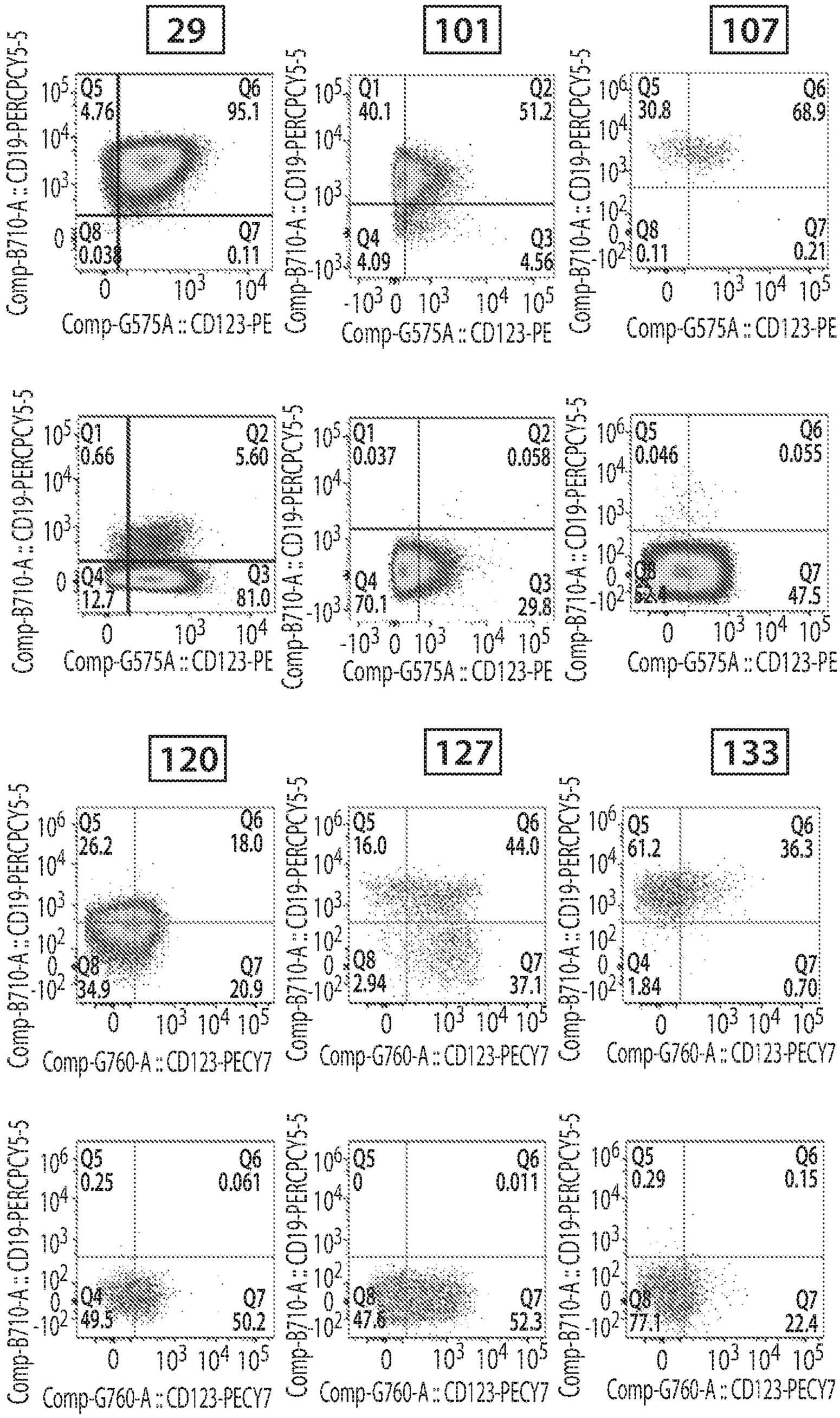

29
101
107
120
127
133
Comp-B710-A :: CD19-PERCPCY5-5
Comp-G575A :: CD123-PE
Comp-G760-A :: CD123-PECY7
Q1 40.1
Q2 51.2
Q4 4.09
Q3 4.56
Q5 30.8
Q6 68.9
Q8 0.11
Q7 0.21
Q1 0.037
Q2 0.058
Q4 70.1
Q3 29.8
Q5 0.046
Q6 0.055
Q7 47.5
Q5 16.0
Q6 44.0
Q8 2.94
Q7 37.1
Q5 61.2
Q6 36.3
Q4 1.84
Q7 0.70
Q5 0
Q6 0.011
Q8 47.6
Q7 52.3
Q5 0.29
Q6 0.15
Q8 77.1
Q7 22.4
CD19
CD123

FIG. 25

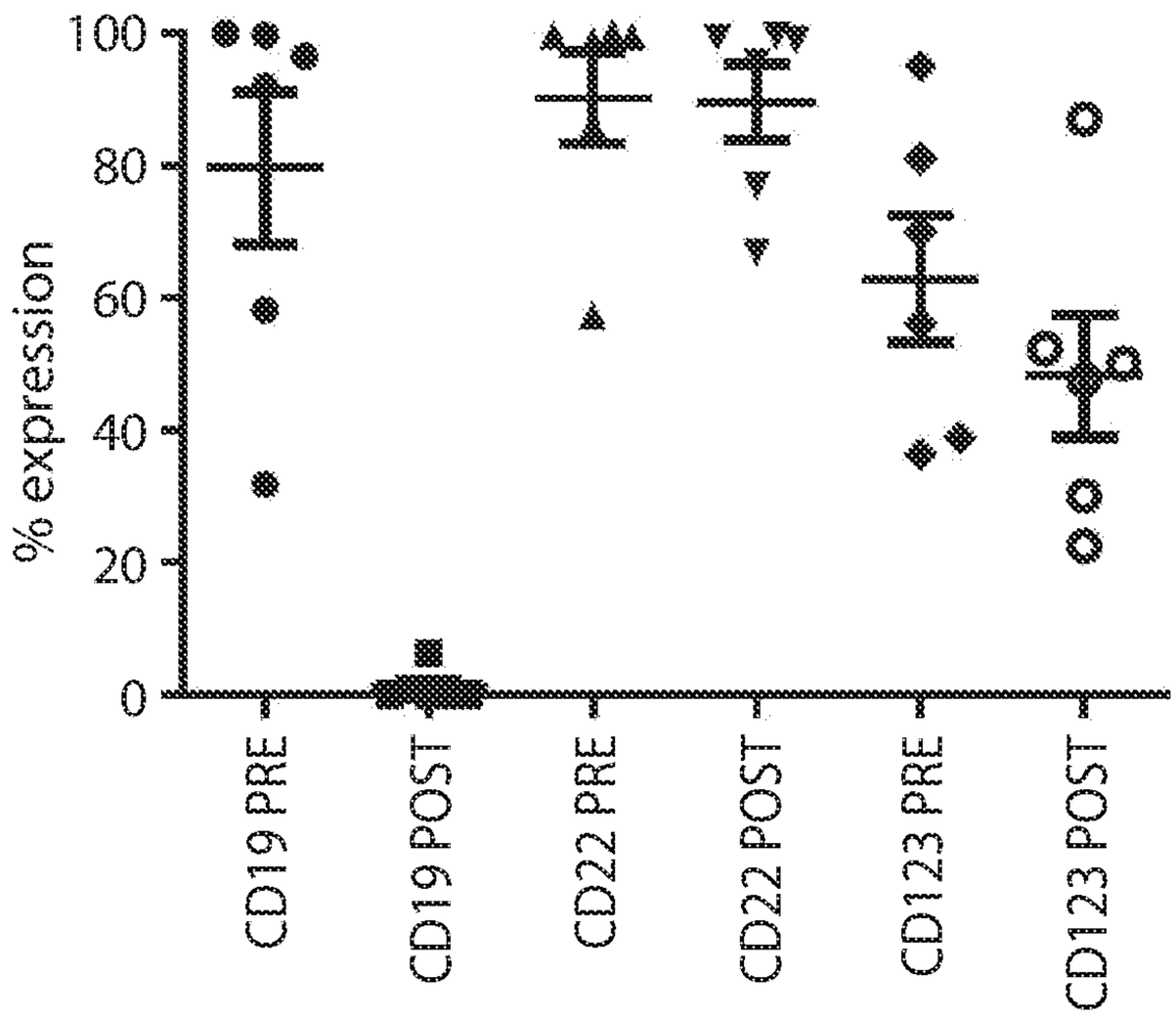

% expression
100
80
60
40
20
0
CD19 PRE
CD19 POST
CD22 PRE
CD22 POST
CD123 PRE
CD123 POST

FIG. 26

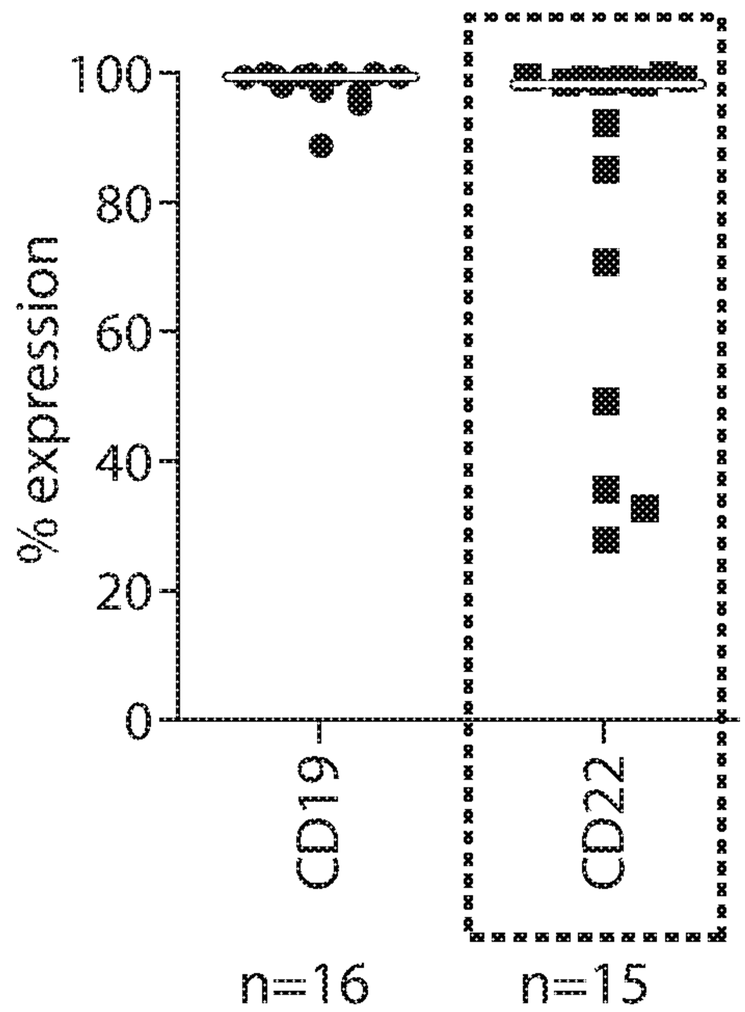


FIG. 27A

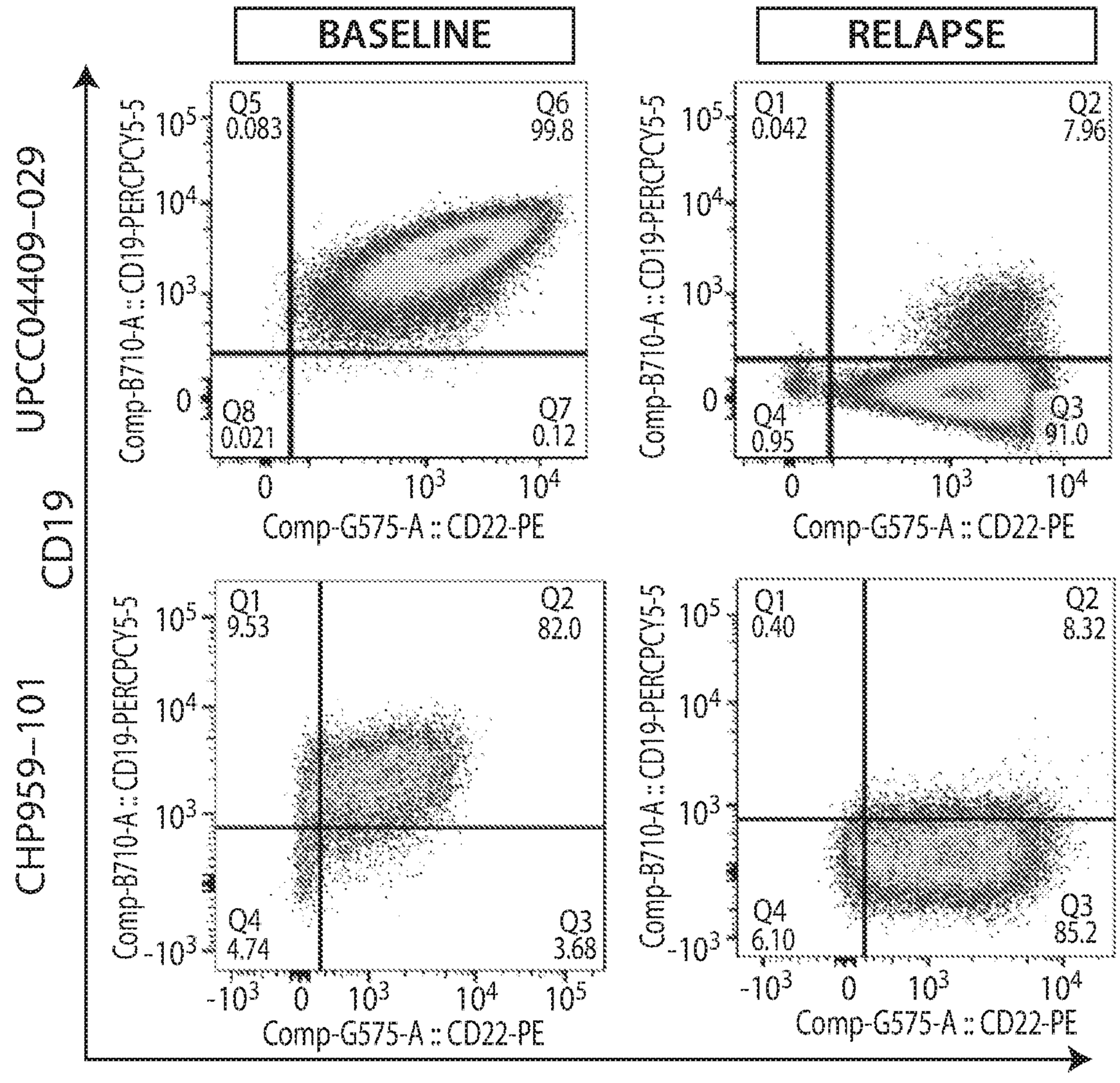


FIG. 27B

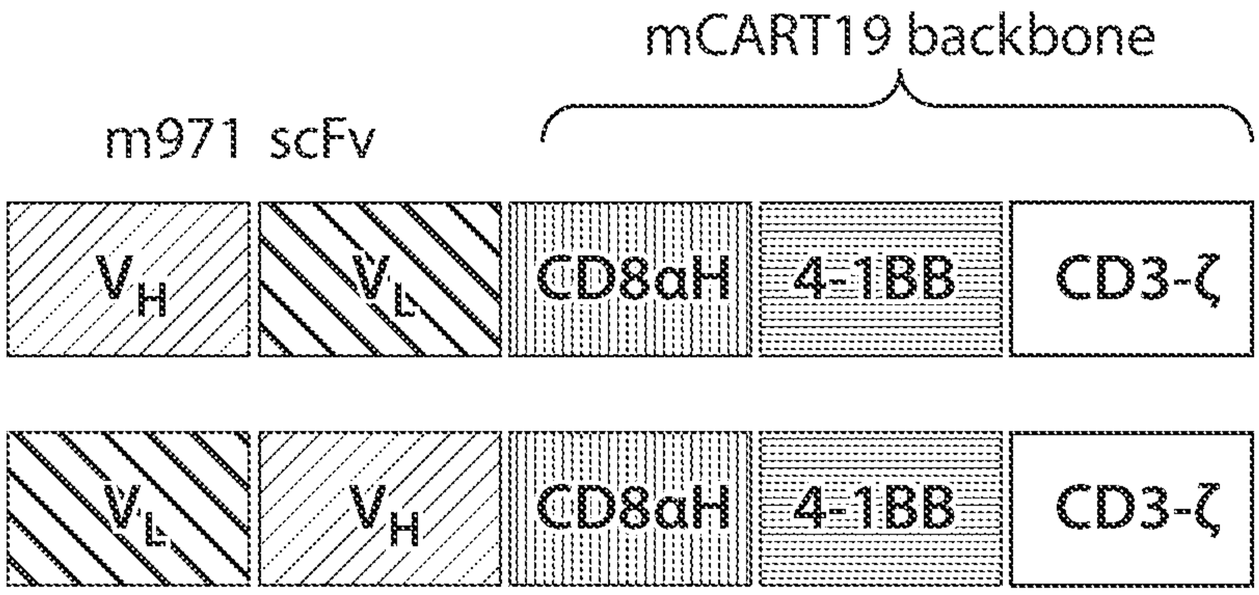


FIG. 28A

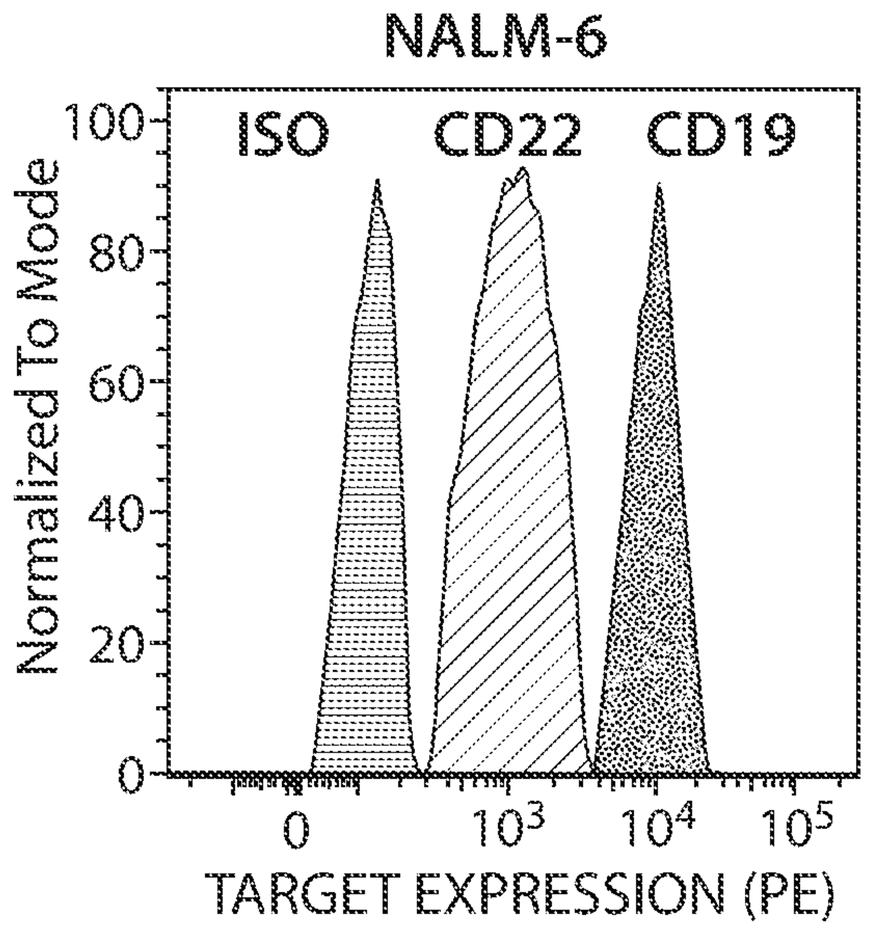


FIG. 28B

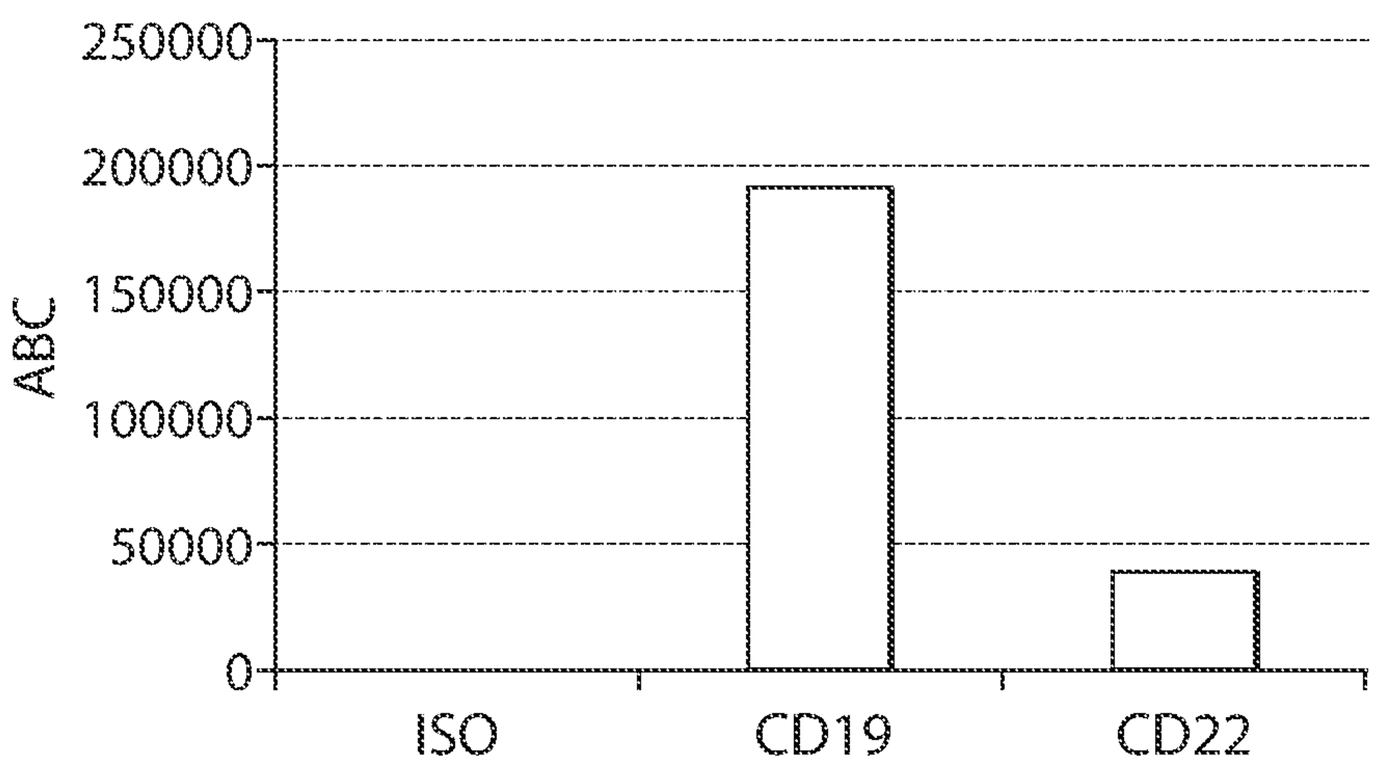


FIG. 28C

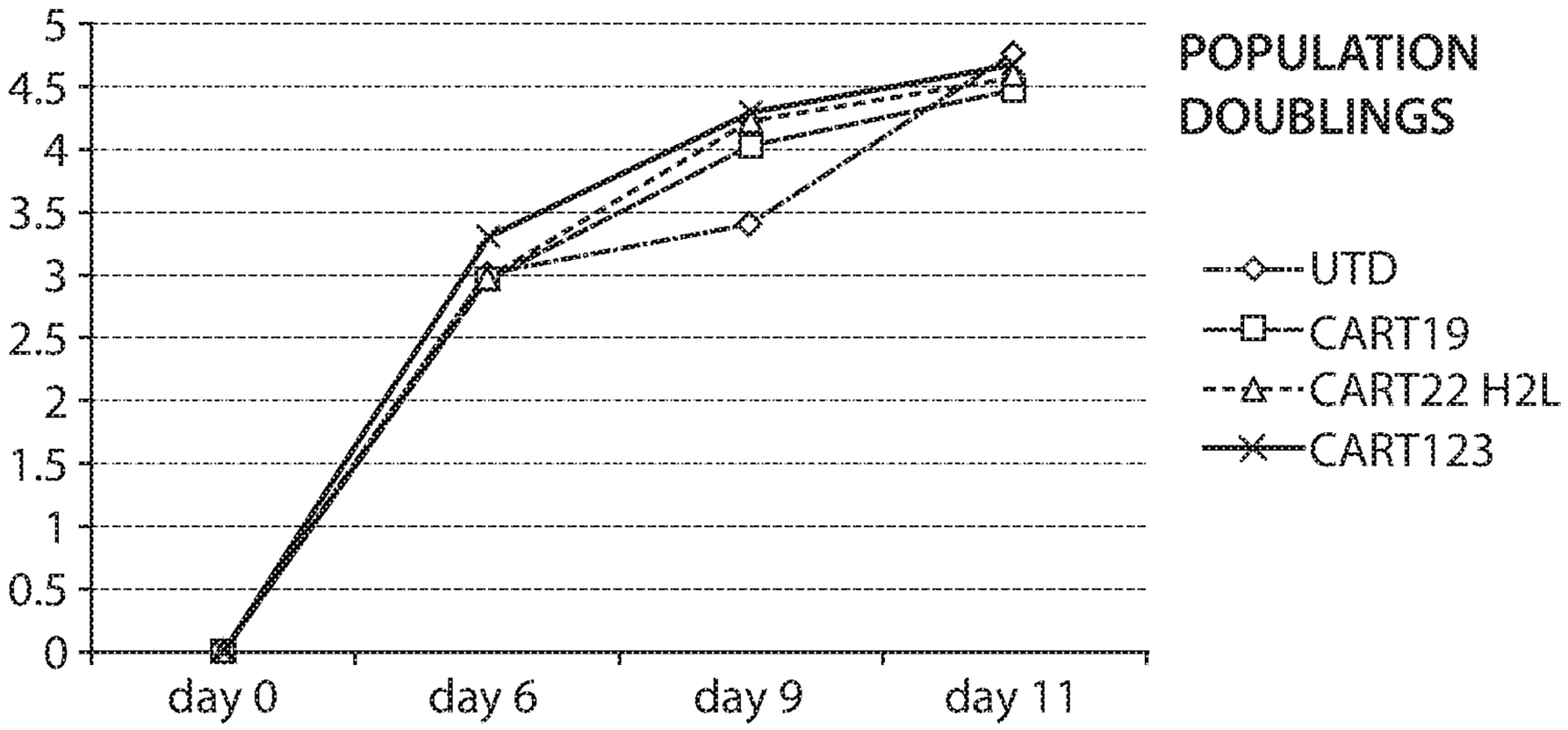
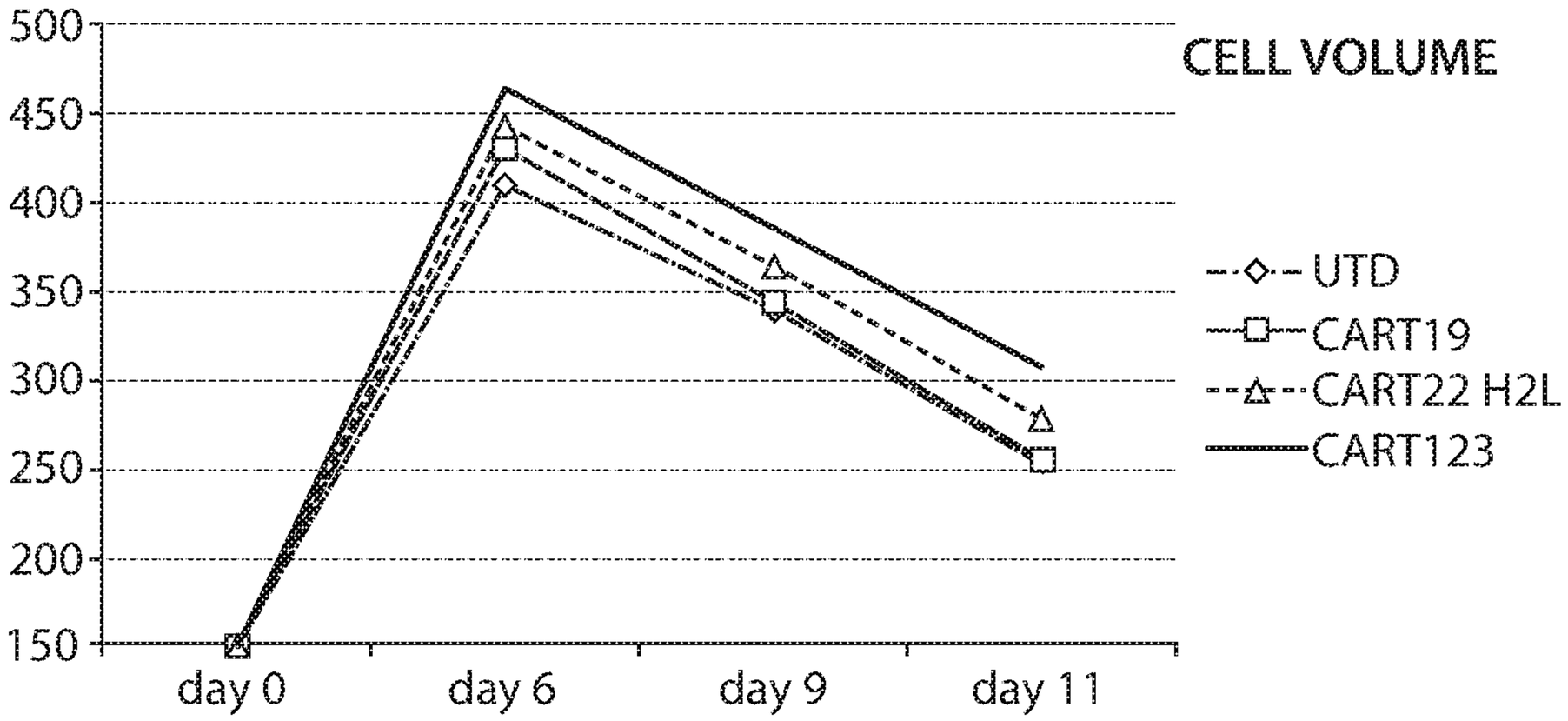


FIG. 29A



ND365

FIG. 29B

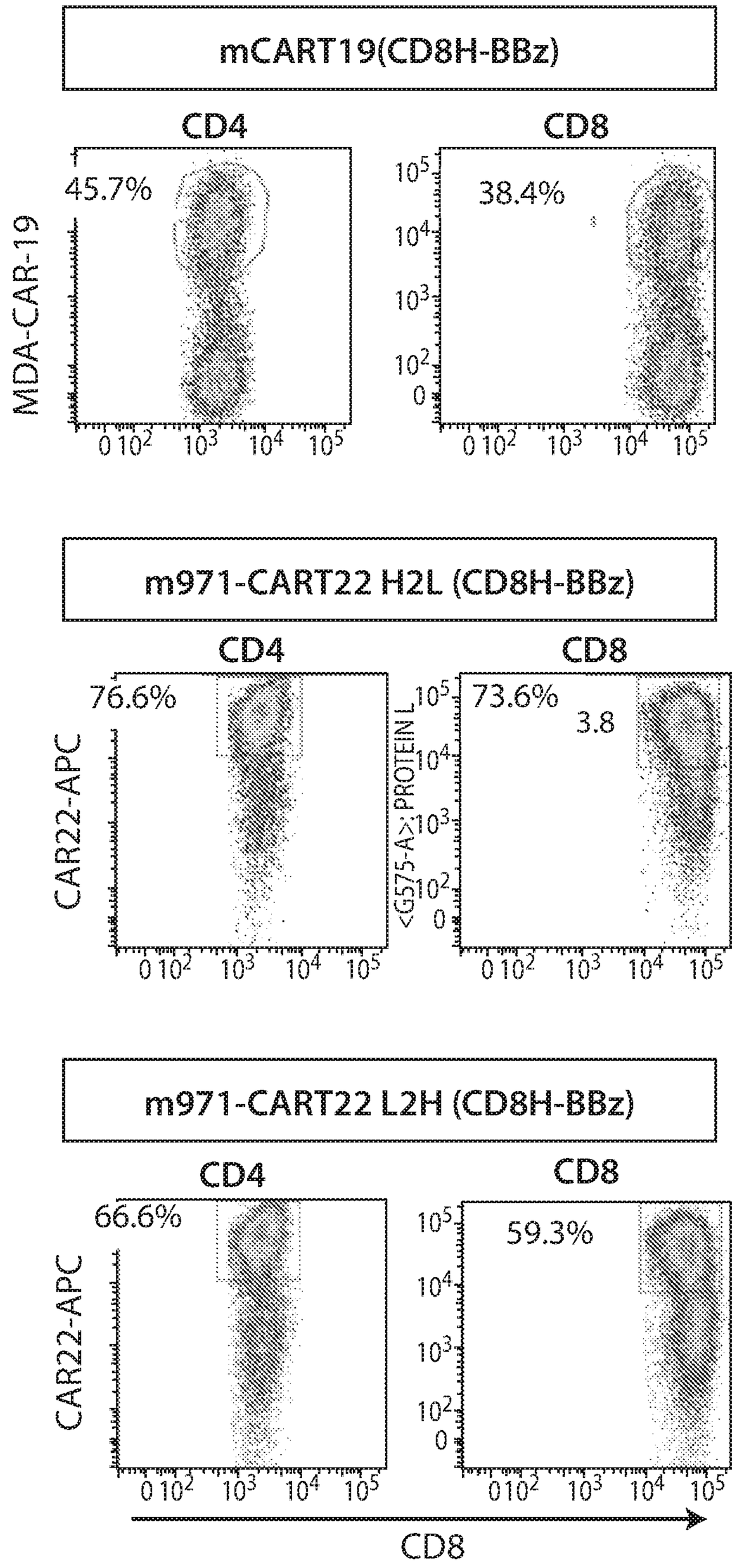

mCART19(CD8H-BBz)
CD4
CD8
MDA-CAR-19
45.7%
38.4%
m971-CART22 H2L (CD8H-BBz)
CD4
CD8
CAR22-APC
76.6%
<G575-A>:PROTEIN L
73.6%
3.8
m971-CART22 L2H (CD8H-BBz)
CD4
CD8
CAR22-APC
66.6%
59.3%
CD8

FIG. 29C

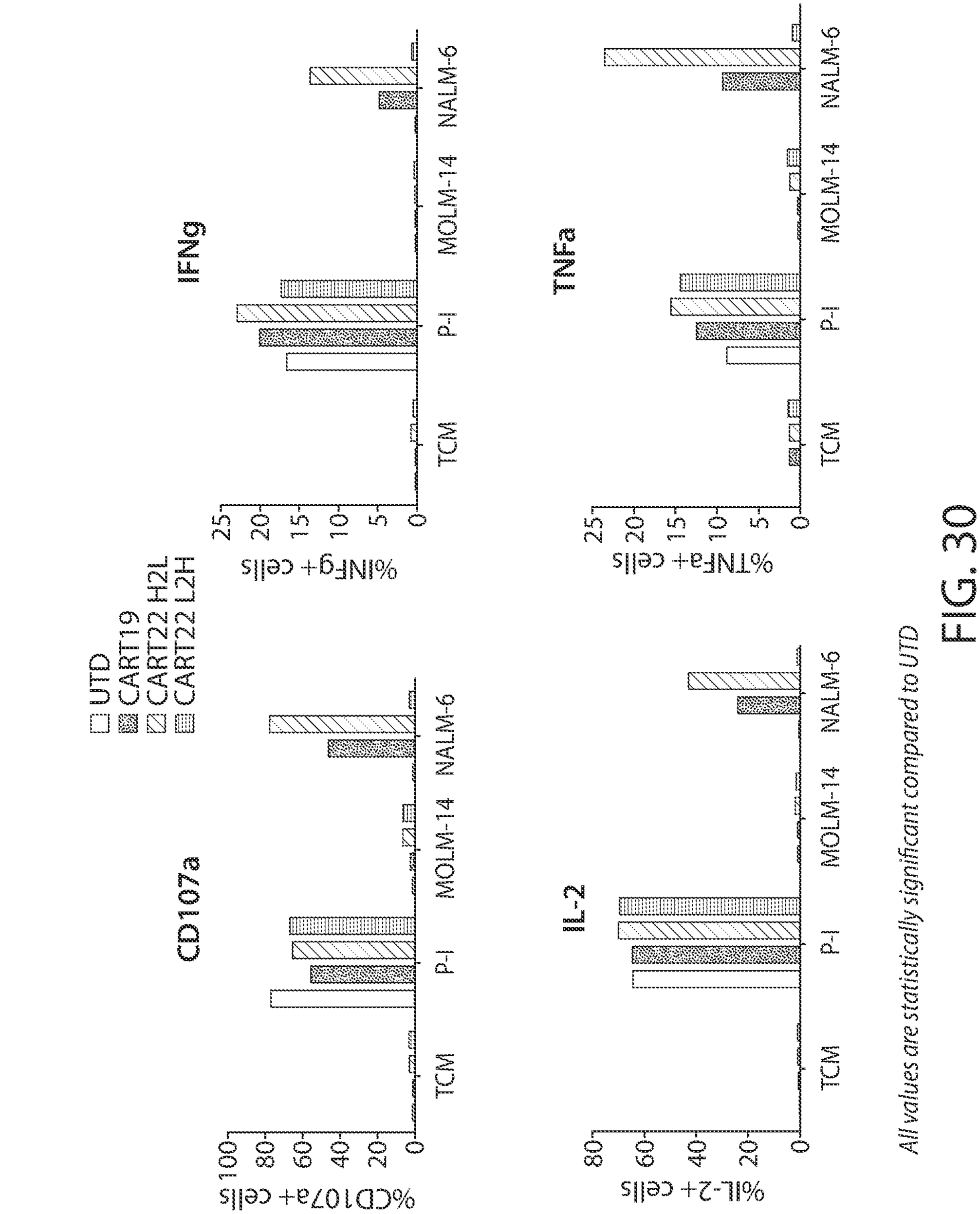


FIG. 30

All values are statistically significant compared to UTD

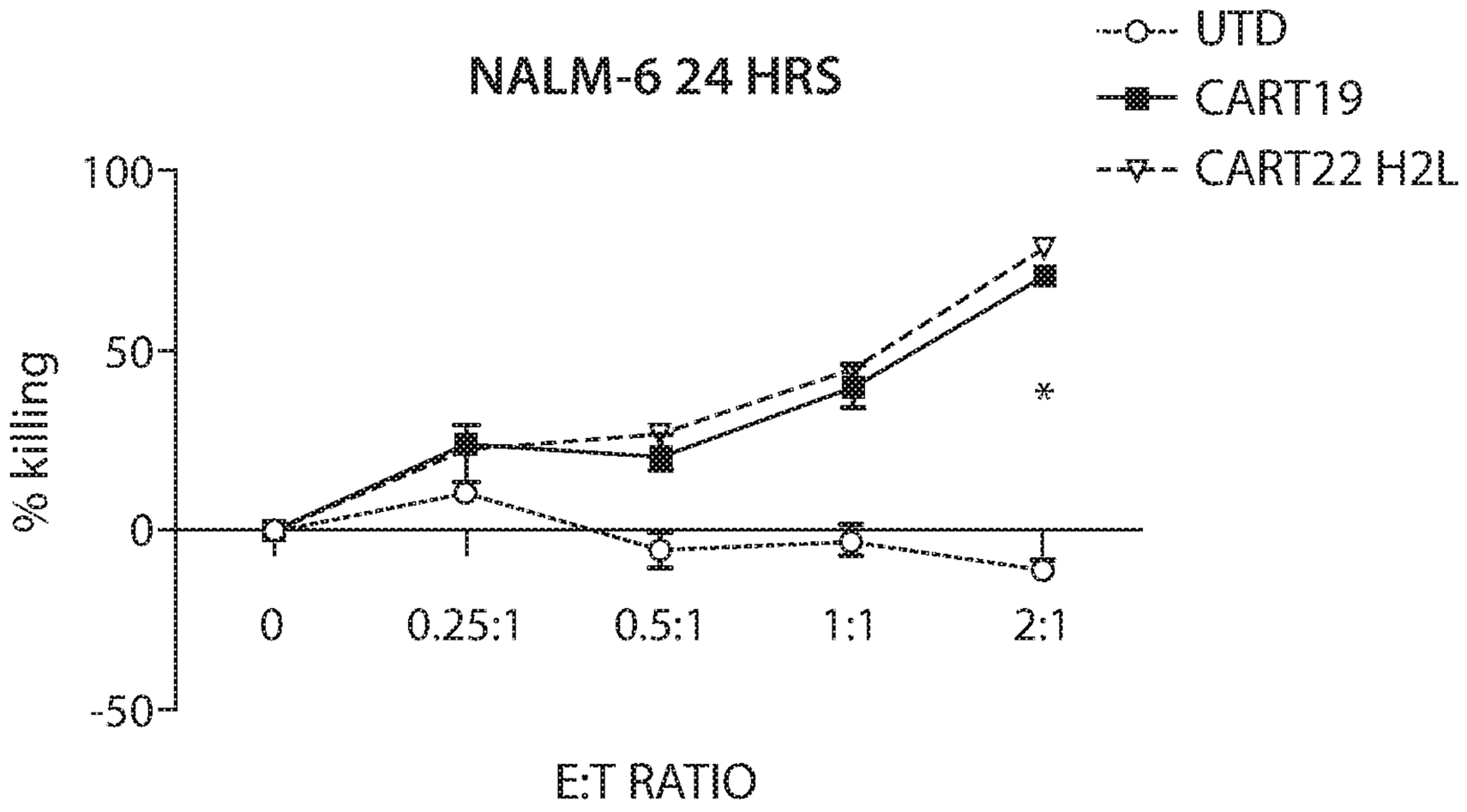

NALM-6 24 HRS
UTD
CART19
CART22 H2L
100
50
0
-50
% killing
0
0.25:1
0.5:1
1:1
2:1
*
E:T RATIO

FIG. 31

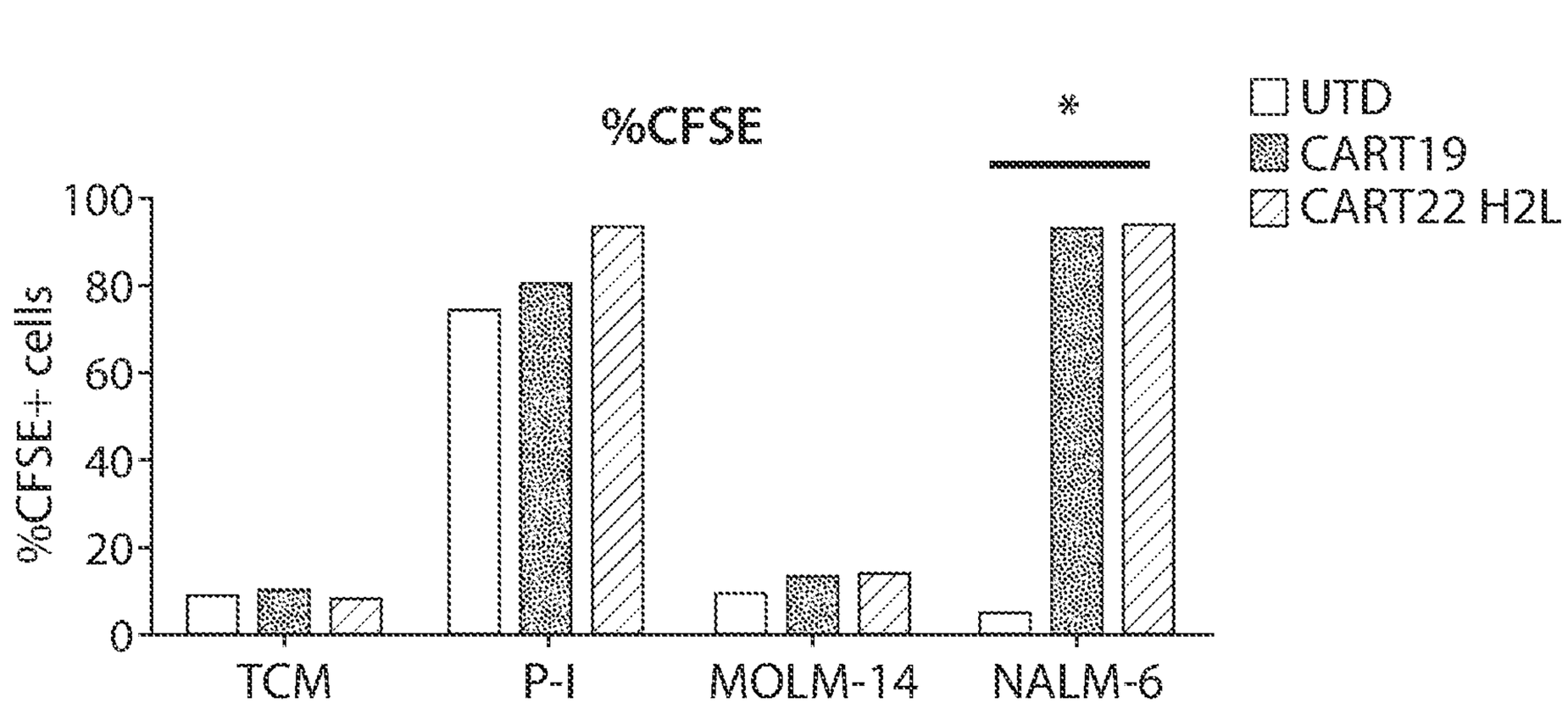


FIG. 32A

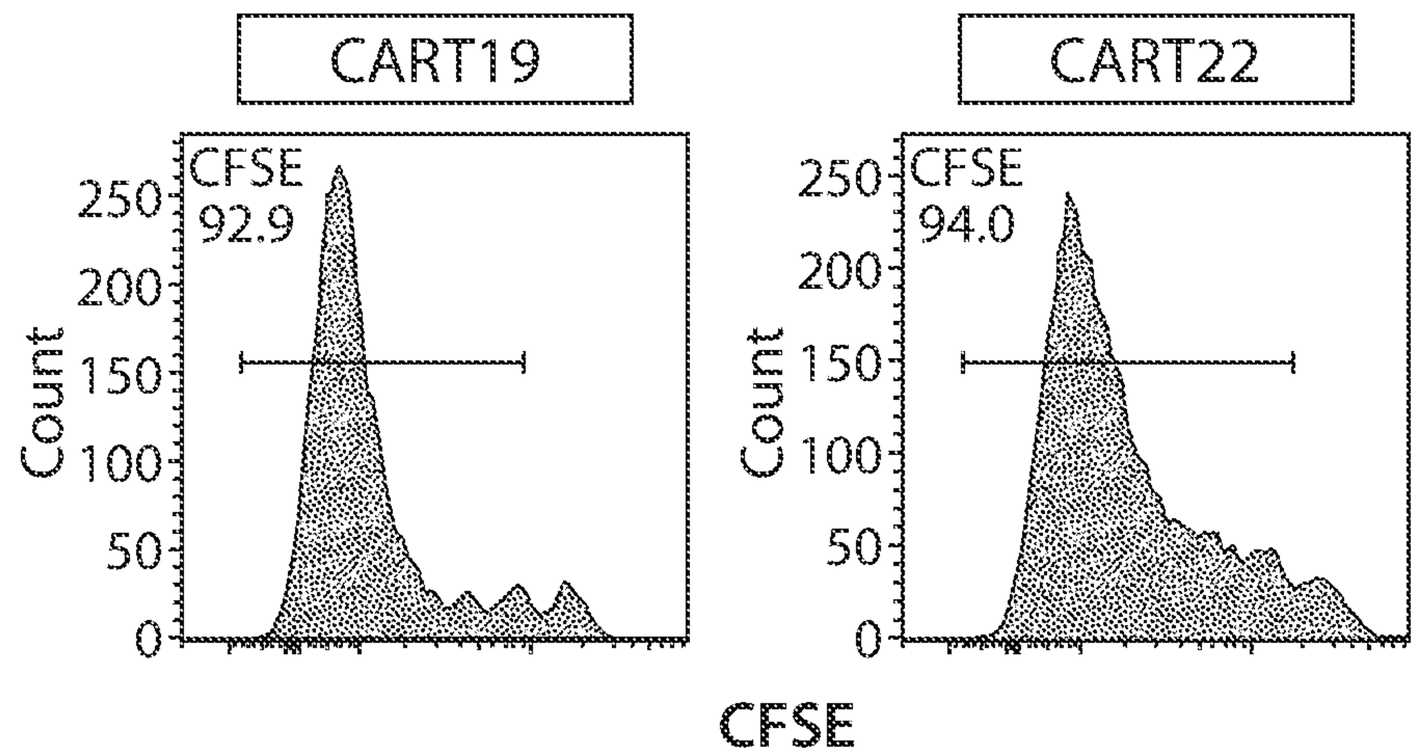


FIG. 32B

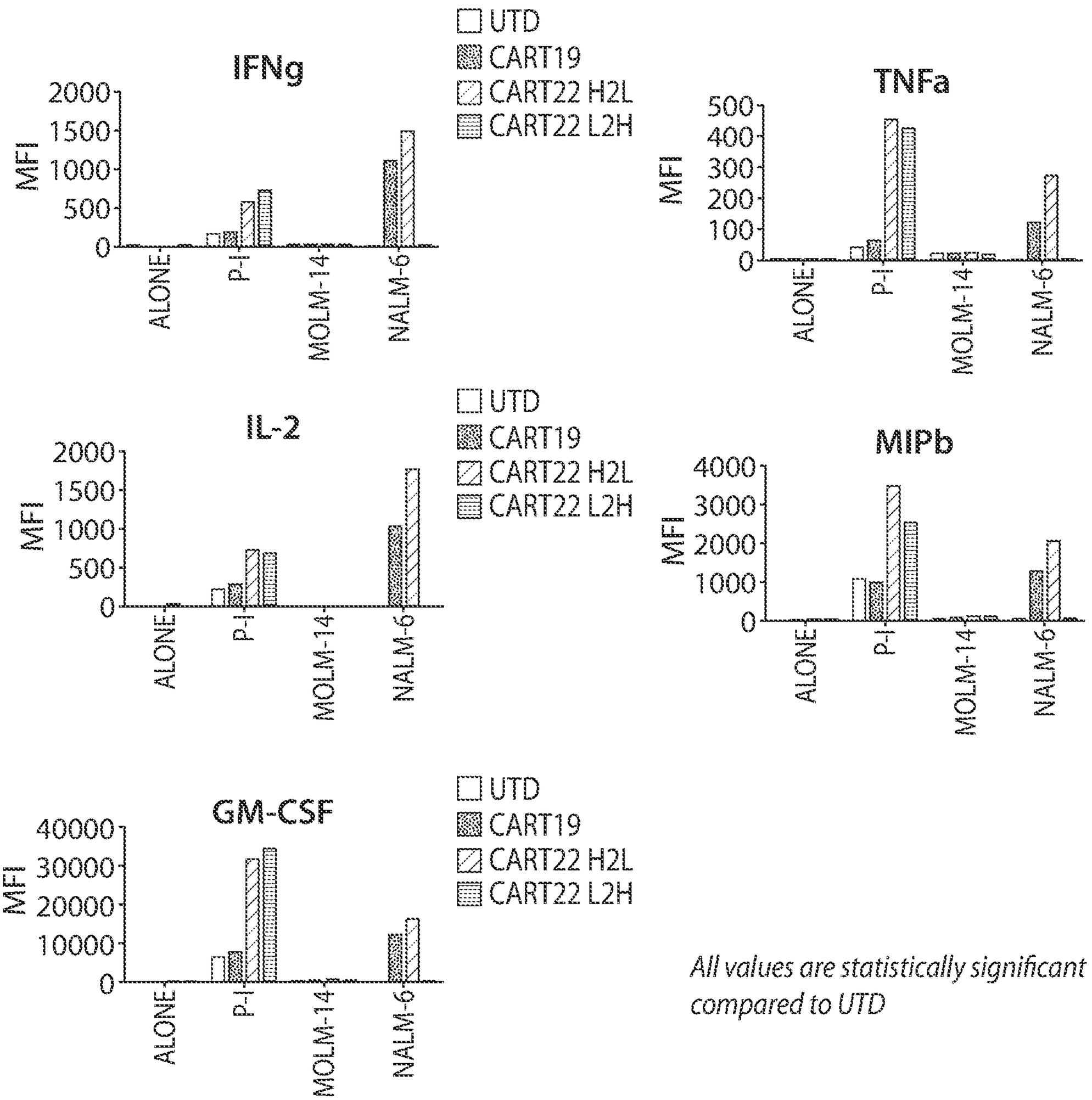

IFNg
MFI
2000
1500
1000
500
0
ALONE
P-I
MOLM-14
NALM-6
UTD
CART19
CART22 H2L
CART22 L2H
TNFa
MFI
500
400
300
200
100
0
ALONE
P-I
MOLM-14
NALM-6
IL-2
MFI
2000
1500
1000
500
0
ALONE
P-I
MOLM-14
NALM-6
UTD
CART19
CART22 H2L
CART22 L2H
MIPb
MFI
4000
3000
2000
1000
0
ALONE
P-I
MOLM-14
NALM-6
GM-CSF
MFI
40000
30000
20000
10000
0
ALONE
P-I
MOLM-14
NALM-6
UTD
CART19
CART22 H2L
CART22 L2H
All values are statistically significant compared to UTD

FIG. 33

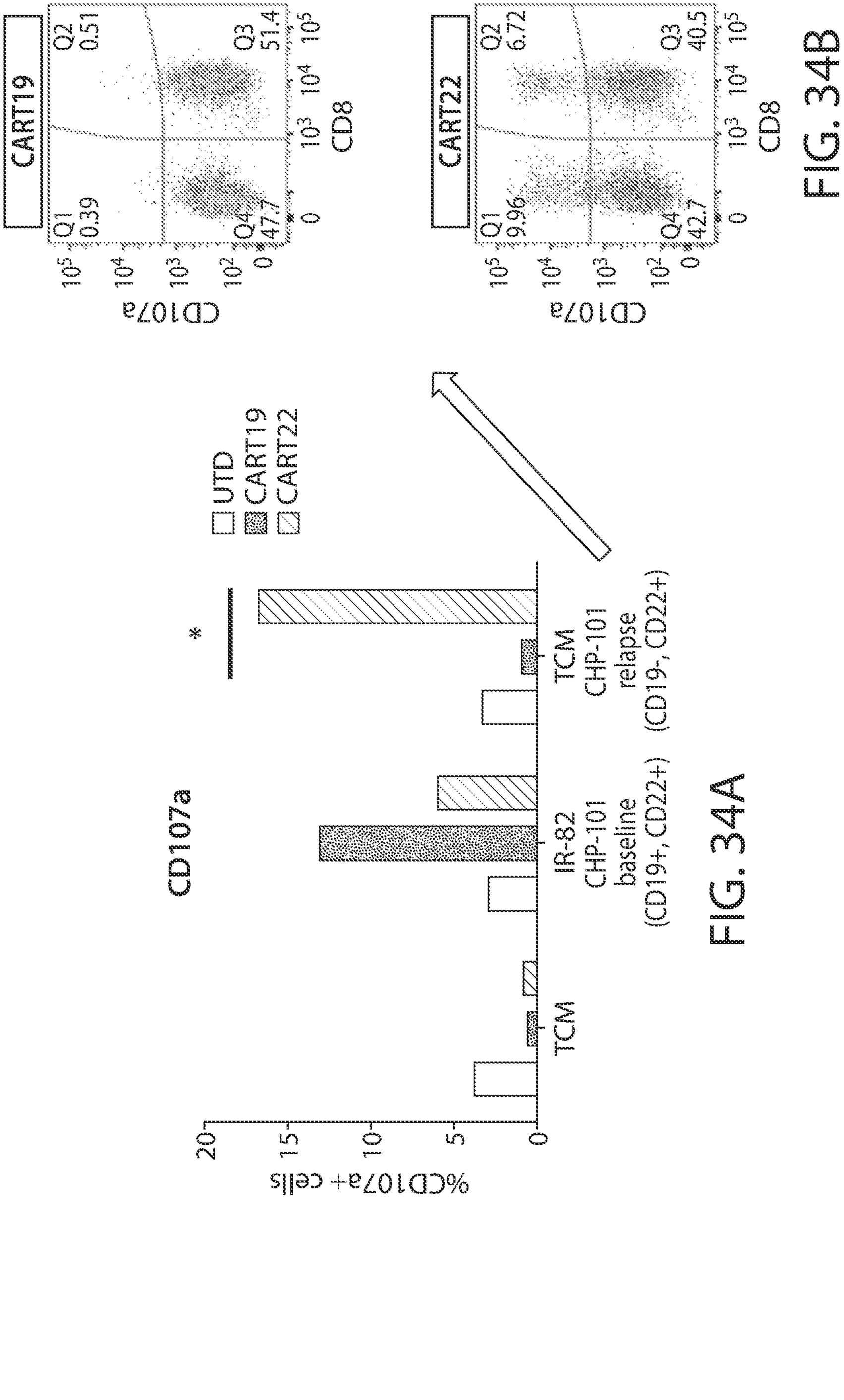


FIG. 34B

FIG. 34A

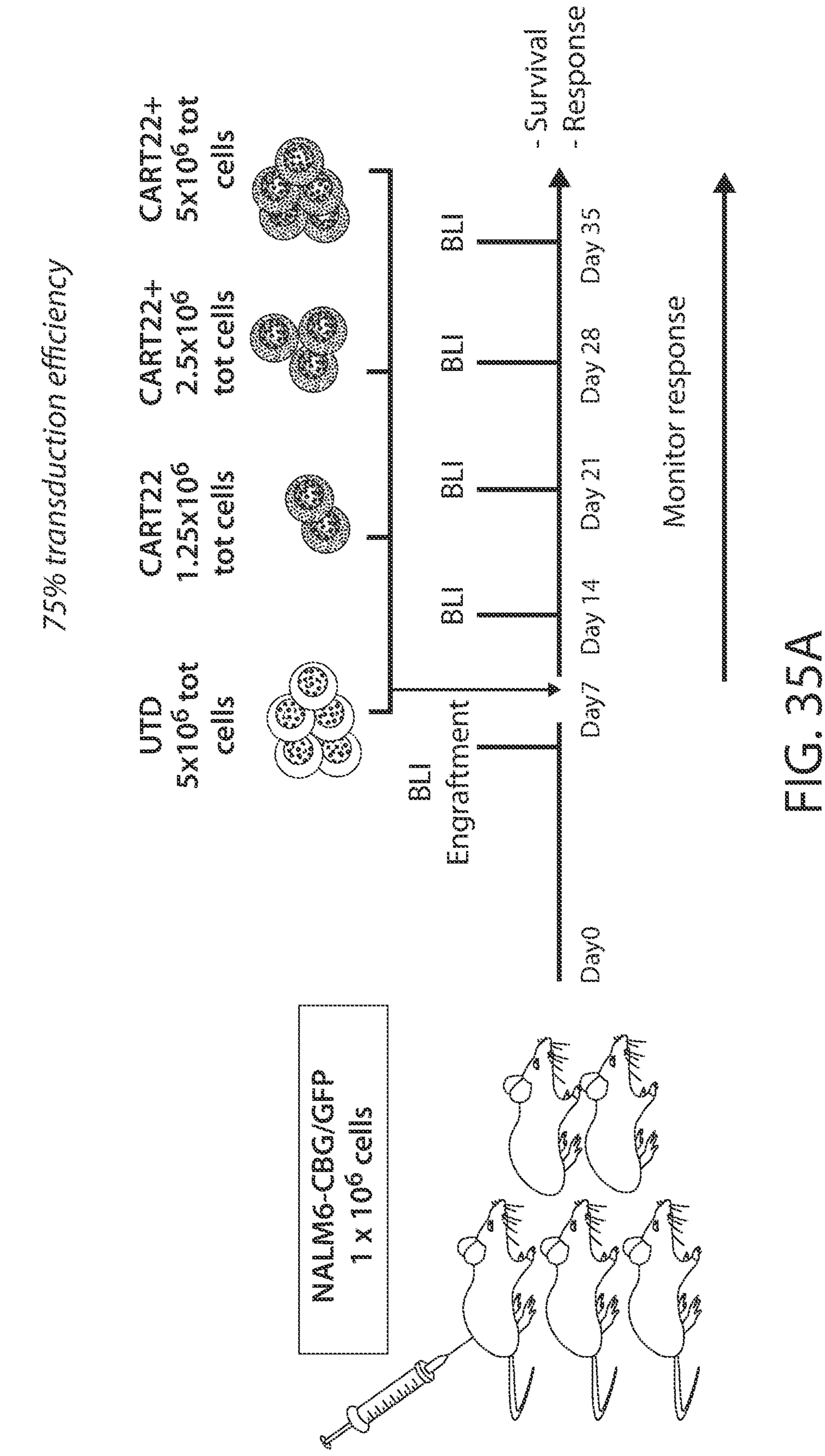
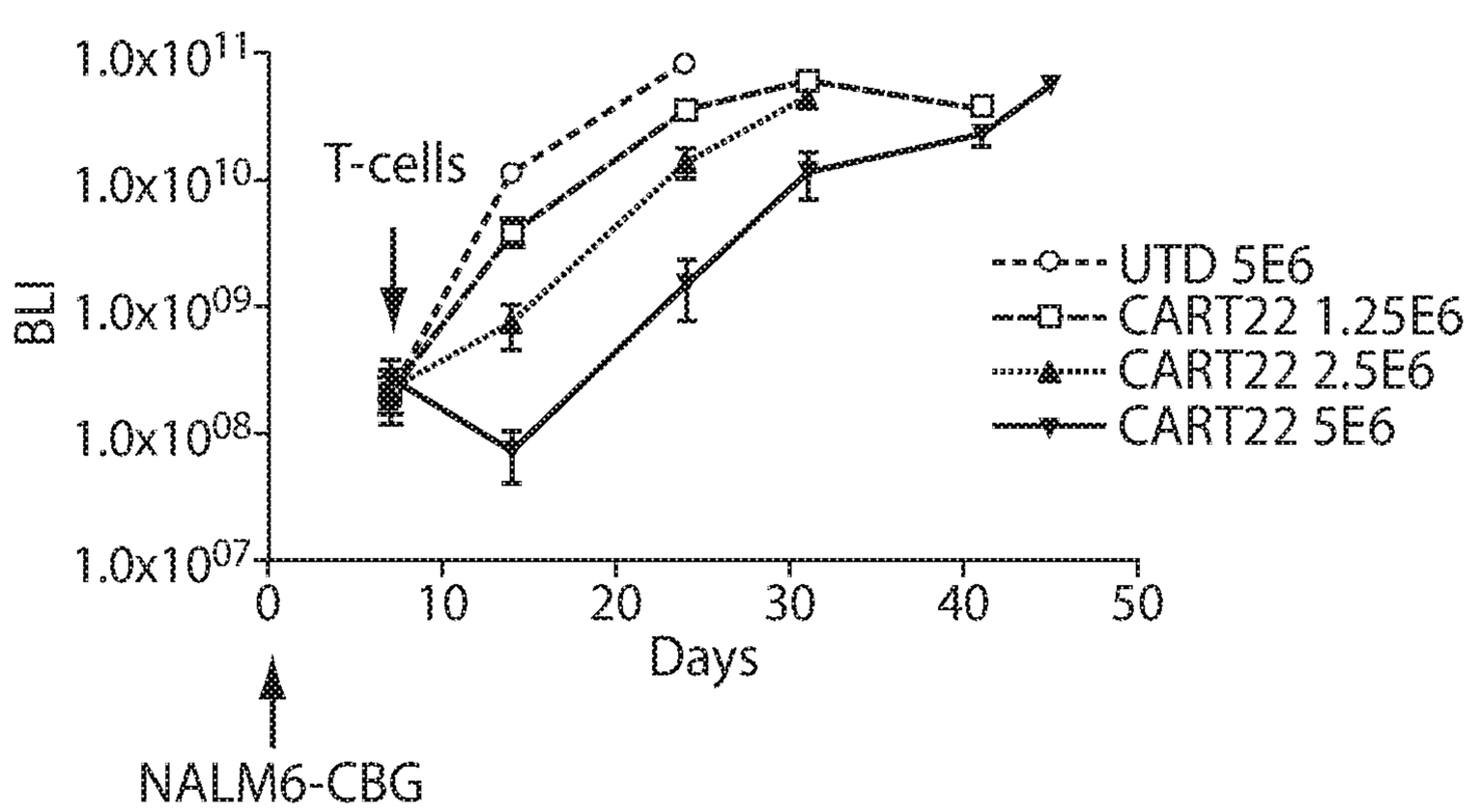

NALM6-CBG/GFP
1 x 10^6 cells
UTD
5x10^6 tot cells
CART22
1.25x10^6 tot cells
CART22+
2.5x10^6 tot cells
CART22+
5x10^6 tot cells
75% transduction efficiency
Day0
Day7
BLI
Engraftment
Monitor response
Day 14
Day 21
Day 28
Day 35
BLI
BLI
BLI
- Survival
- Response

FIG. 35A



ND365 *75% transduction efficiency*

FIG. 35B

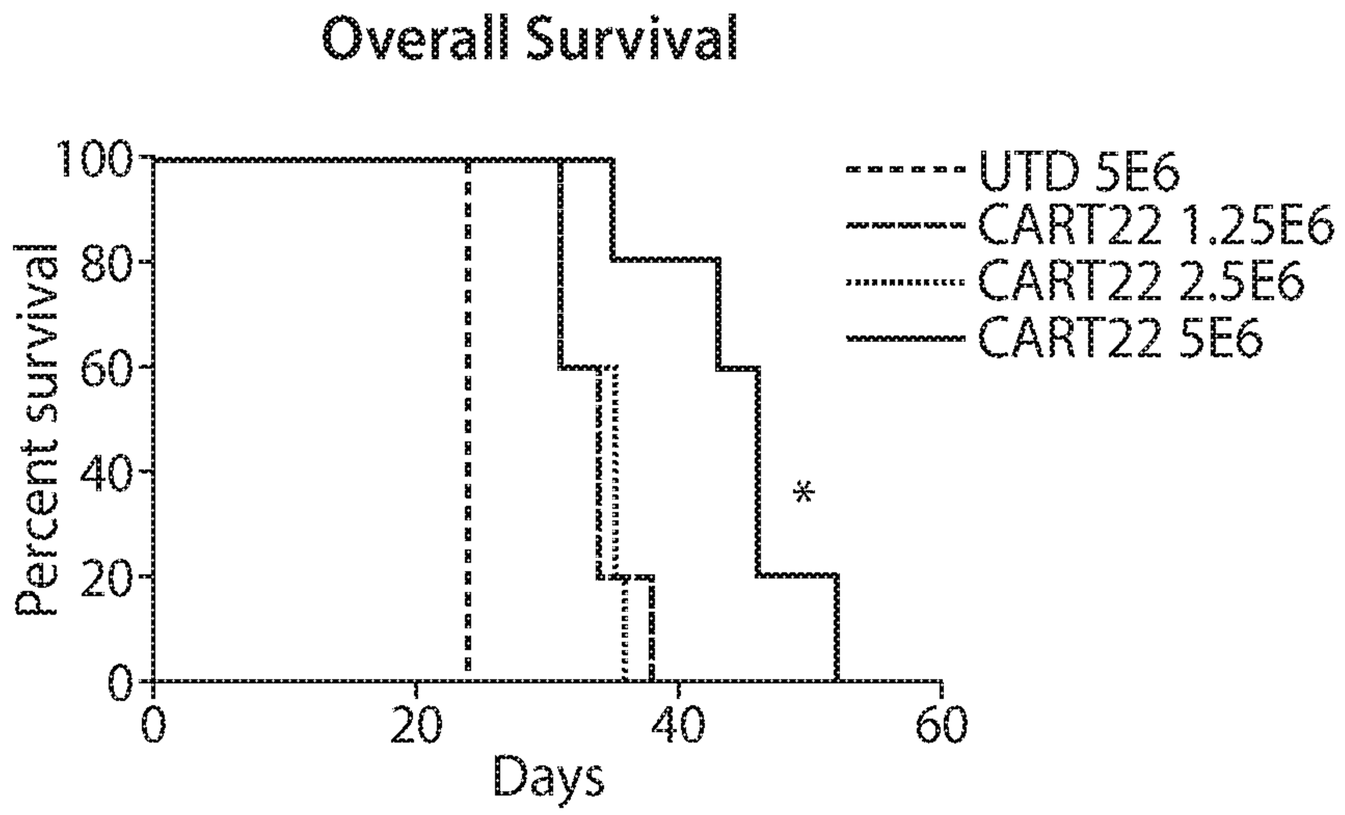


FIG. 35C

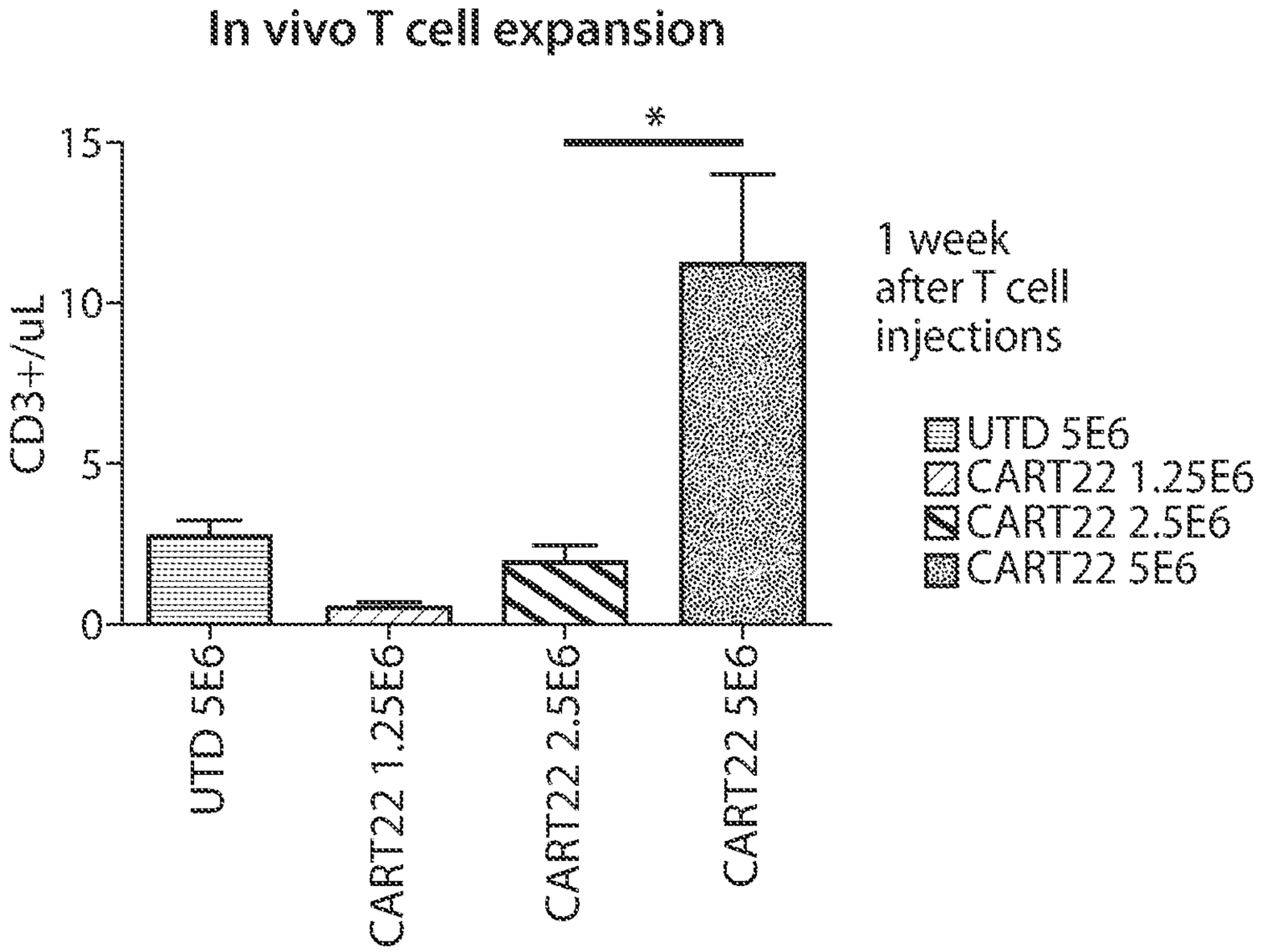

In vivo T cell expansion
CD3+/uL
15
10
5
0
UTD 5E6
CART22 1.25E6
CART22 2.5E6
CART22 5E6
*
1 week
after T cell
injections
UTD 5E6
CART22 1.25E6
CART22 2.5E6
CART22 5E6

FIG. 35D

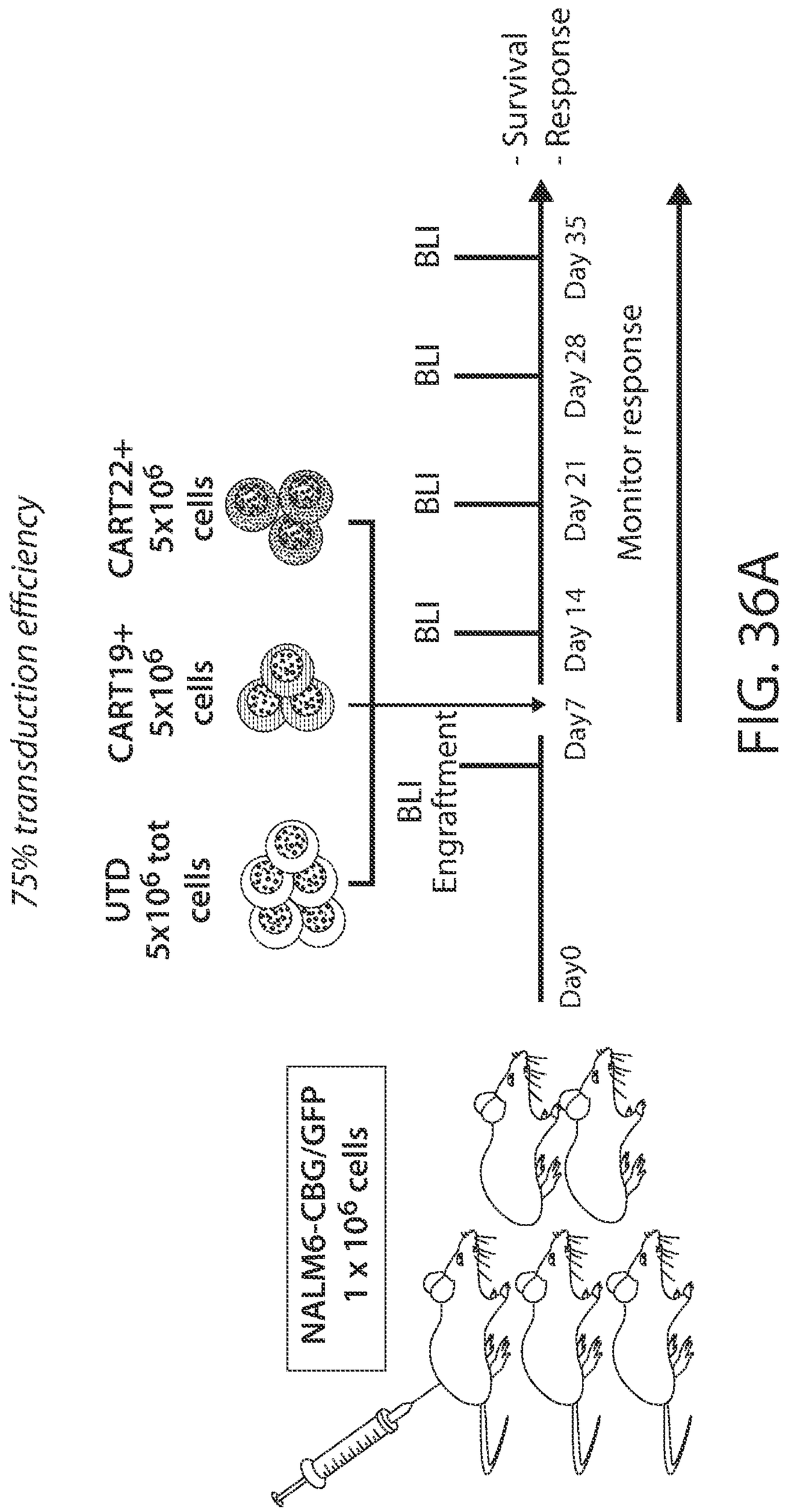

NALM6-CBG/GFP
1 x 10^6 cells
75% transduction efficiency
UTD
5x10^6 tot cells
CART19+
5x10^6 cells
CART22+
5x10^6 cells
BLI
Engraftment
BLI
BLI
BLI
Day0
Day7
Day 14
Day 21
Day 28
Day 35
Monitor response
- Survival
- Response

FIG. 36A

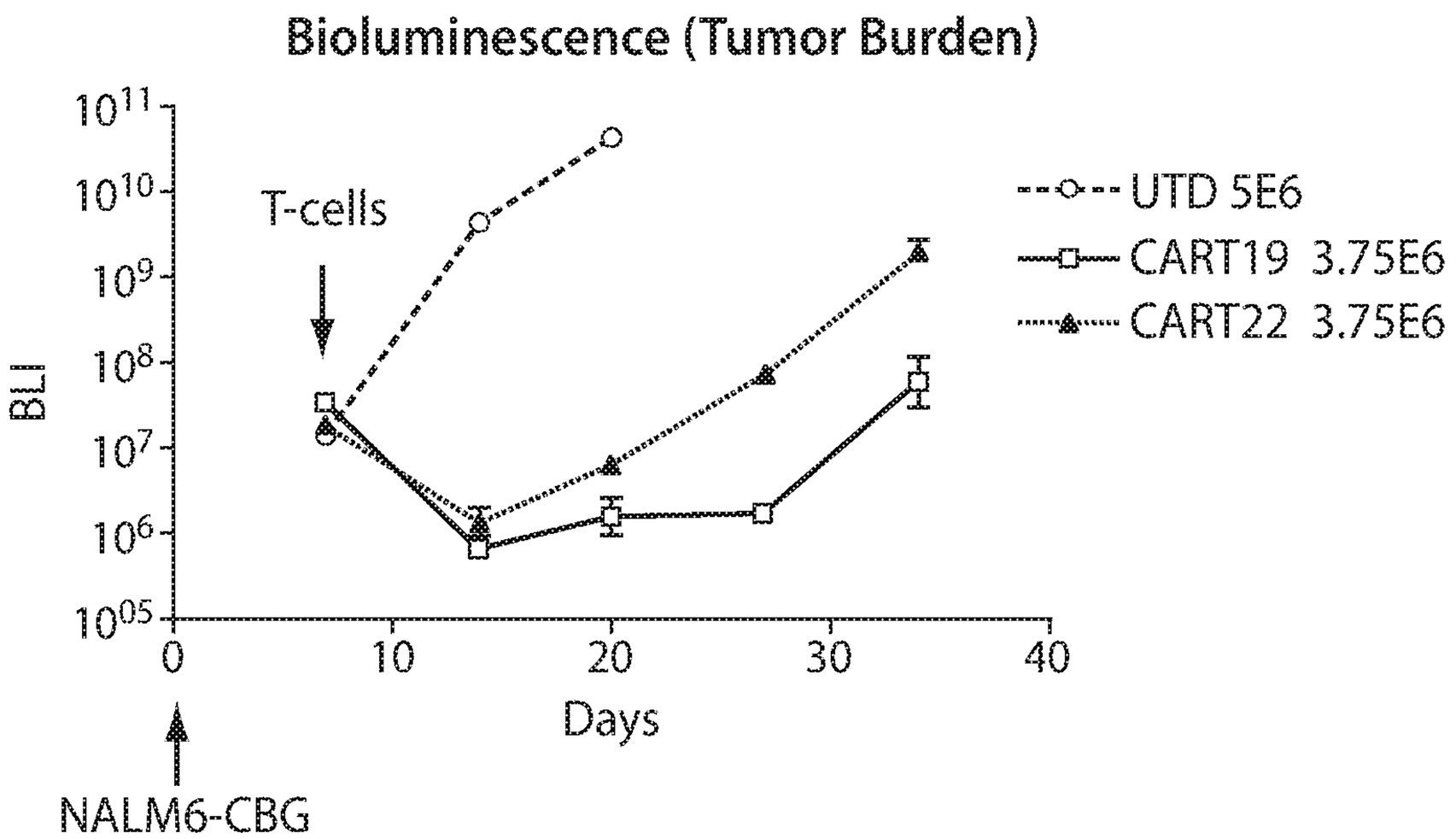
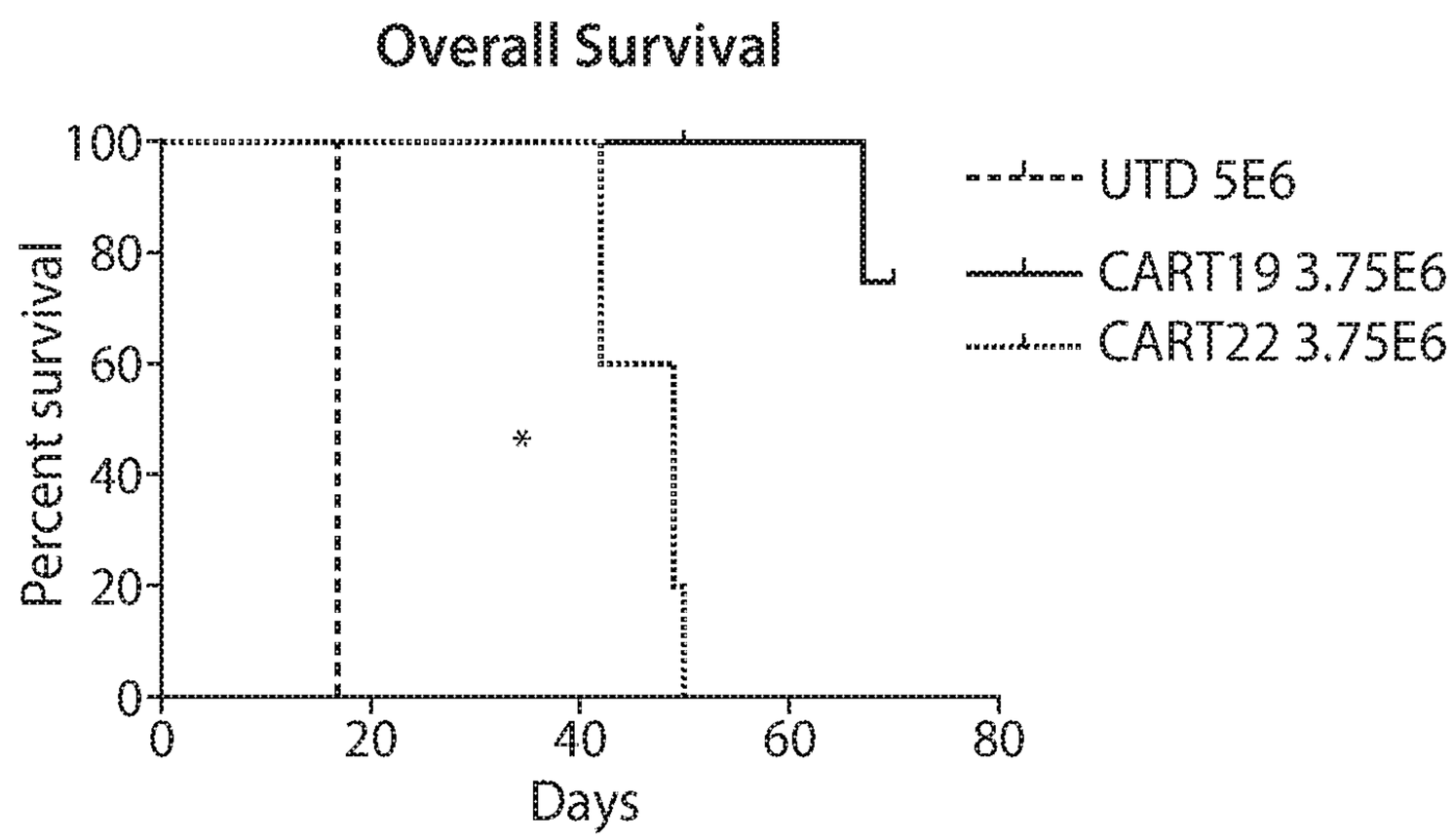


FIG. 36B



** CARTs were normalized to same number of CAR-pos (3.75e6/mouse)*

FIG. 36C

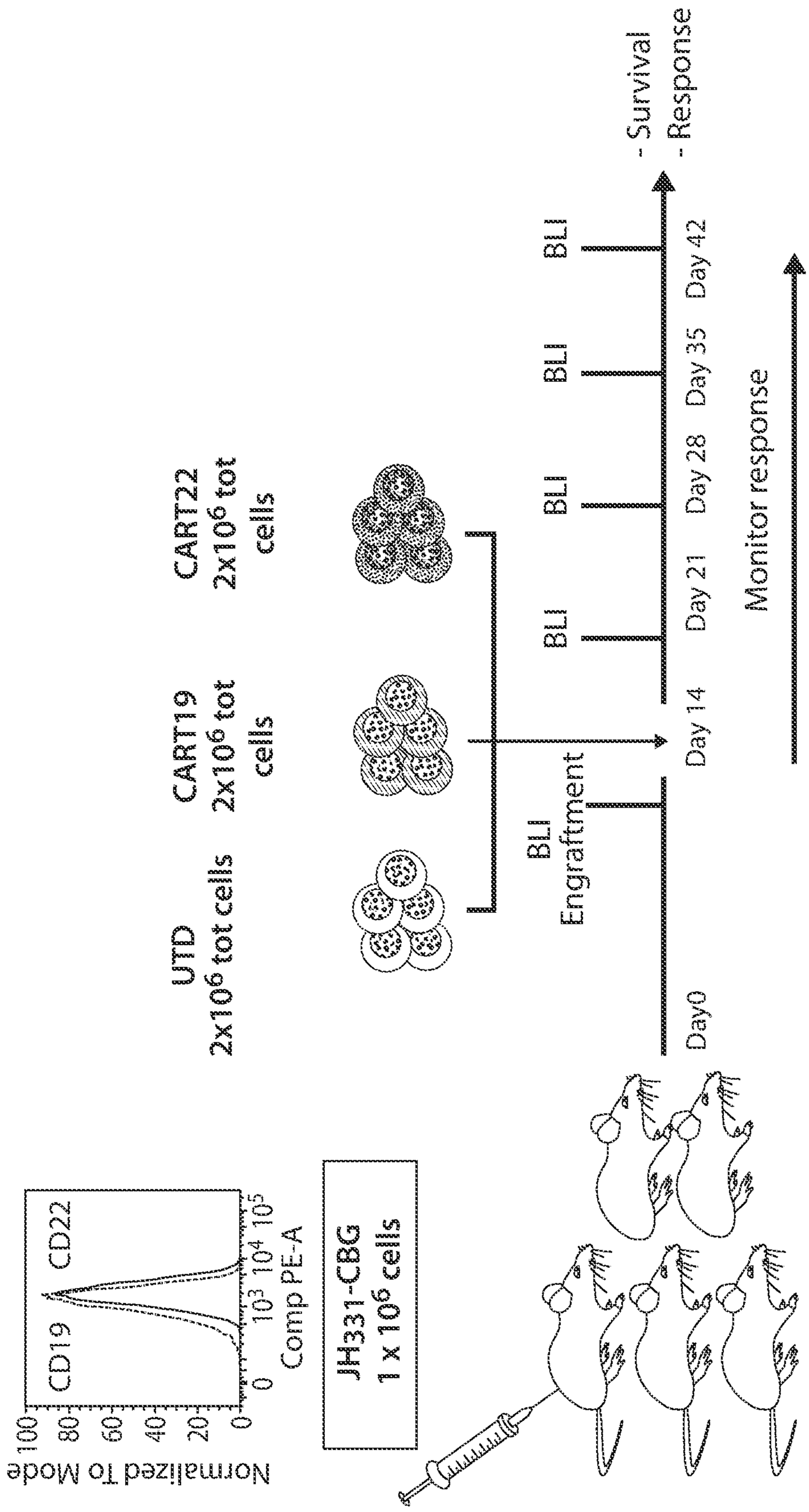

- Survival
- Response
BLI
BLI
BLI
BLI
Day 21
Day 28
Day 35
Day 42
Monitor response
CART22
2x10^6 tot
cells
CART19
2x10^6 tot
cells
UTD
2x10^6 tot cells
BLI
Engraftment
Day 14
Day0
JH331-CBG
1 x 10^6 cells
CD19
CD22
Normalized To Mode
Comp PE-A

FIG. 37A

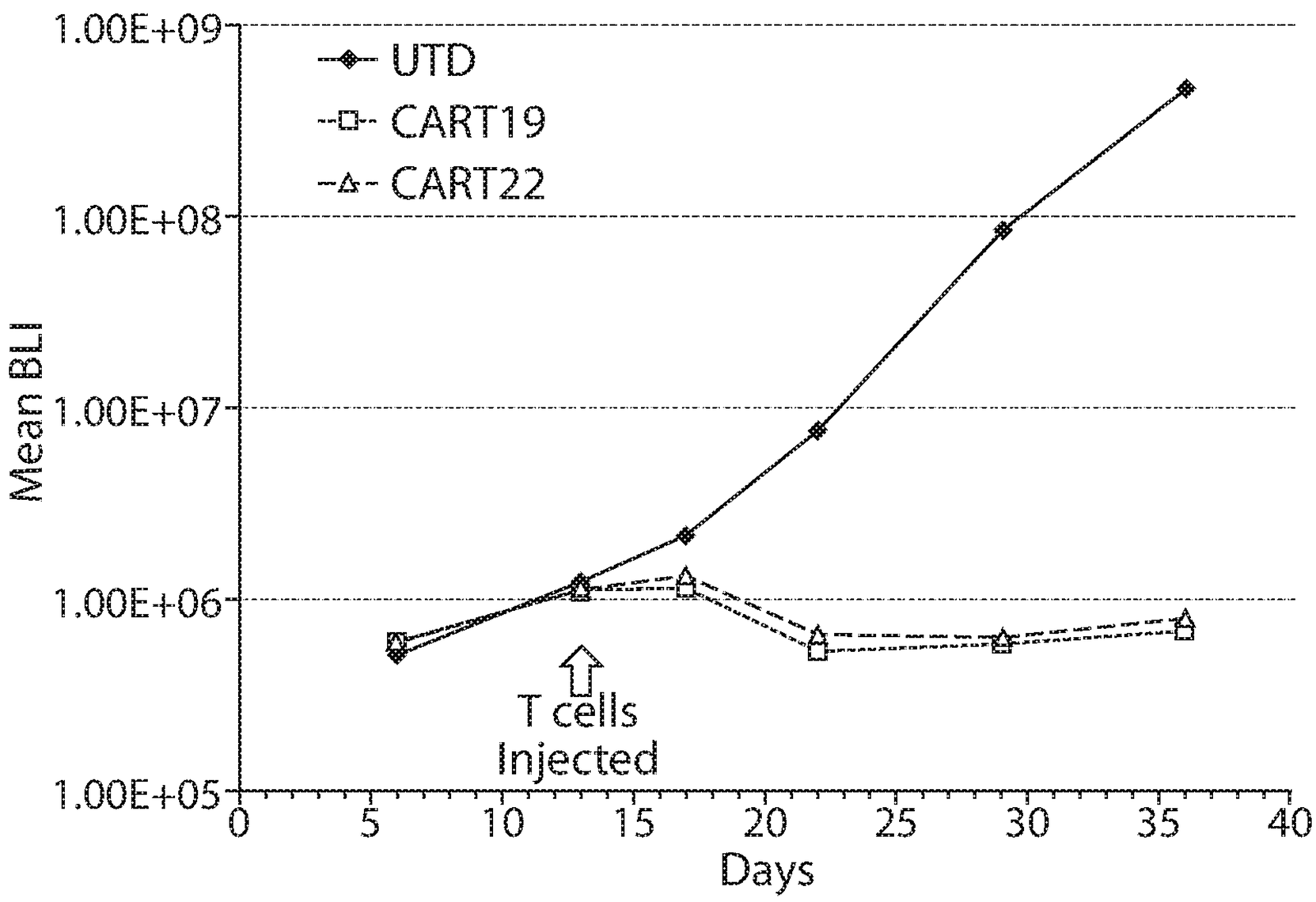

1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
Mean BLI
UTD
CART19
CART22
T cells
Injected
0
5
10
15
20
25
30
35
40
Days

FIG. 37B

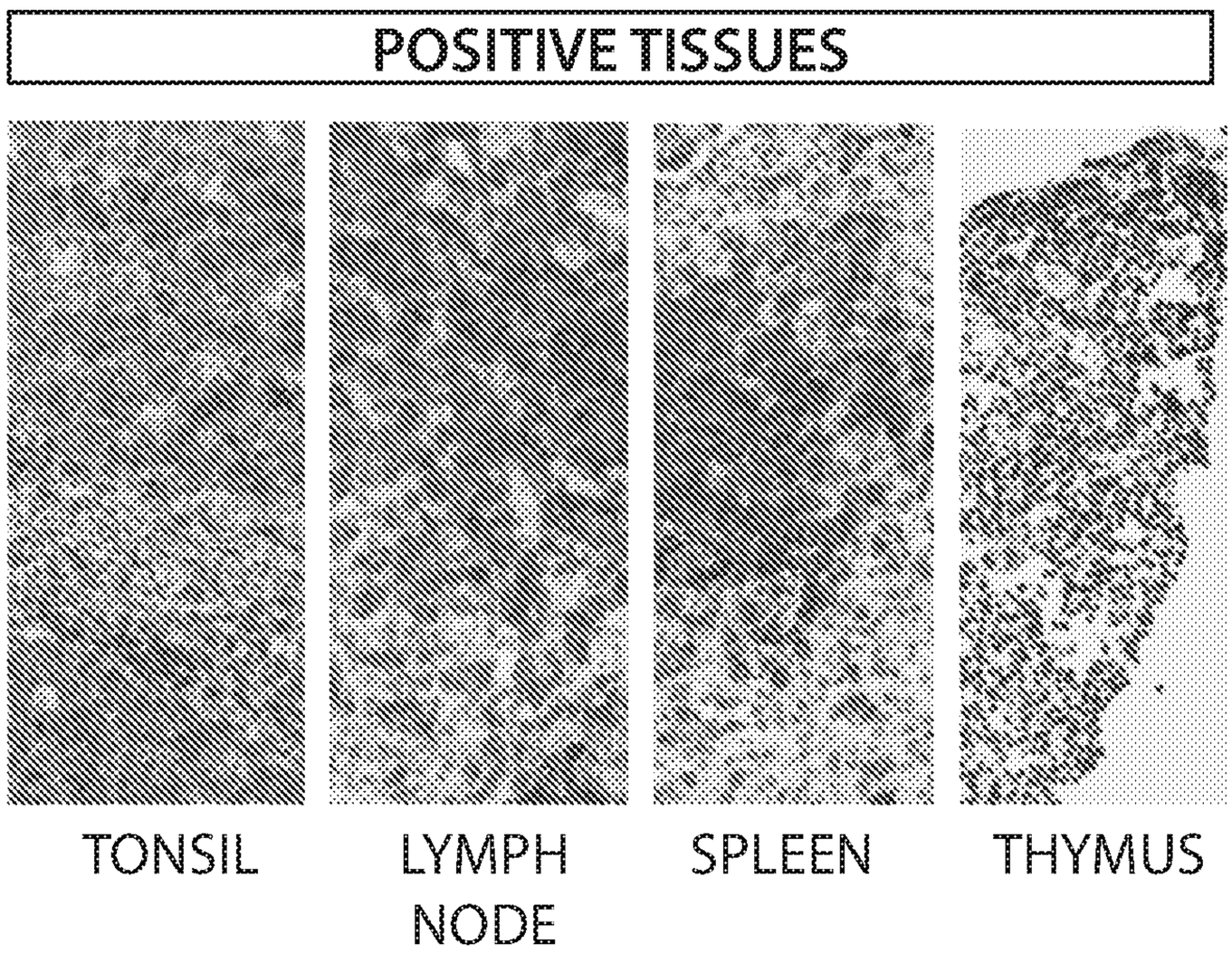
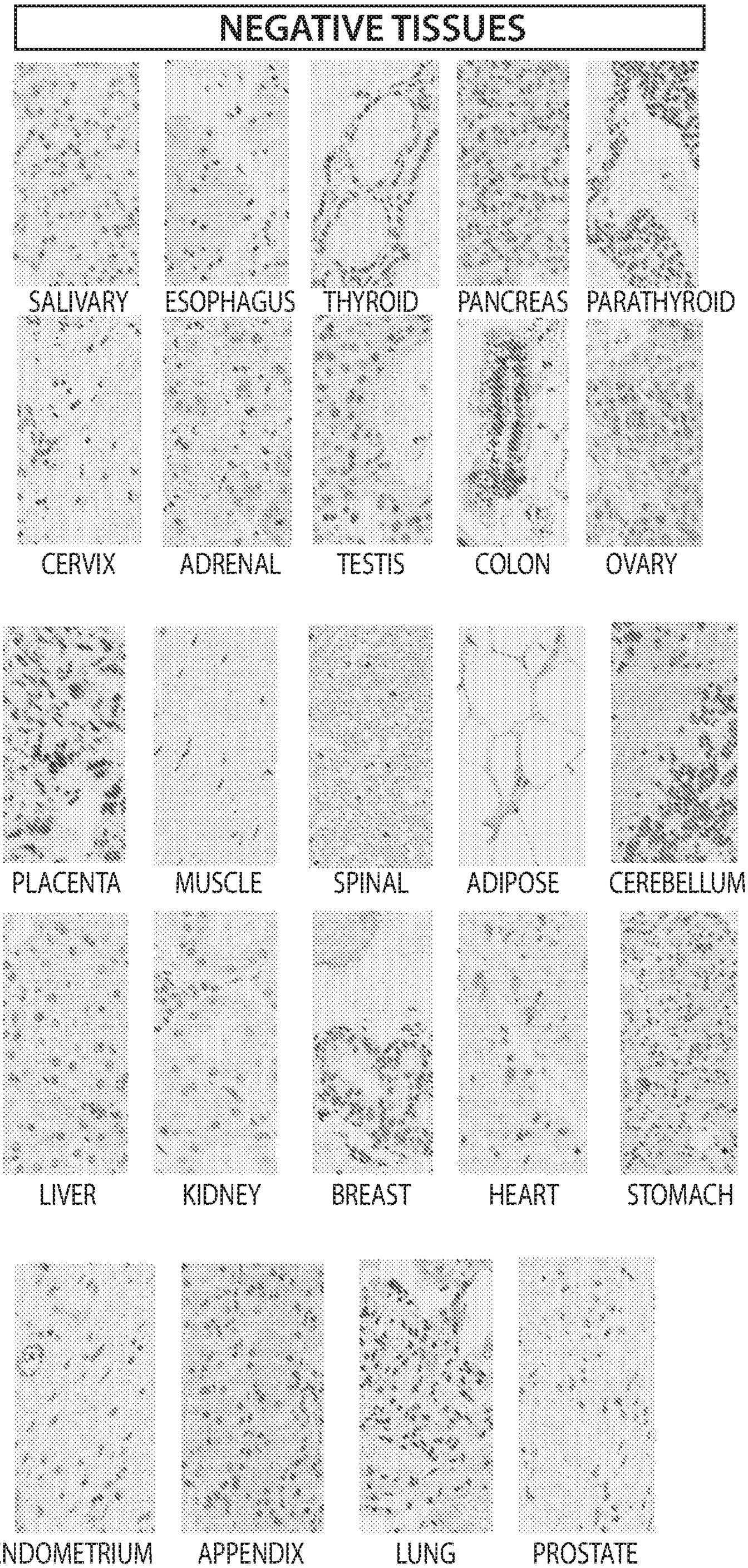


FIG. 38A


NEGATIVE TISSUES
SALIVARY
ESOPHAGUS
THYROID
PANCREAS
PARATHYROID
CERVIX
ADRENAL
TESTIS
COLON
OVARY
PLACENTA
MUSCLE
SPINAL
ADIPOSE
CEREBELLUM
LIVER
KIDNEY
BREAST
HEART
STOMACH
ENDOMETRIUM
APPENDIX
LUNG
PROSTATE

20X

FIG. 38B

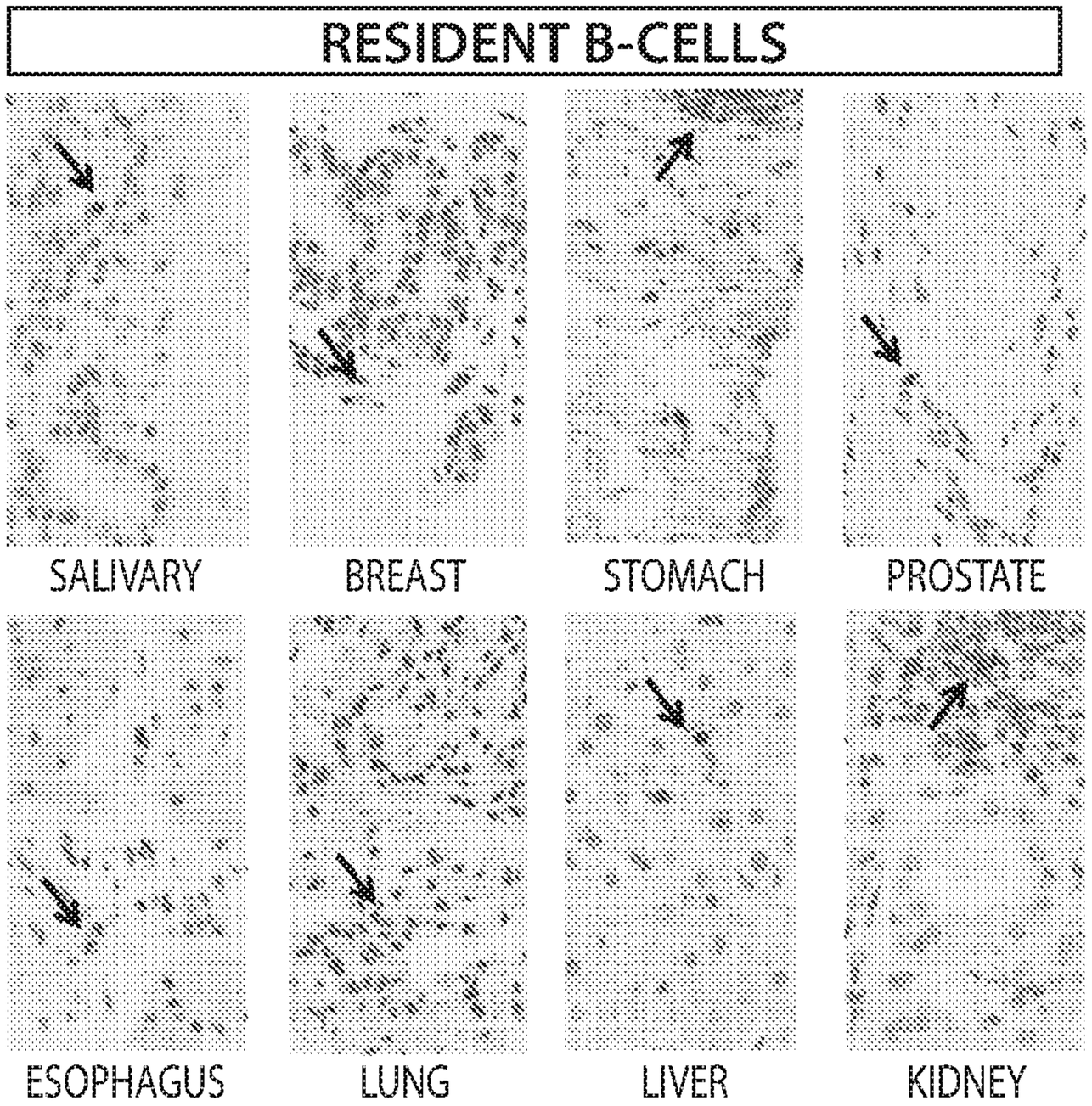

RESIDENT B-CELLS
SALIVARY
BREAST
STOMACH
PROSTATE
ESOPHAGUS
LUNG
LIVER
KIDNEY

FIG. 38C

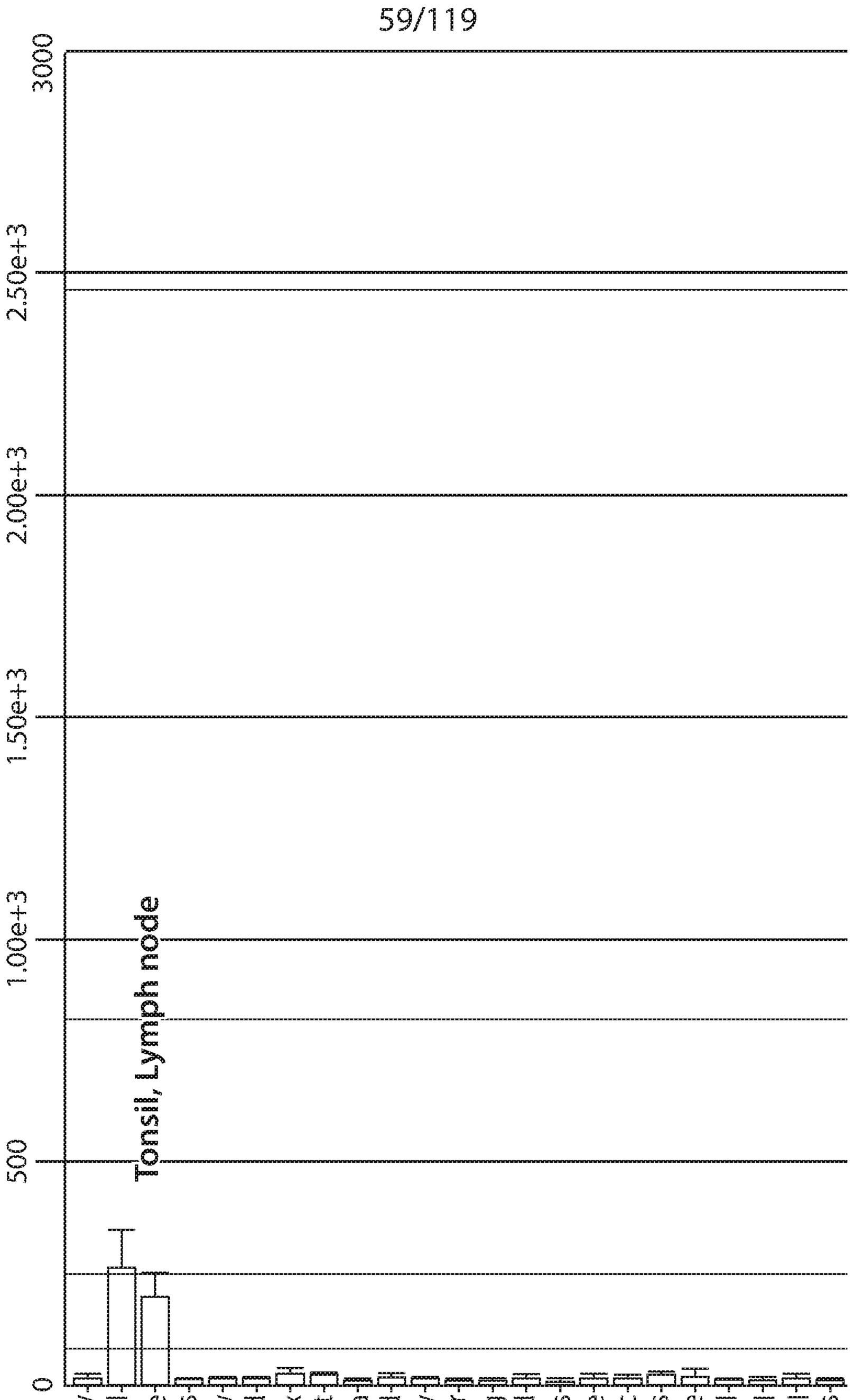

3000
2.50e+3
2.00e+3
1.50e+3
1.00e+3
500
0
Tonsil, Lymph node
Kidney
Tonsil
Lymph node
Thymus
Bone marrow
Adrenal gland
Adrenal Cortex
Olfactory Cult
Trachea
Salivary gland
Pituitary
Fetal liver
Fetal lung
Fetal Thyroid
Uterus
Adipocyte
Pancreatic
Pancreas
Testis Seminiferous Tubule
Testis Leydig Cell
Testis Interstitial Cell
Testis Germ Cell
Testis

FIG. 39-1

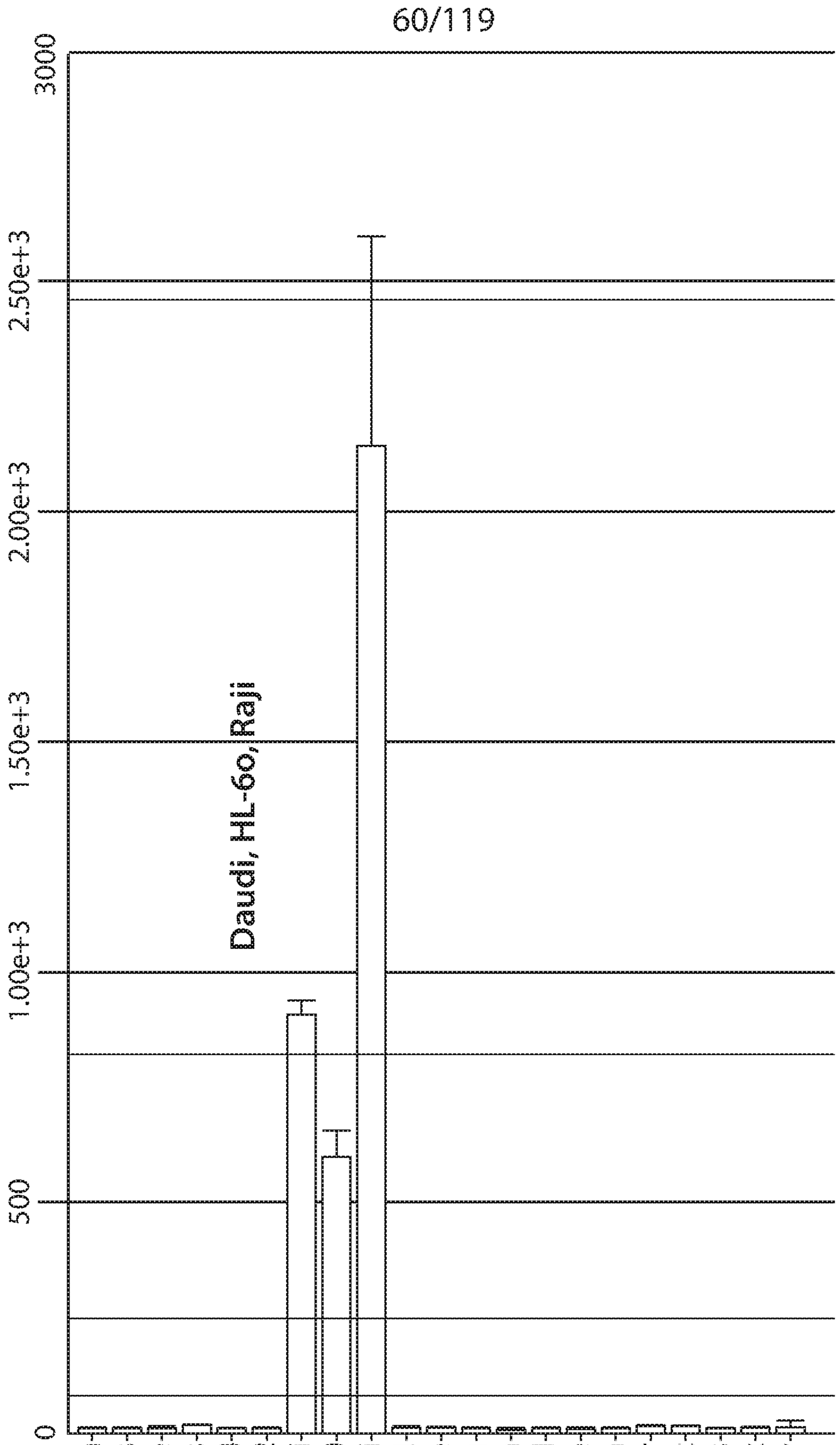

60/119
3000
2.50e+3
2.00e+3
1.50e+3
1.00e+3
500
0
Daudi, HL-60, Raji
Colorectal adenocarcinoma
Bronchial Epithelial Cells
Smooth Muscle
Cardiac Myocytes
Leukemia lymphoblastoid MOLM14
Leukemia_chronic myelogenous K562
Lymphoma_burkitt Daudi
Leukemia_promyelocytic HL-60
Lymphoma_burkitt Raji
Thyroid
Prostate
Lung
Placenta
CD71+ Early Erythroid
Small intestine
Colon
Liver
Heart
Uterus Corpus
Appendix
Ovary

FIG. 39-2

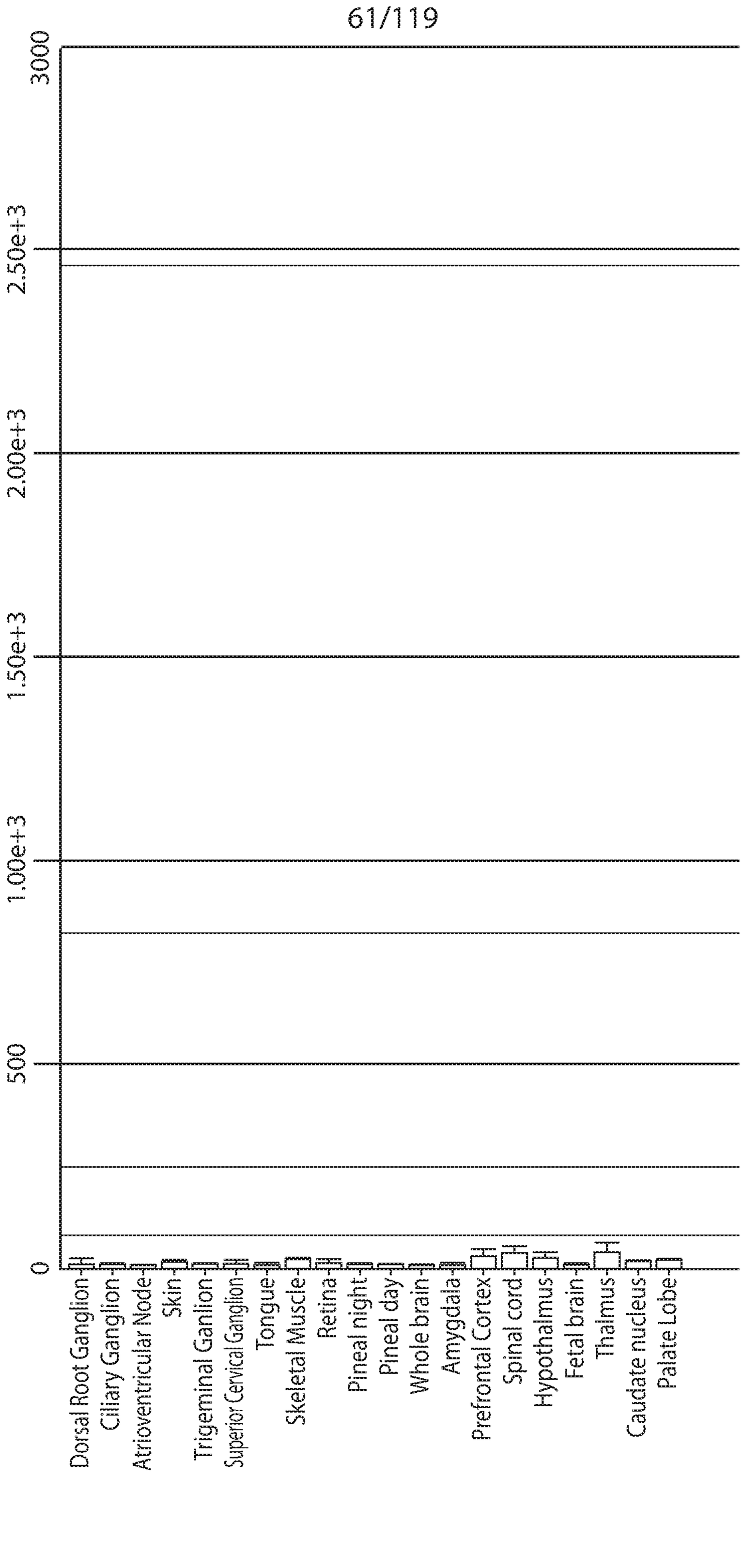

61/119
3000
2.50e+3
2.00e+3
1.50e+3
1.00e+3
500
0
Dorsal Root Ganglion
Ciliary Ganglion
Atrioventricular Node
Skin
Trigeminal Ganlion
Superior Cervical Ganglion
Tongue
Skeletal Muscle
Retina
Pineal night
Pineal day
Whole brain
Amygdala
Prefrontal Cortex
Spinal cord
Hypothalmus
Fetal brain
Thalmus
Caudate nucleus
Palate Lobe

FIG. 39-3

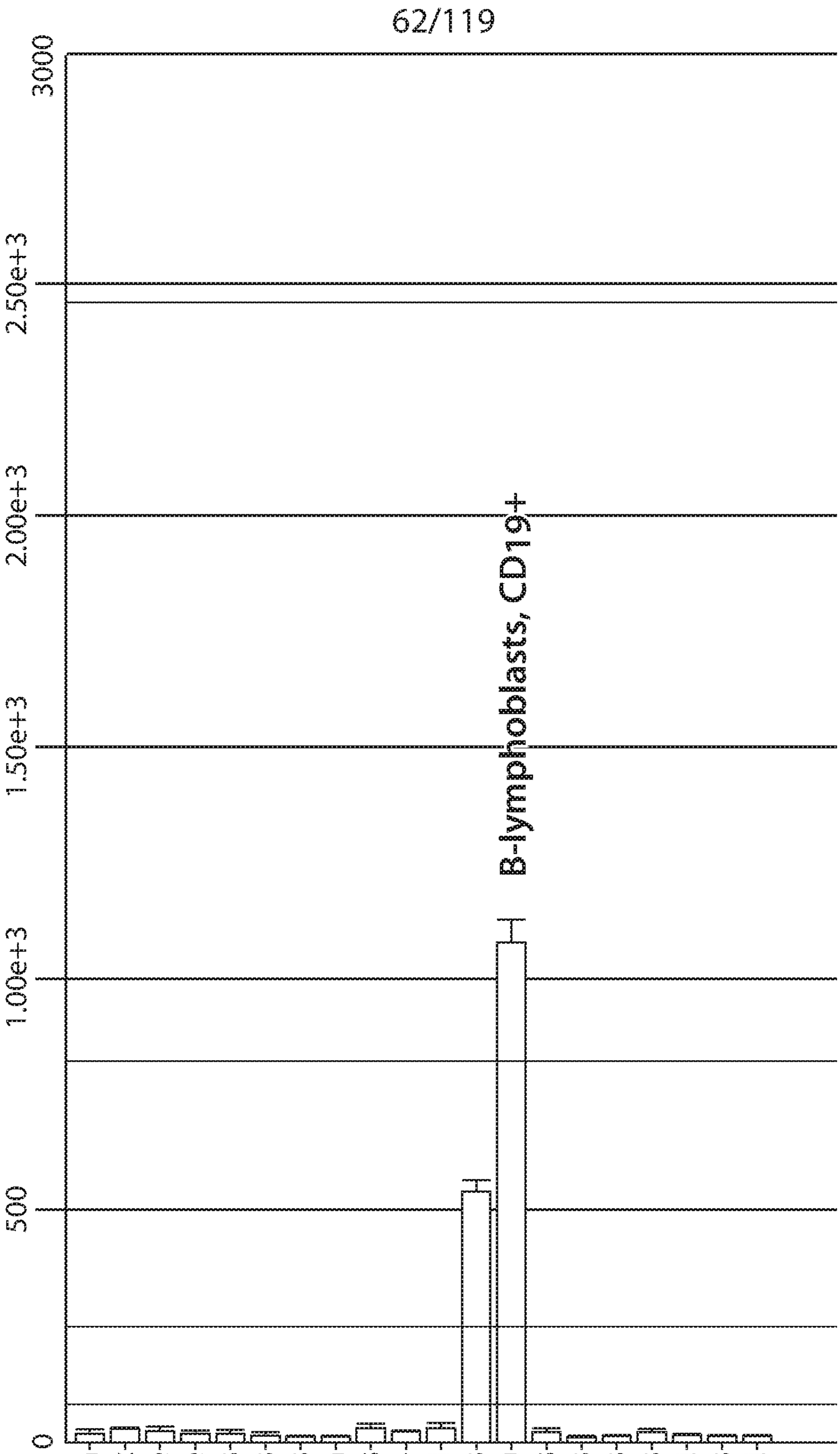

3000
2.50e+3
2.00e+3
1.50e+3
1.00e+3
500
0
B-lymphoblasts, CD19+
Medulla Oblongata
Cingulate Cortex
Occipital Lobe
Temporal Lobe
Subthalamic Nucleus
Fons
Globus Pallidus
Cerebellum
Cerebellum Peduncles
CD34+
CD105+ Endothelial
721 B Lymphoblasts
CD19+ BCells(neg.sel.)
BDCA4+_Dendritic Cells
CD8+_T cells
CD4+_T cells
CD55+_NK Cells
CD33+ Myeloid
CD14+ Monocytes
Whole Blood
Source: BioGPS.org, GeneAtlas U133A, gcrma

FIG. 39-4

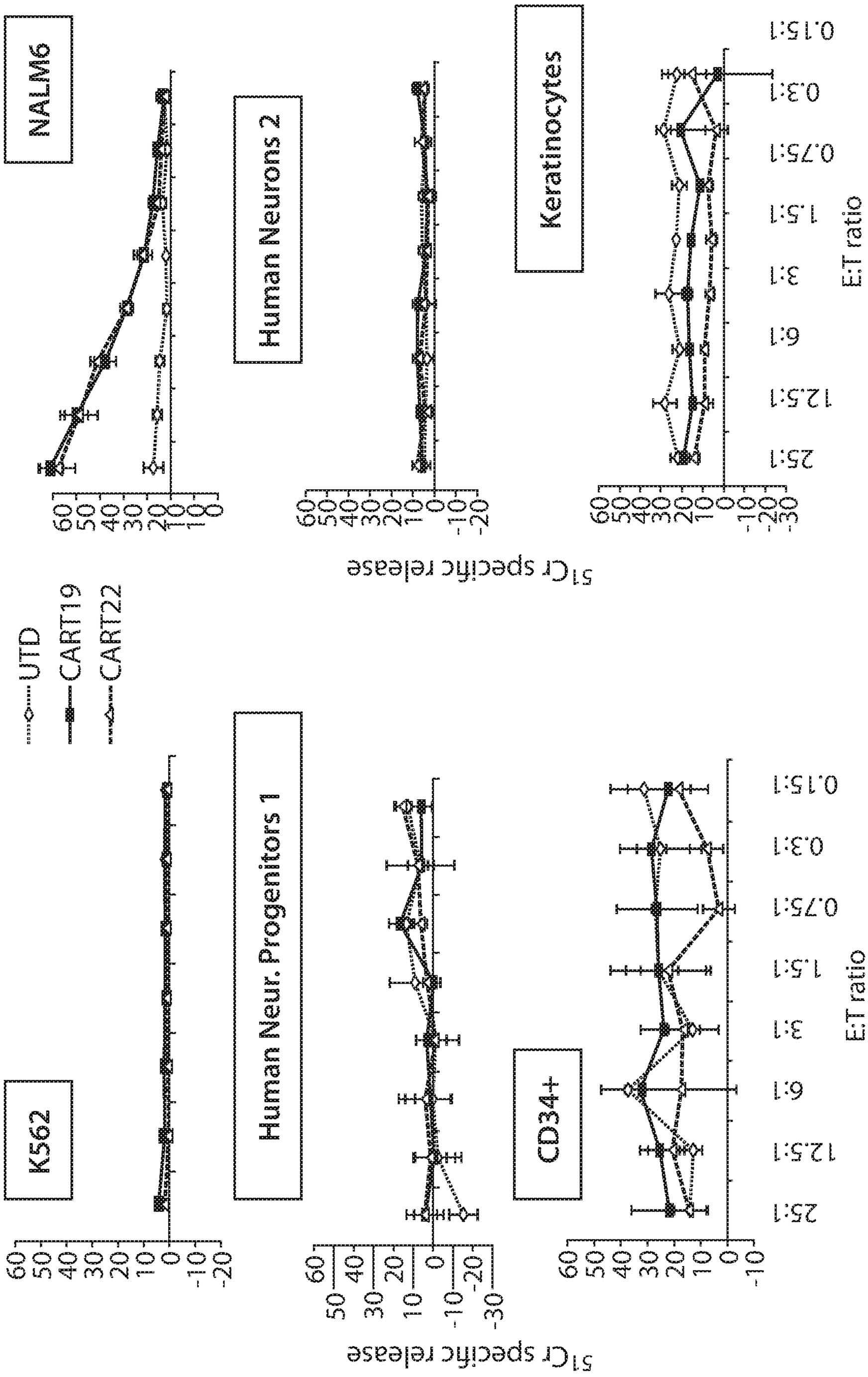

NALM6
Human Neuron. Progenitors 2
Keratinocytes
K562
Human Neur. Progenitors 1
CD34+
UTD
CART19
CART22
51Cr specific release
E:T ratio
0.15:1
0.3:1
0.75:1
1.5:1
3:1
6:1
12.5:1
25:1

FIG. 40

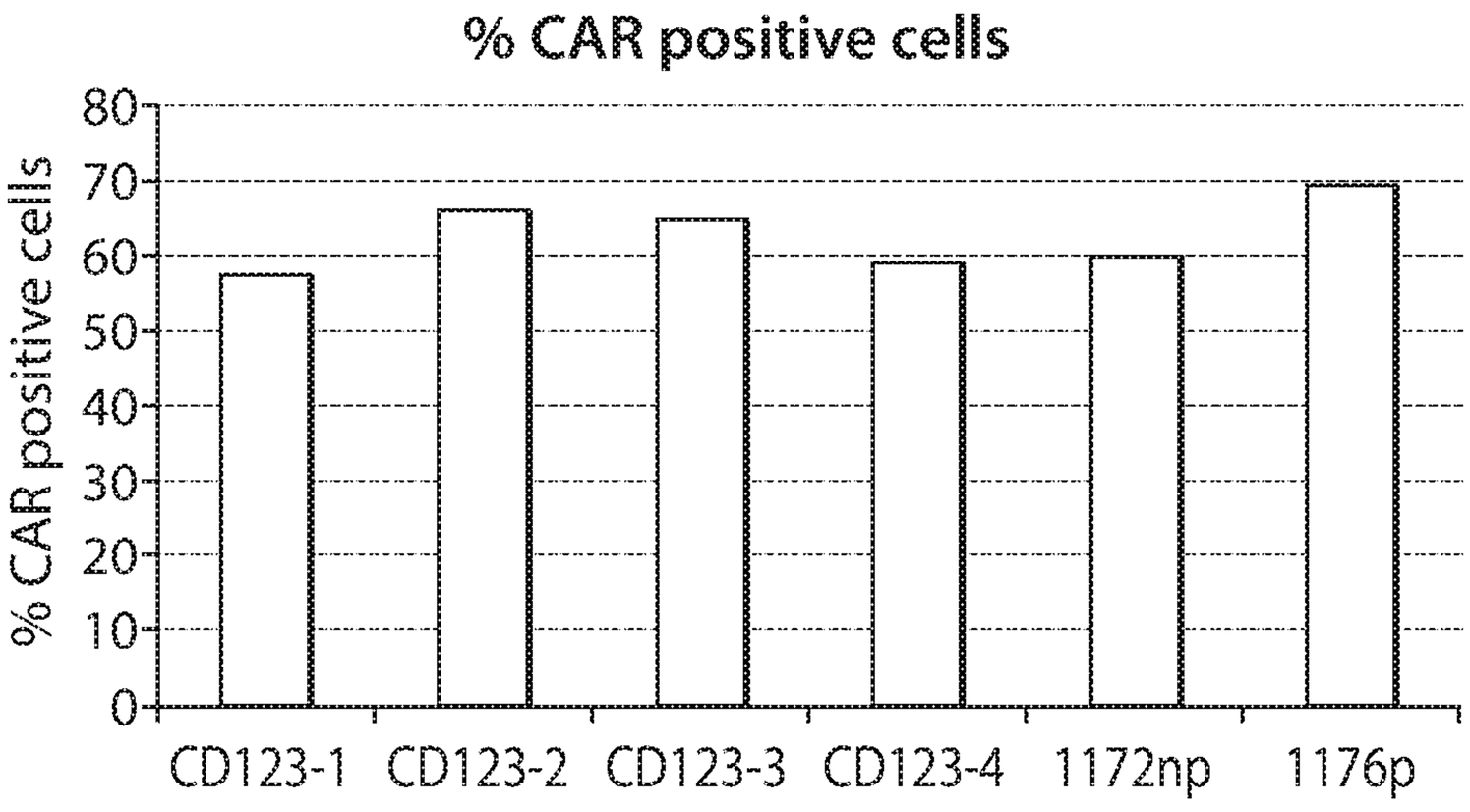

% CAR positive cells
% CAR positive cells
80
70
60
50
40
30
20
10
0
CD123-1
CD123-2
CD123-3
CD123-4
1172np
1176p

FIG. 41

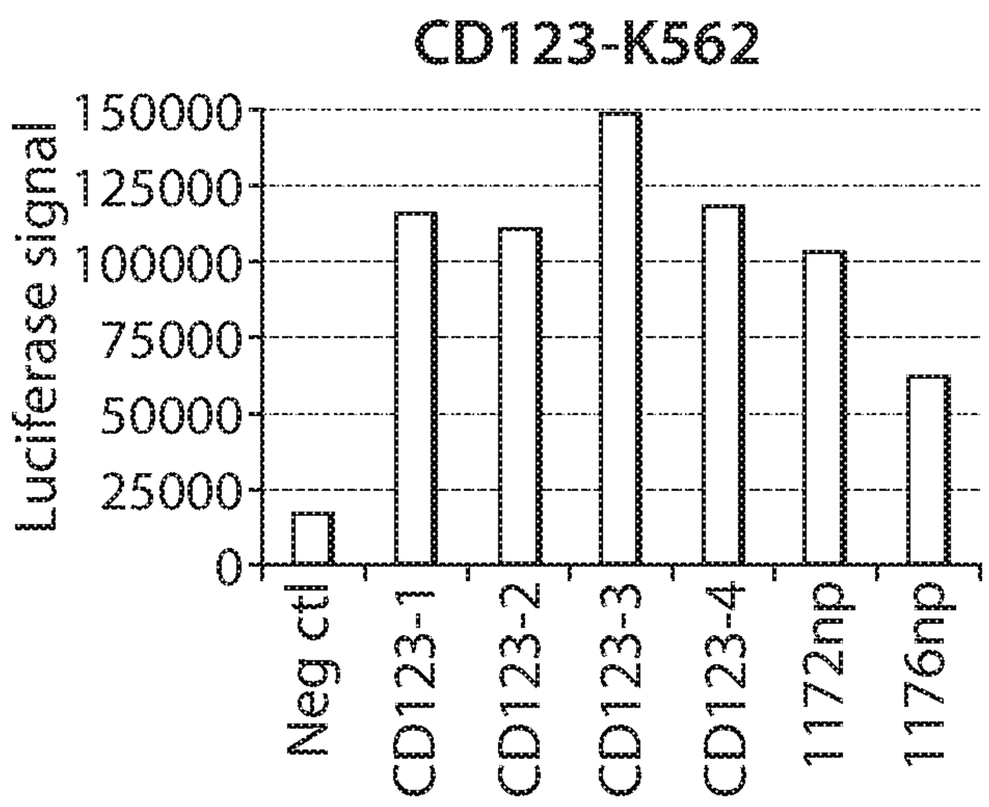


FIG. 42A

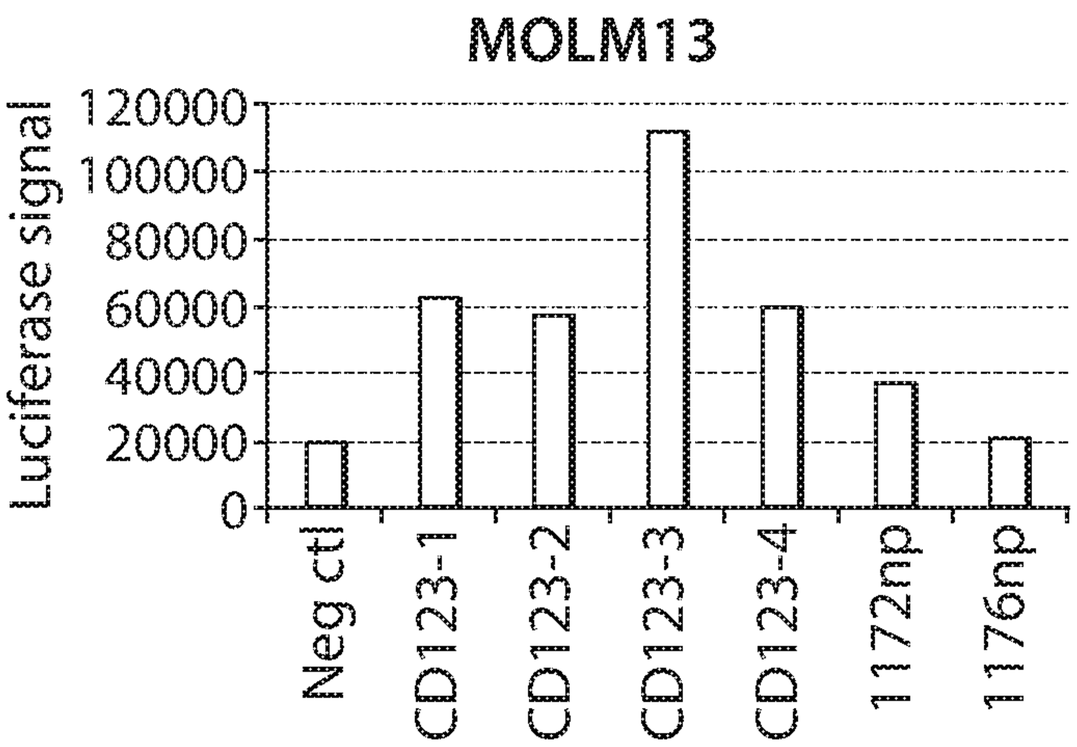


FIG. 42B

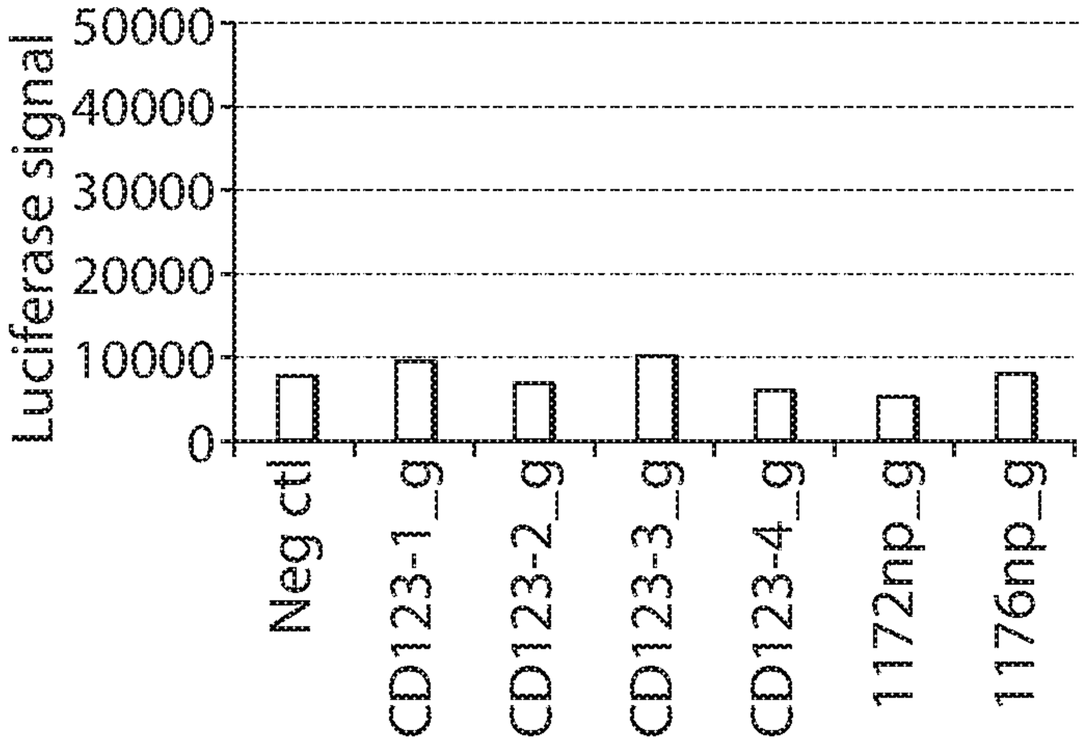


FIG. 42C

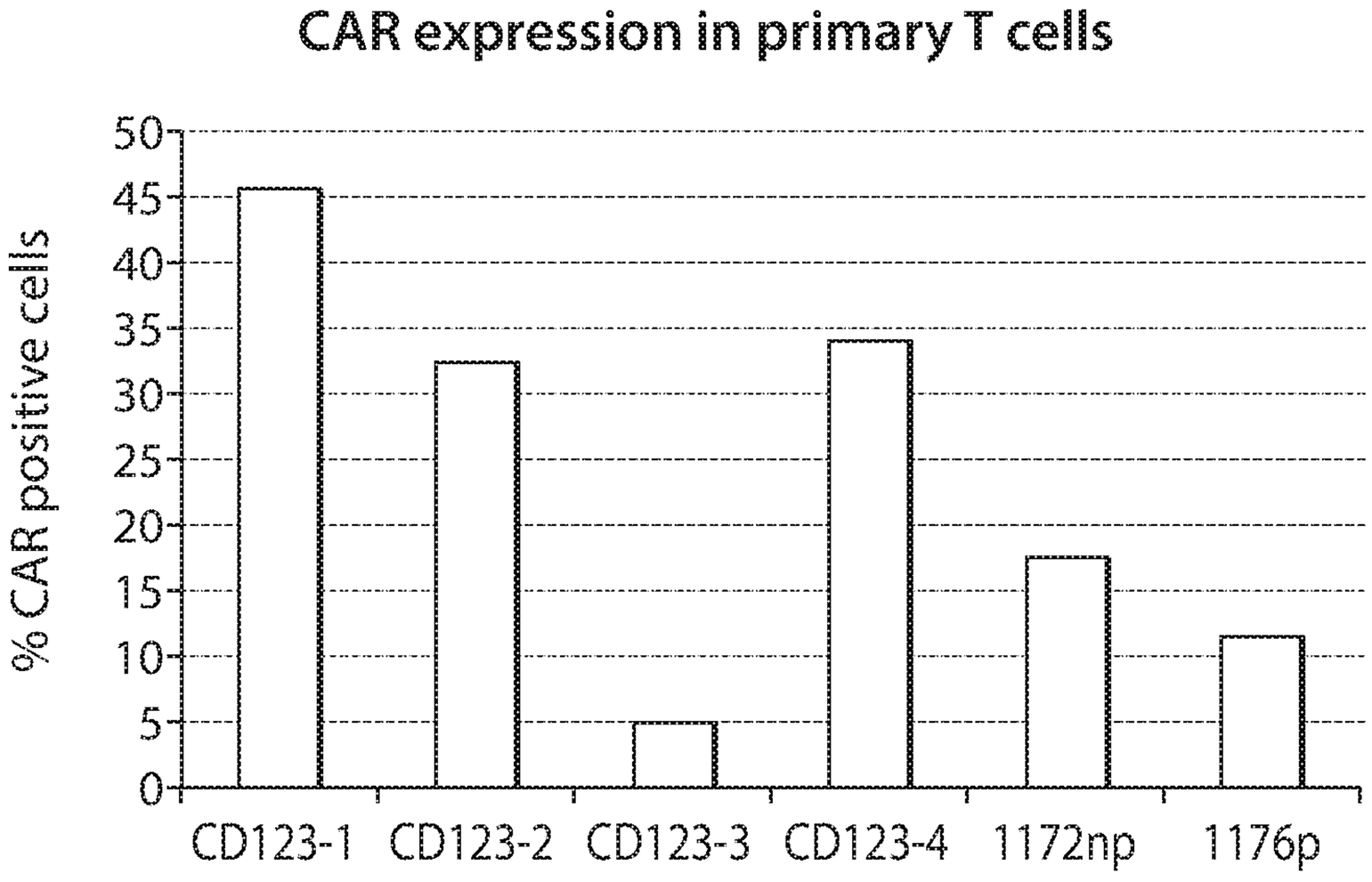

CAR expression in primary T cells
% CAR positive cells
50
45
40
35
30
25
20
15
10
5
0
CD123-1
CD123-2
CD123-3
CD123-4
1172np
1176p

FIG. 43A

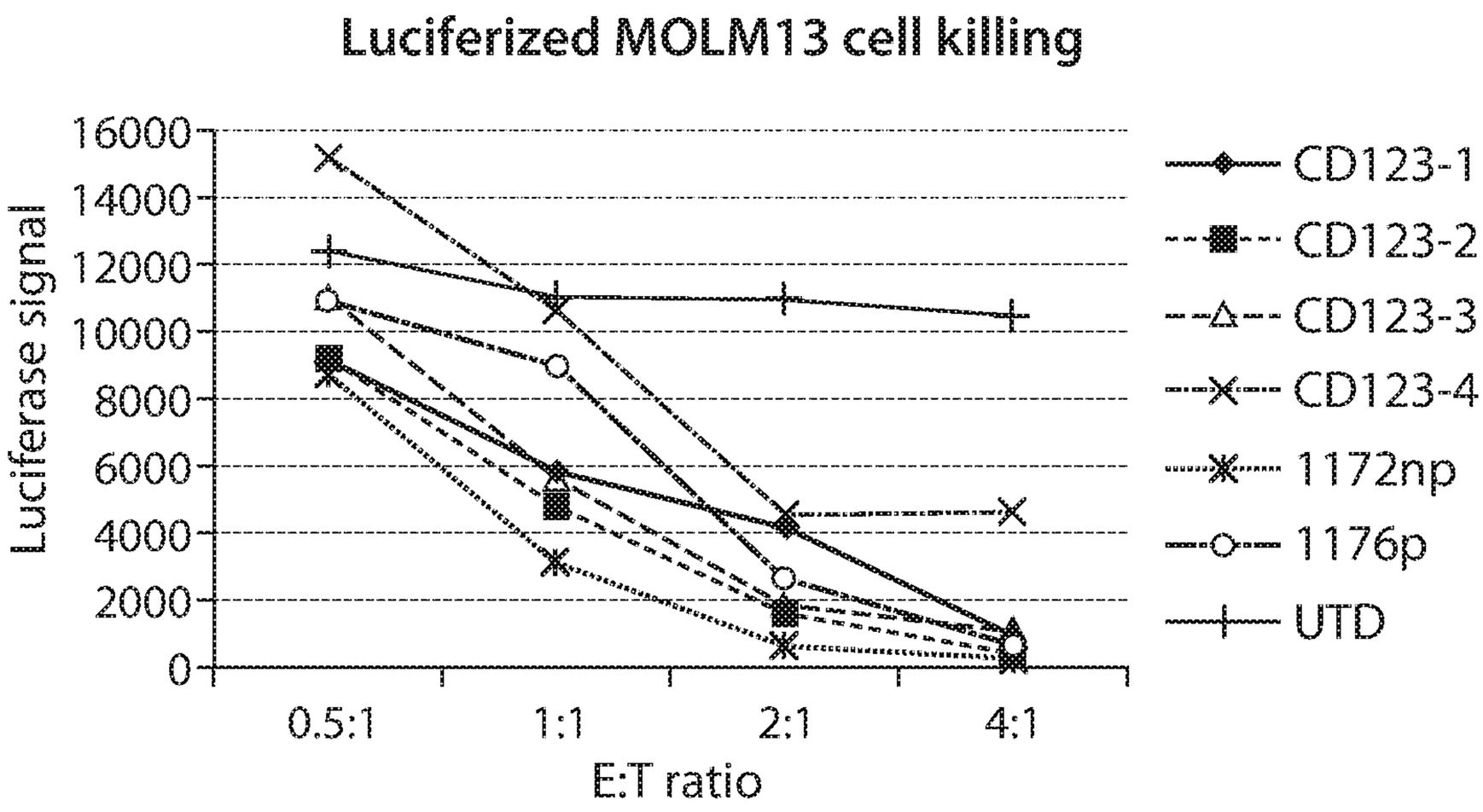
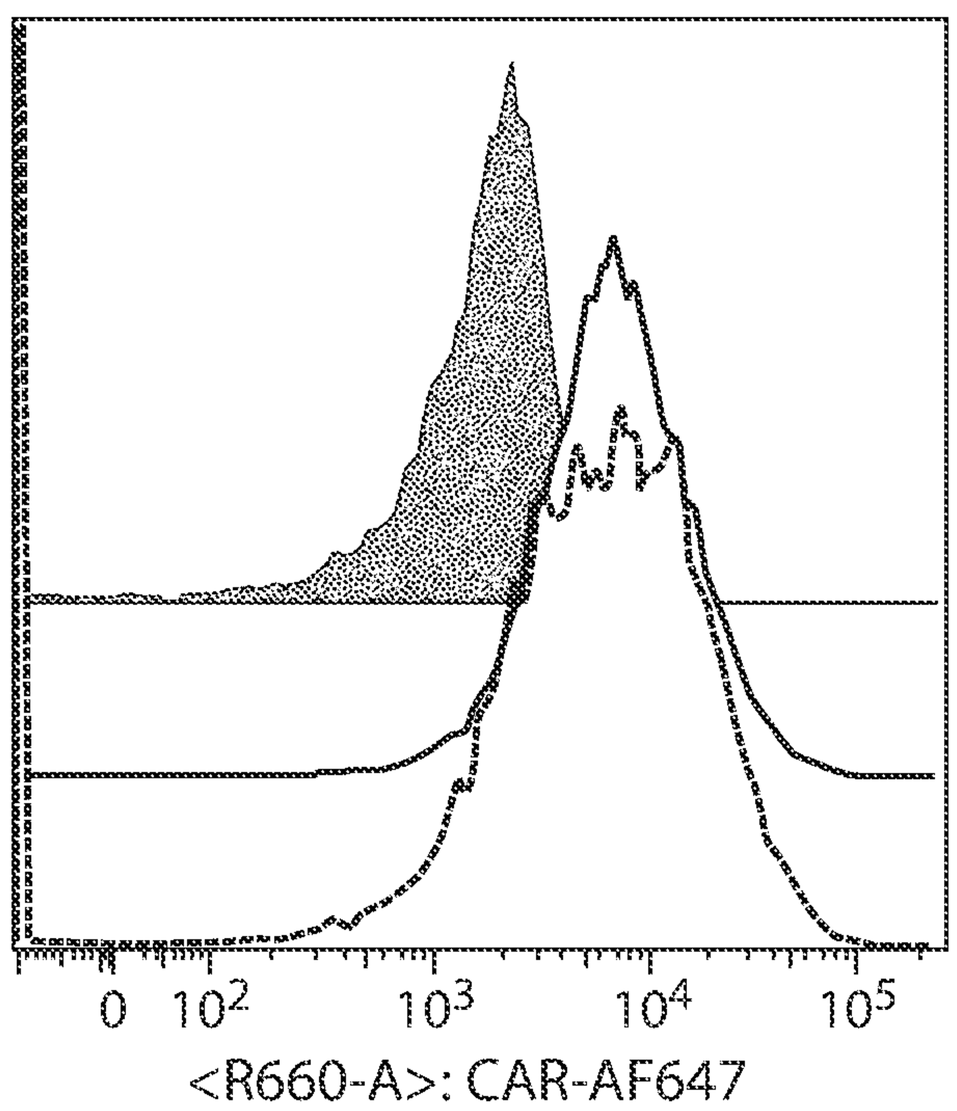
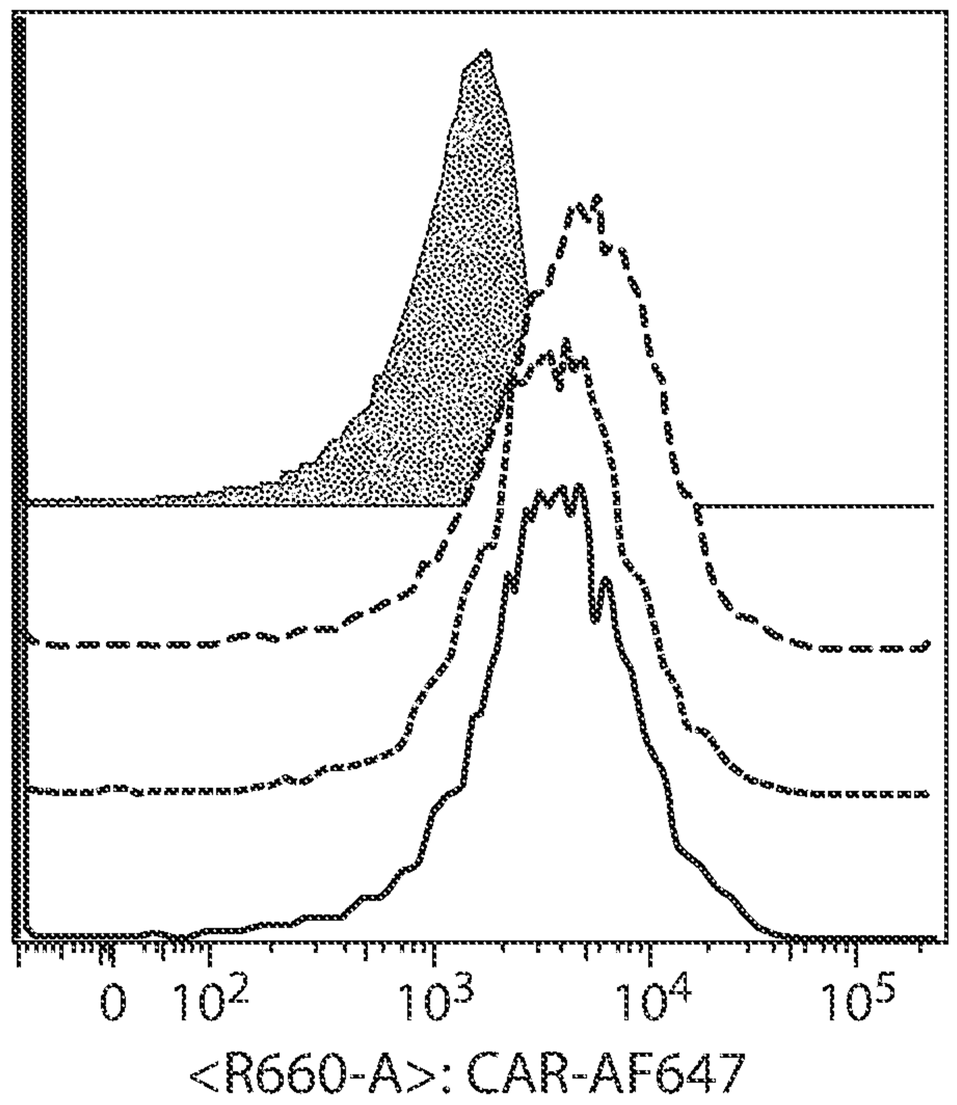

Luciferized MOLM13 cell killing
Luciferase signal
16000
14000
12000
10000
8000
6000
4000
2000
0
0.5:1
1:1
2:1
4:1
E:T ratio
CD123-1
CD123-2
CD123-3
CD123-4
1172np
1176p
UTD

FIG. 43B



	Sample	Median:<R660-A>
------	1176	5965
———	1172	6996
▒	UTD	1603

FIG. 44A



	Sample	Median:<R660-A>
———	NVS4	3323
- - - - -	NVS3	3356
— — —	NVS2	4265
▒	UTD IGG	1289

FIG. 44B

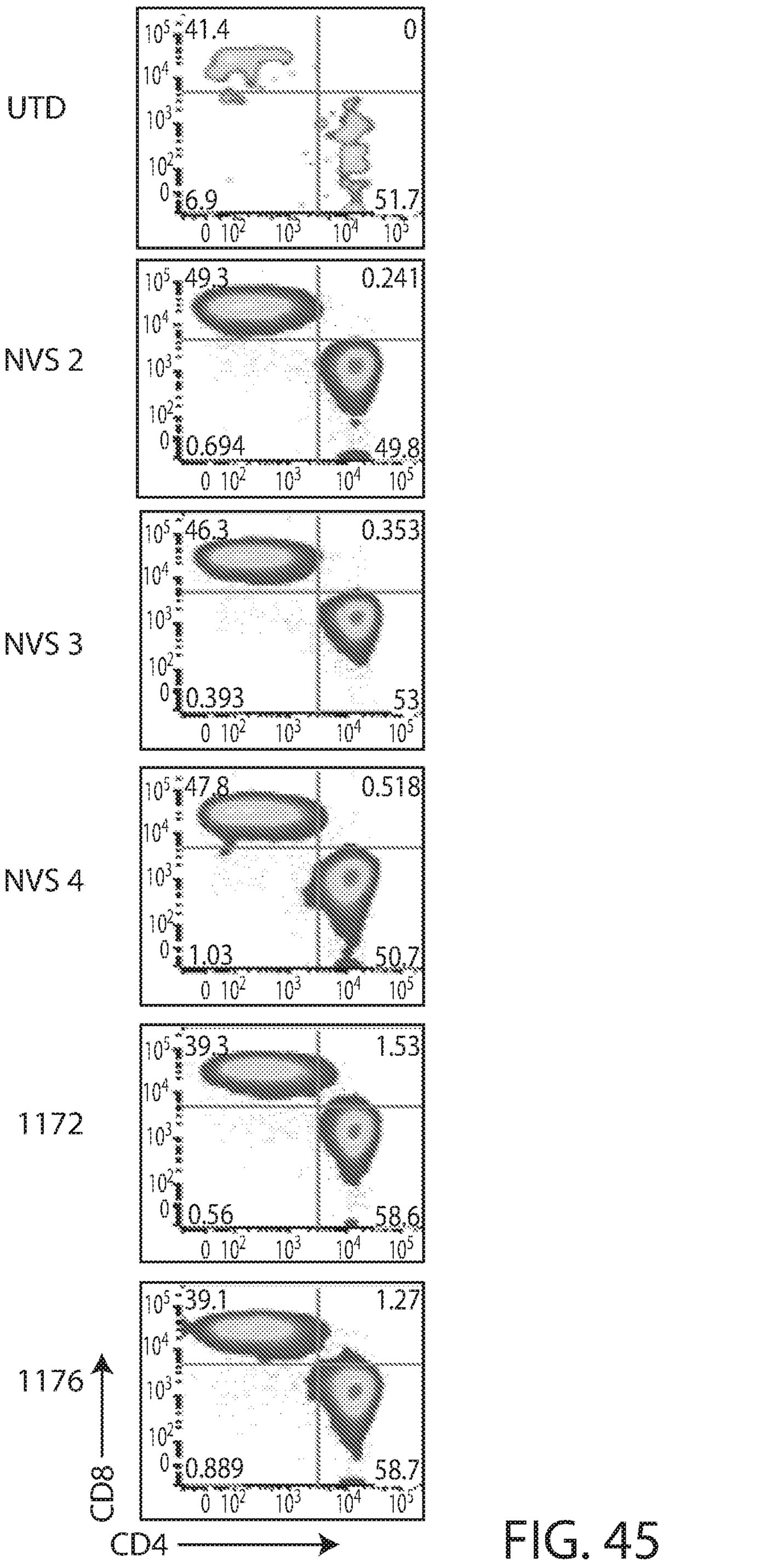

UTD
41.4
0
6.9
51.7
NVS 2
49.3
0.241
0.694
49.8
NVS 3
46.3
0.353
0.393
53
NVS 4
47.8
0.518
1.03
50.7
1172
39.3
1.53
0.56
58.6
1176
39.1
1.27
0.889
58.7
CD8
CD4
0
10^2
10^3
10^4
10^5

FIG. 45

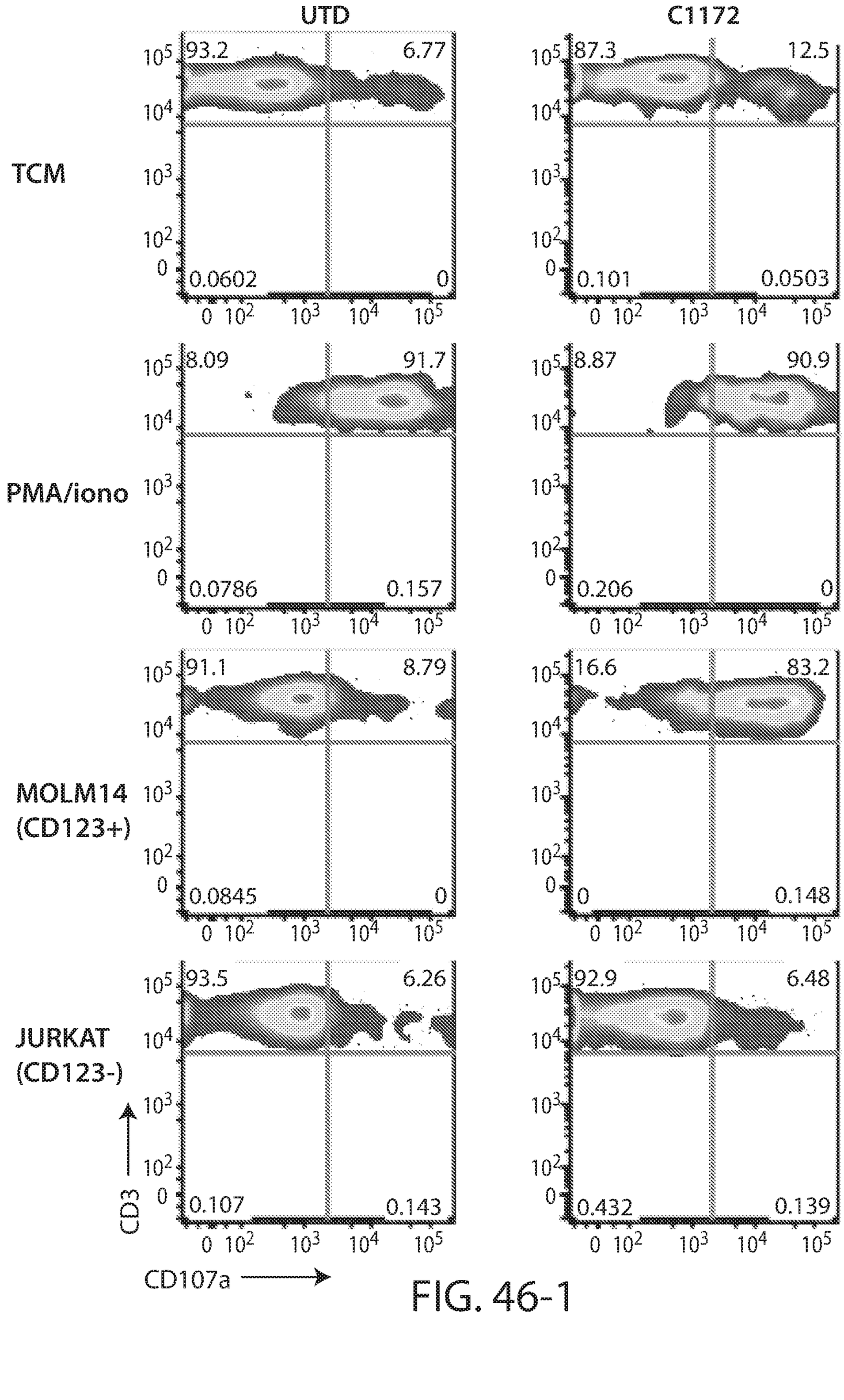

UTD
C1172
TCM
PMA/iono
MOLM14 (CD123+)
JURKAT (CD123-)
93.2
6.77
0.0602
0
87.3
12.5
0.101
0.0503
8.09
91.7
0.0786
0.157
8.87
90.9
0.206
0
91.1
8.79
0.0845
0
16.6
83.2
0
0.148
93.5
6.26
0.107
0.143
92.9
6.48
0.432
0.139
CD3
CD107a
0 10^2 10^3 10^4 10^5

FIG. 46-1

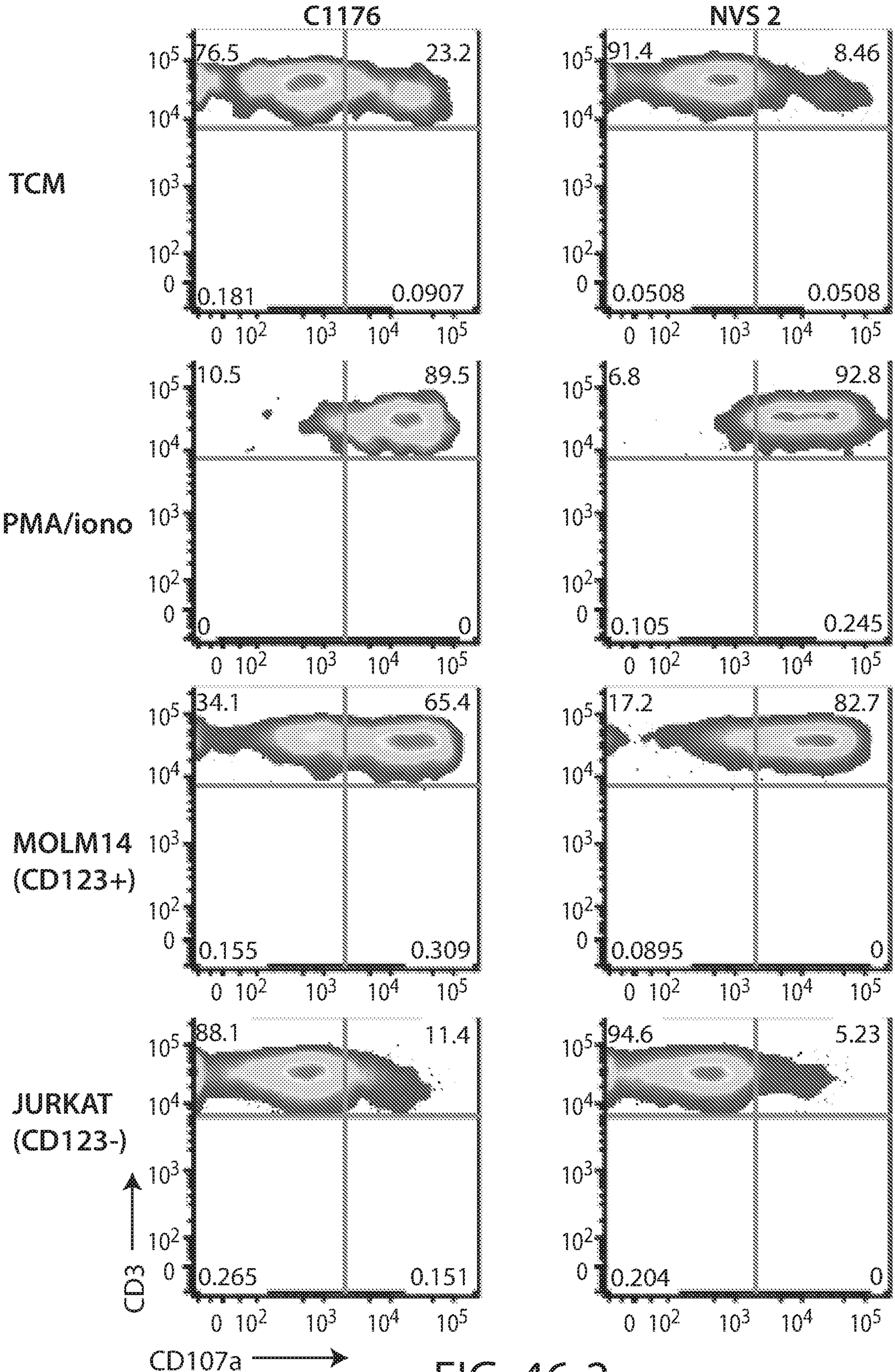

C1176
NVS 2
TCM
PMA/iono
MOLM14 (CD123+)
JURKAT (CD123-)
CD3
CD107a
76.5
23.2
0.181
0.0907
91.4
8.46
0.0508
0.0508
10.5
89.5
0
0
6.8
92.8
0.105
0.245
34.1
65.4
0.155
0.309
17.2
82.7
0.0895
0
88.1
11.4
0.265
0.151
94.6
5.23
0.204
0

FIG. 46-2

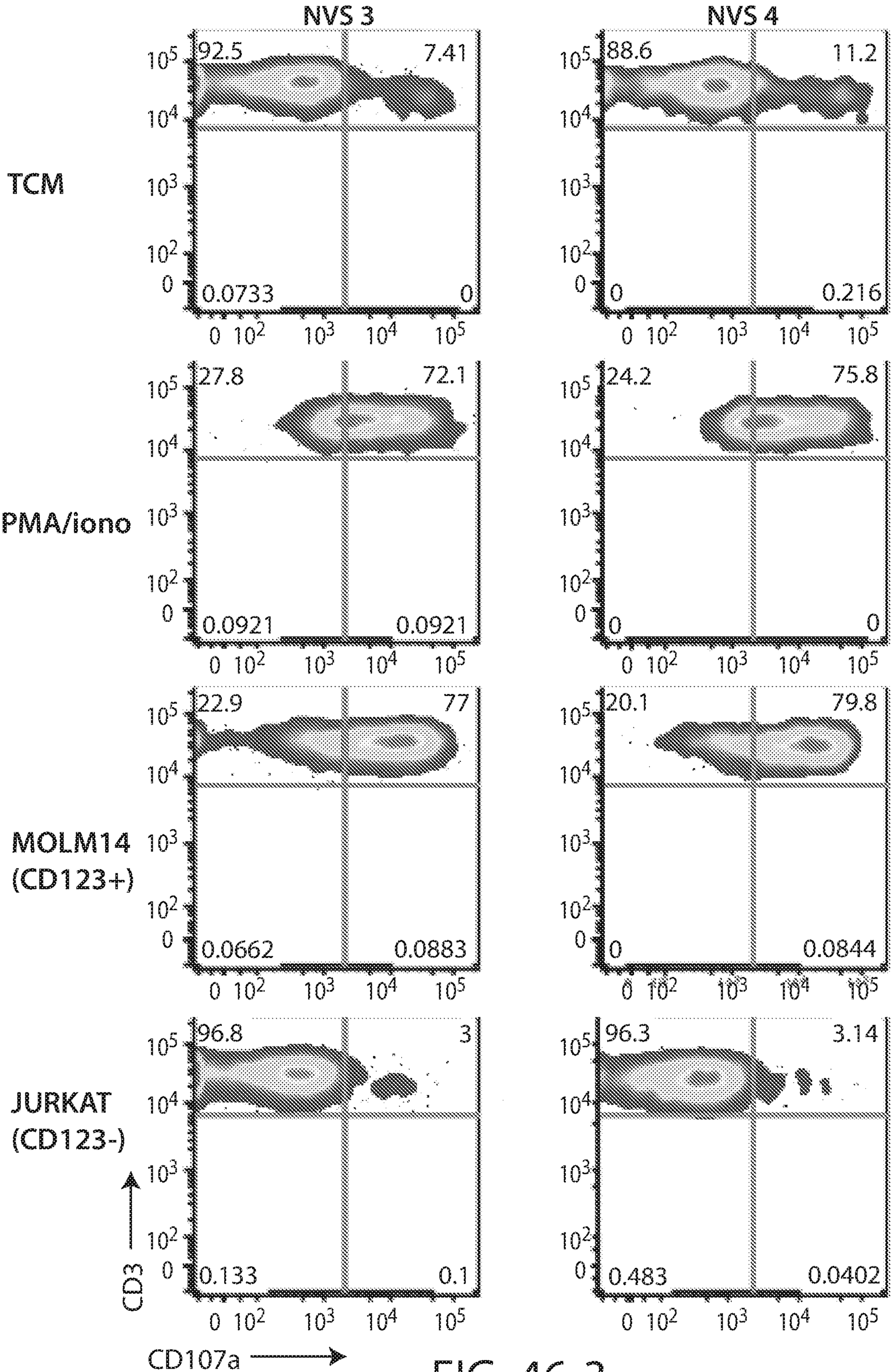

NVS 3
NVS 4
TCM
92.5
7.41
0.0733
0
88.6
11.2
0
0.216
PMA/iono
27.8
72.1
0.0921
0.0921
24.2
75.8
0
0
MOLM14
(CD123+)
22.9
77
0.0662
0.0883
20.1
79.8
0
0.0844
JURKAT
(CD123-)
96.8
3
0.133
0.1
96.3
3.14
0.483
0.0402
CD3
CD107a
0 10^2 10^3 10^4 10^5

FIG. 46-3

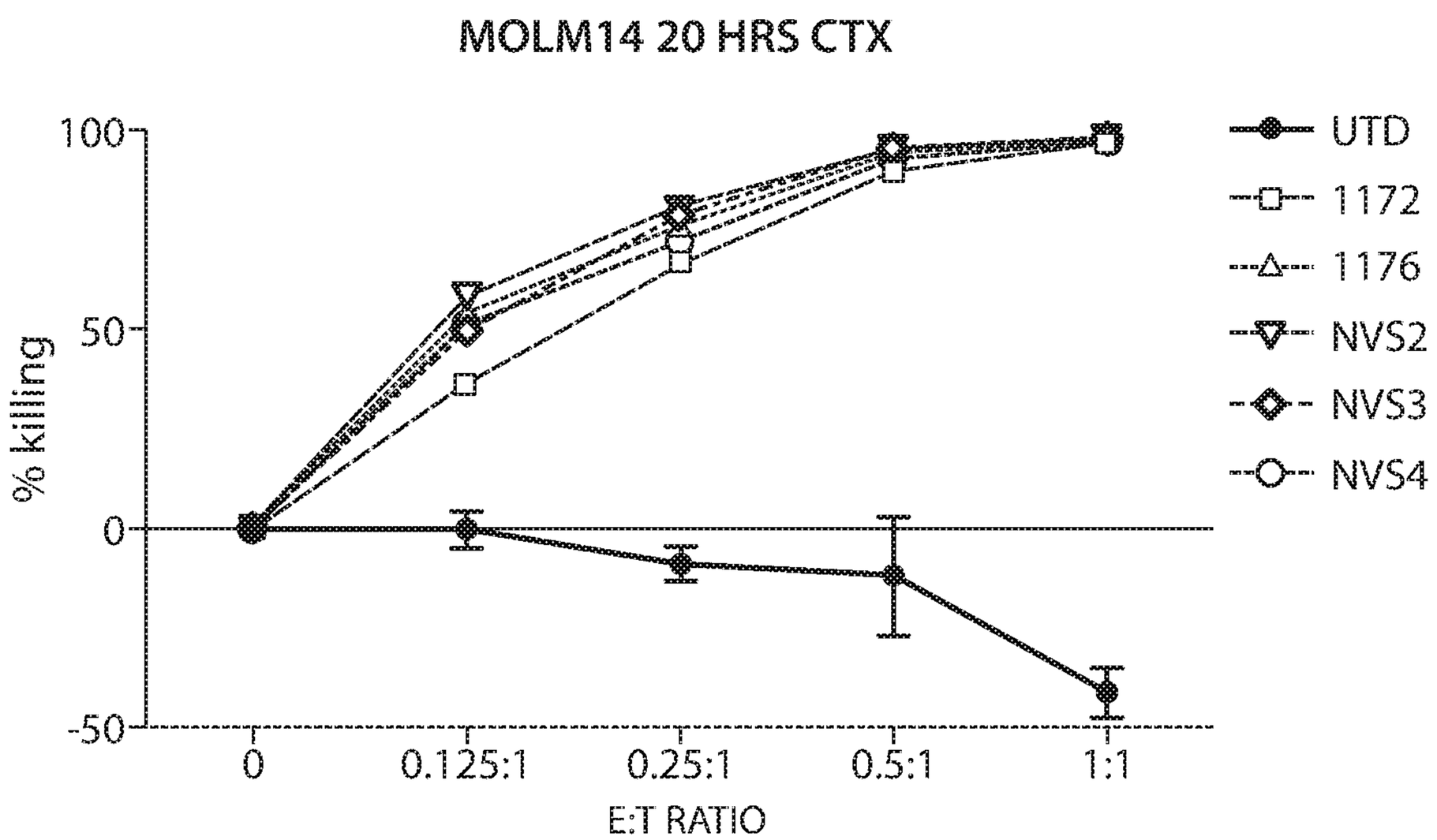

MOLM14 20 HRS CTX
% killing
100
50
0
-50
0
0.125:1
0.25:1
0.5:1
1:1
E:T RATIO
UTD
1172
1176
NVS2
NVS3
NVS4

FIG. 47

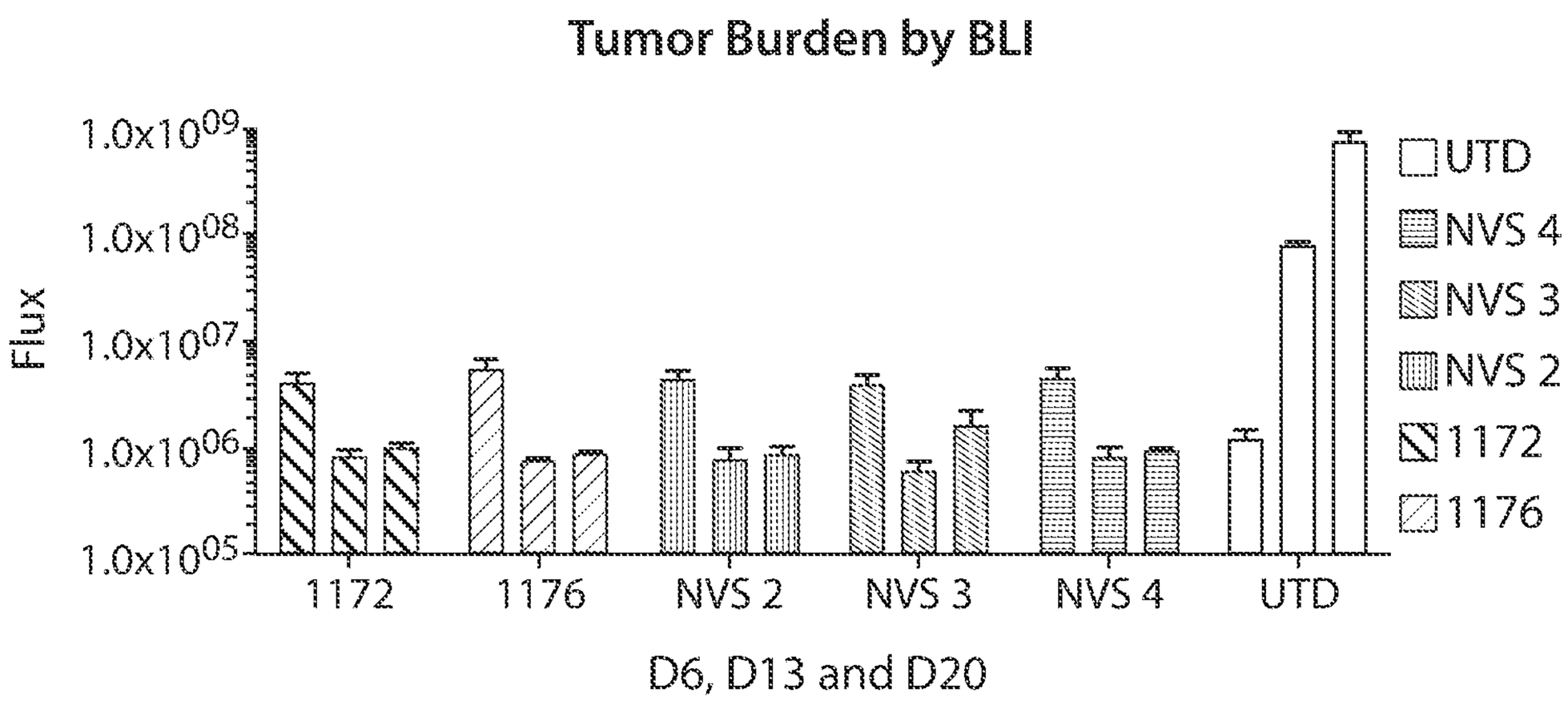

Tumor Burden by BLI
Flux
1.0x10^09
1.0x10^08
1.0x10^07
1.0x10^06
1.0x10^05
1172
1176
NVS 2
NVS 3
NVS 4
UTD
D6, D13 and D20
UTD
NVS 4
NVS 3
NVS 2
1172
1176

FIG. 48

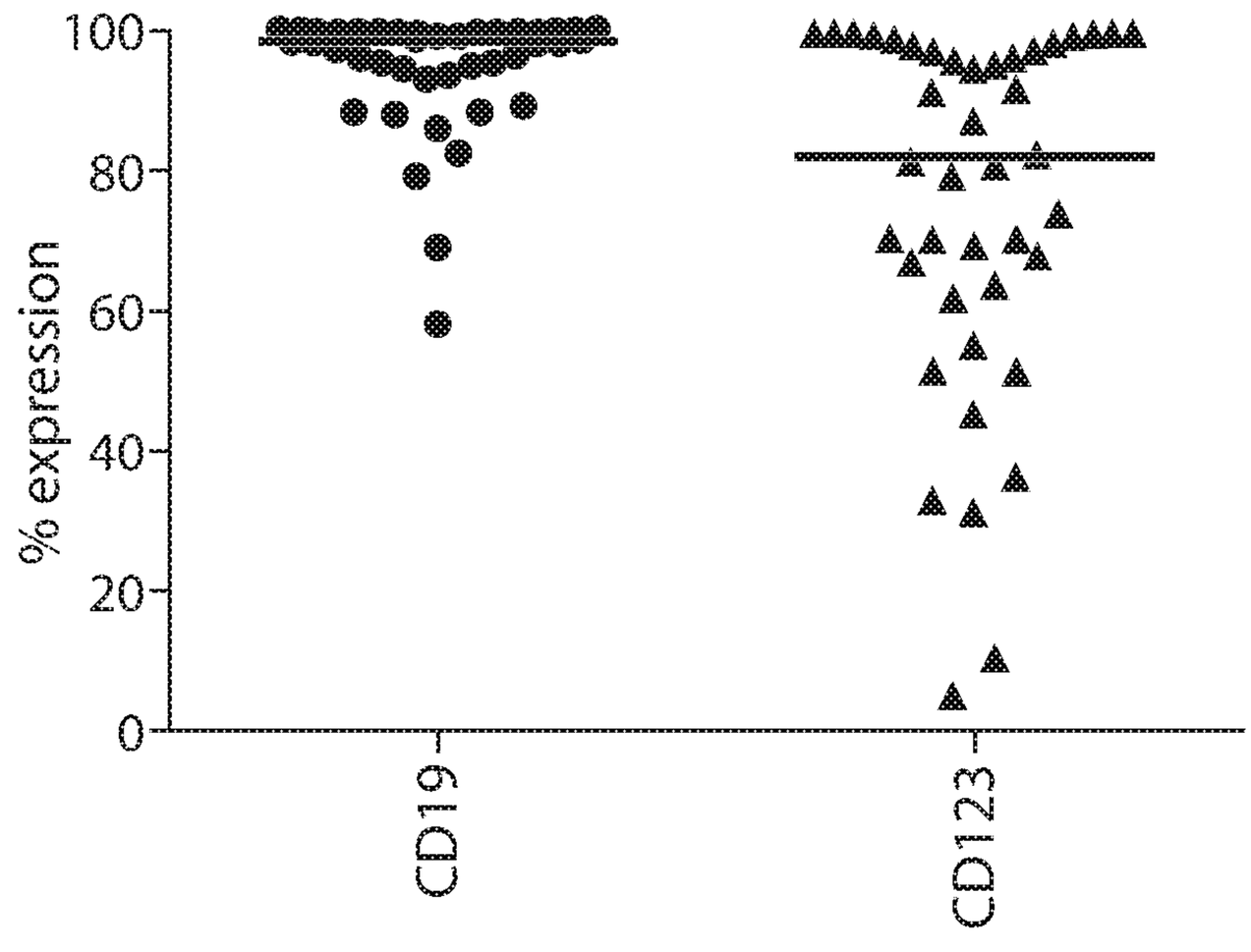


FIG. 49A

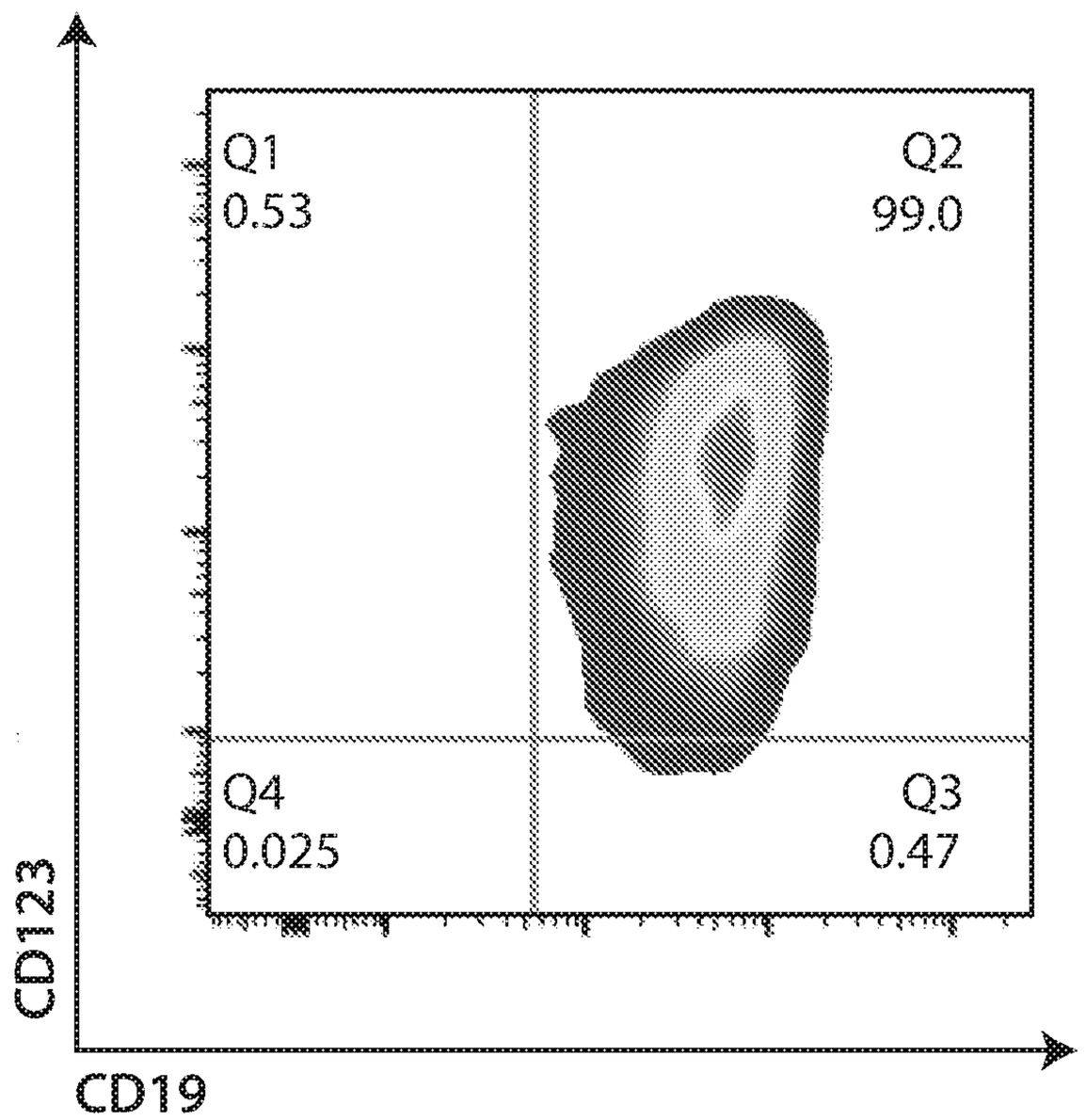


FIG. 49B

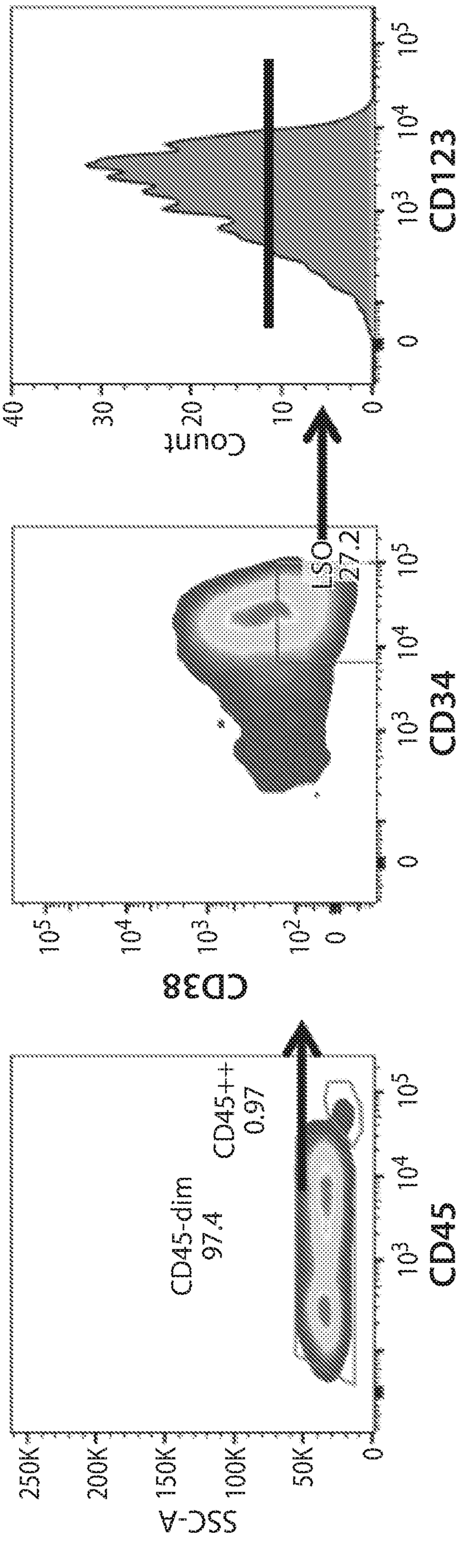


FIG. 49C

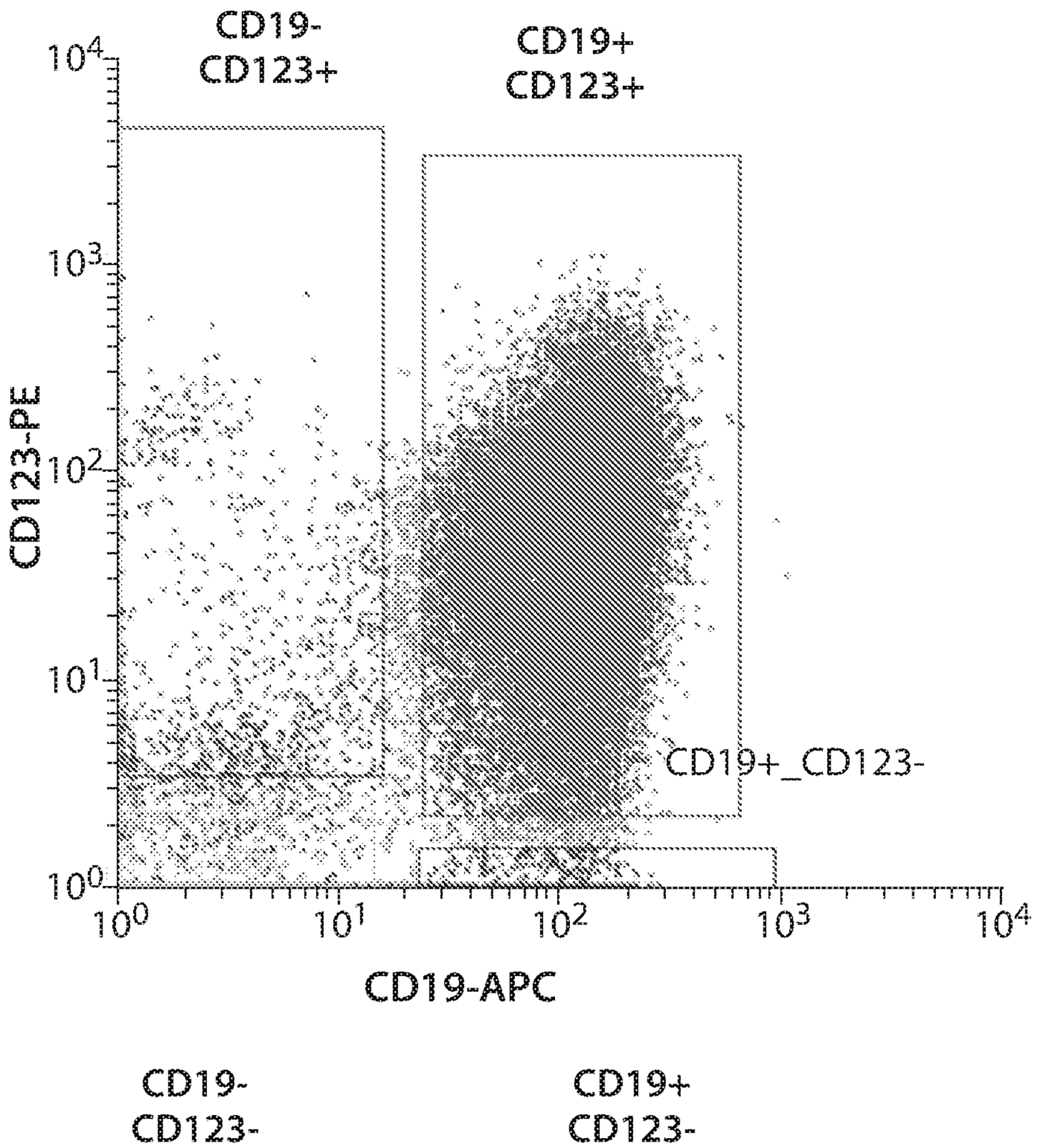

CD19-
CD123+
CD19+
CD123+
CD123-PE
CD19+_CD123-
CD19-APC
CD19-
CD123-
CD19+
CD123-
NEGATIVE
POSITIVE
ATYPICAL
POSITIVE

FIG. 49D

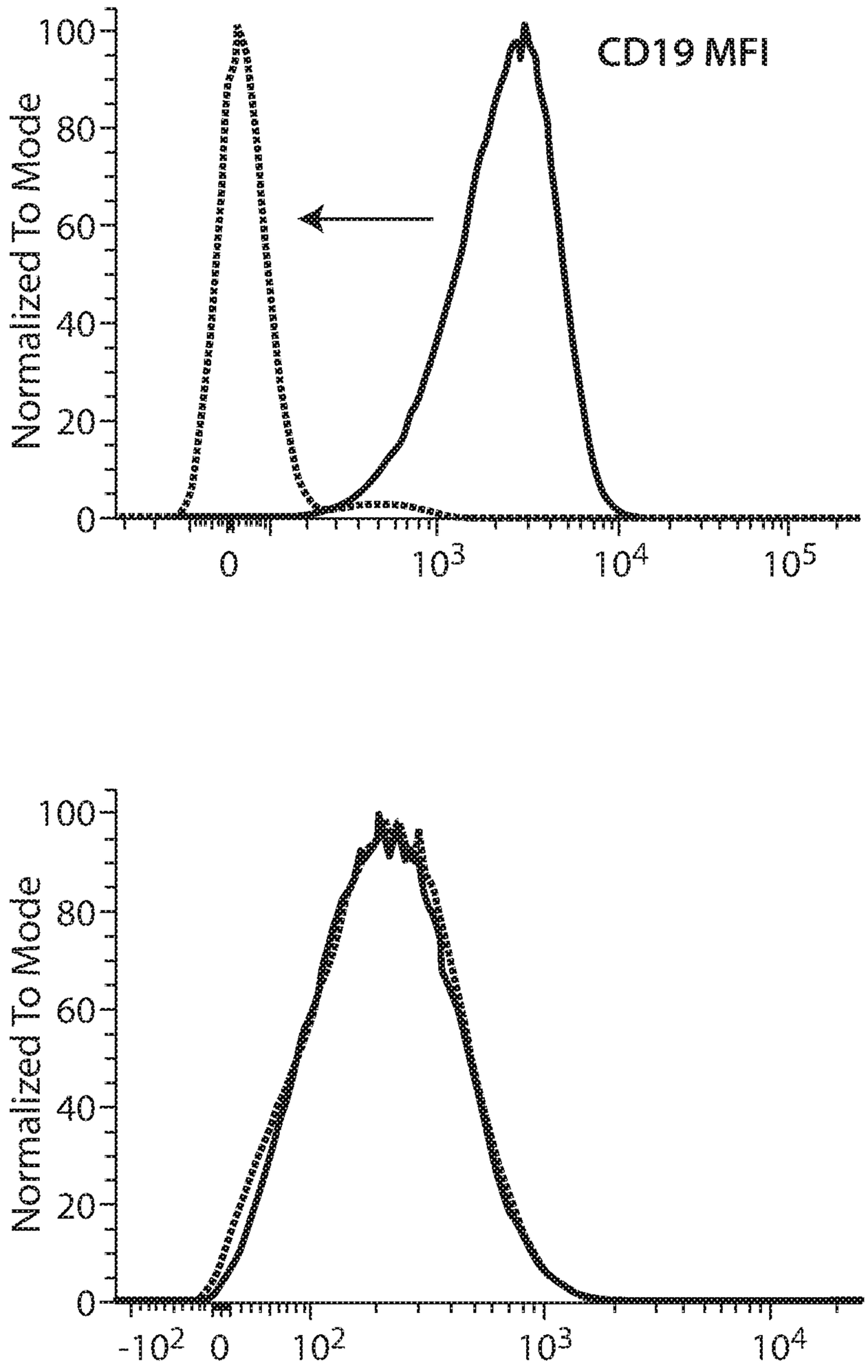

CD19 MFI
Normalized To Mode
100
80
60
40
20
0
0
10^3
10^4
10^5
Normalized To Mode
100
80
60
40
20
0
-10^2
0
10^2
10^3
10^4

FIG. 49E

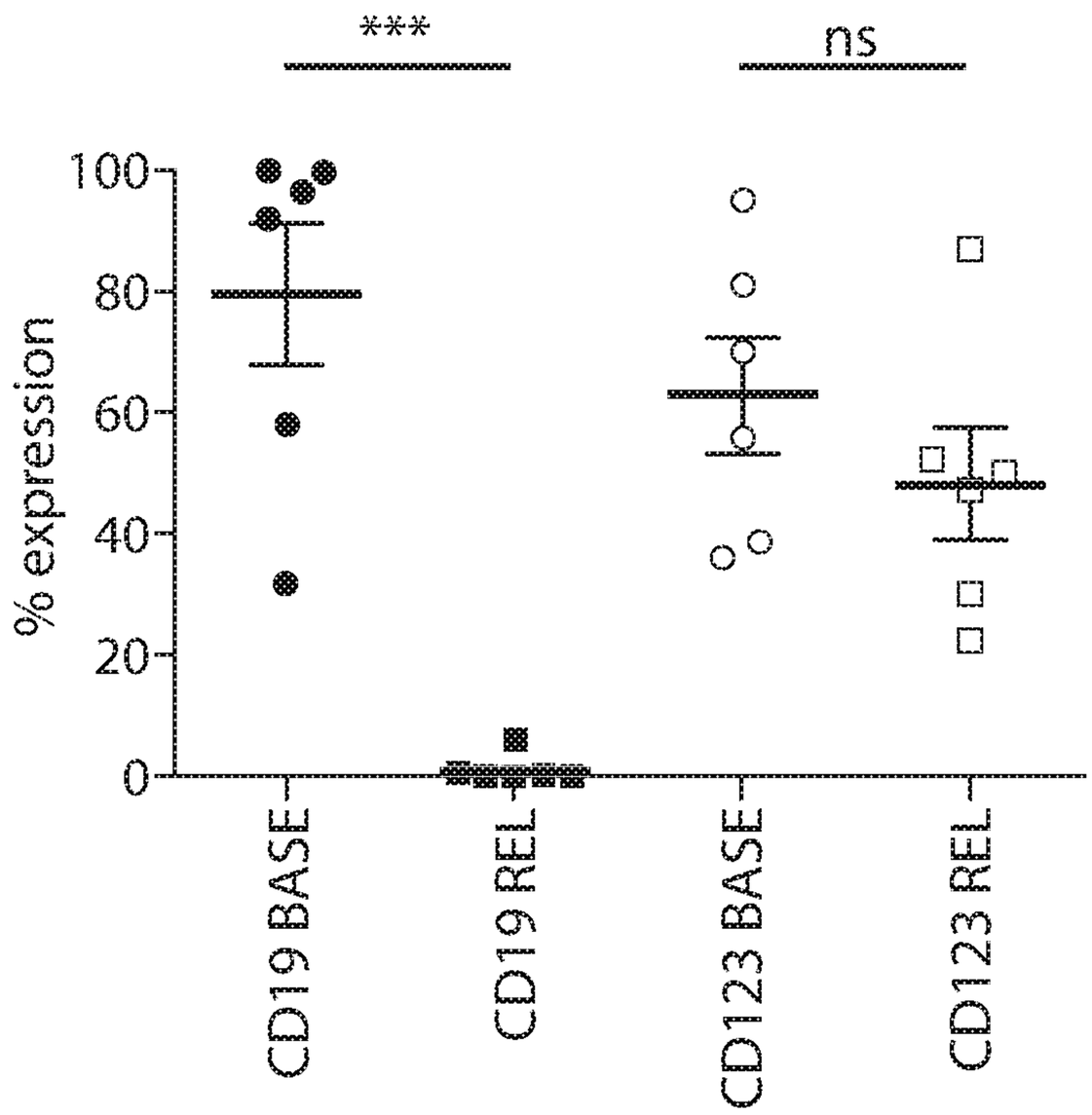

***
ns
100
80
60
40
20
0
% expression
CD19 BASE
CD19 REL
CD123 BASE
CD123 REL

FIG. 49F

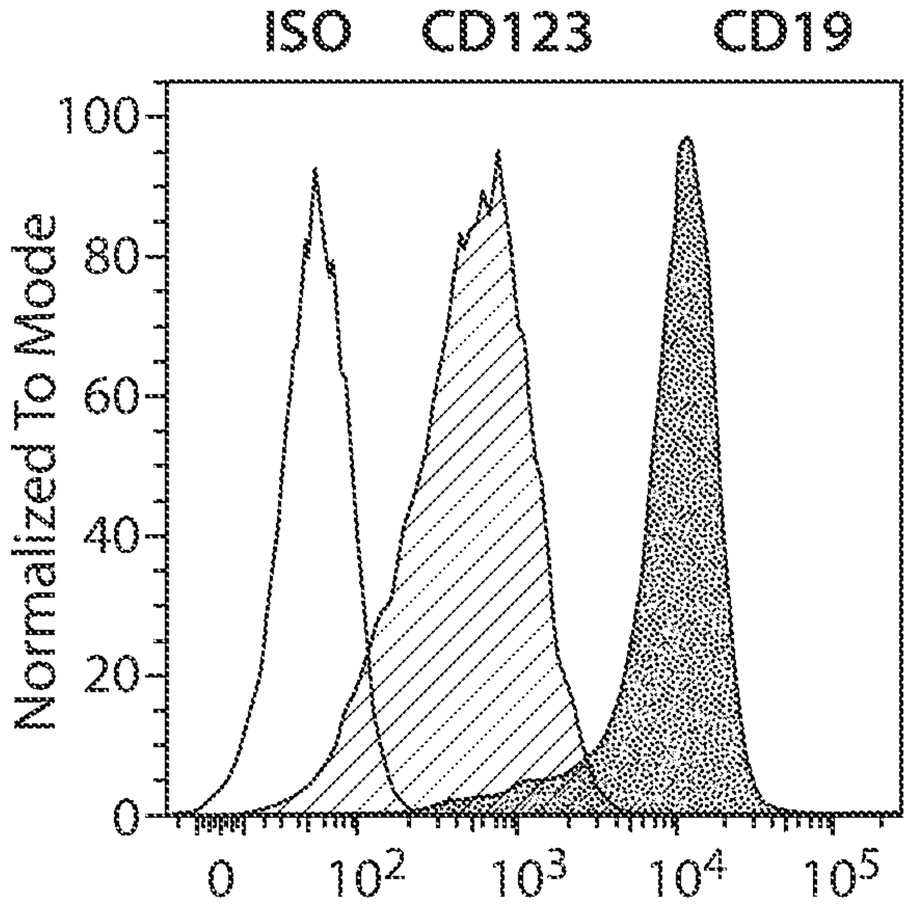


FIG. 50A

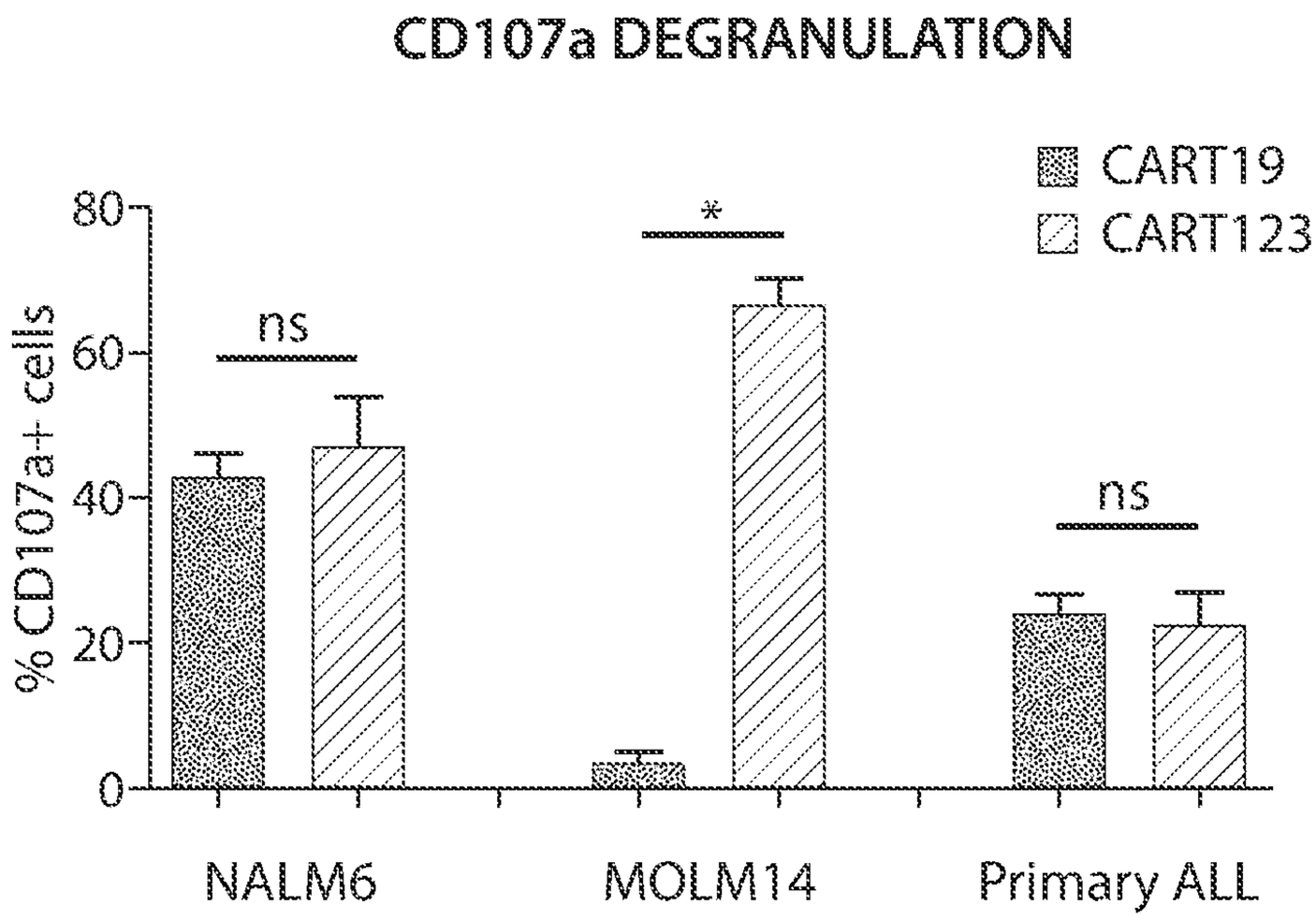


FIG. 50B

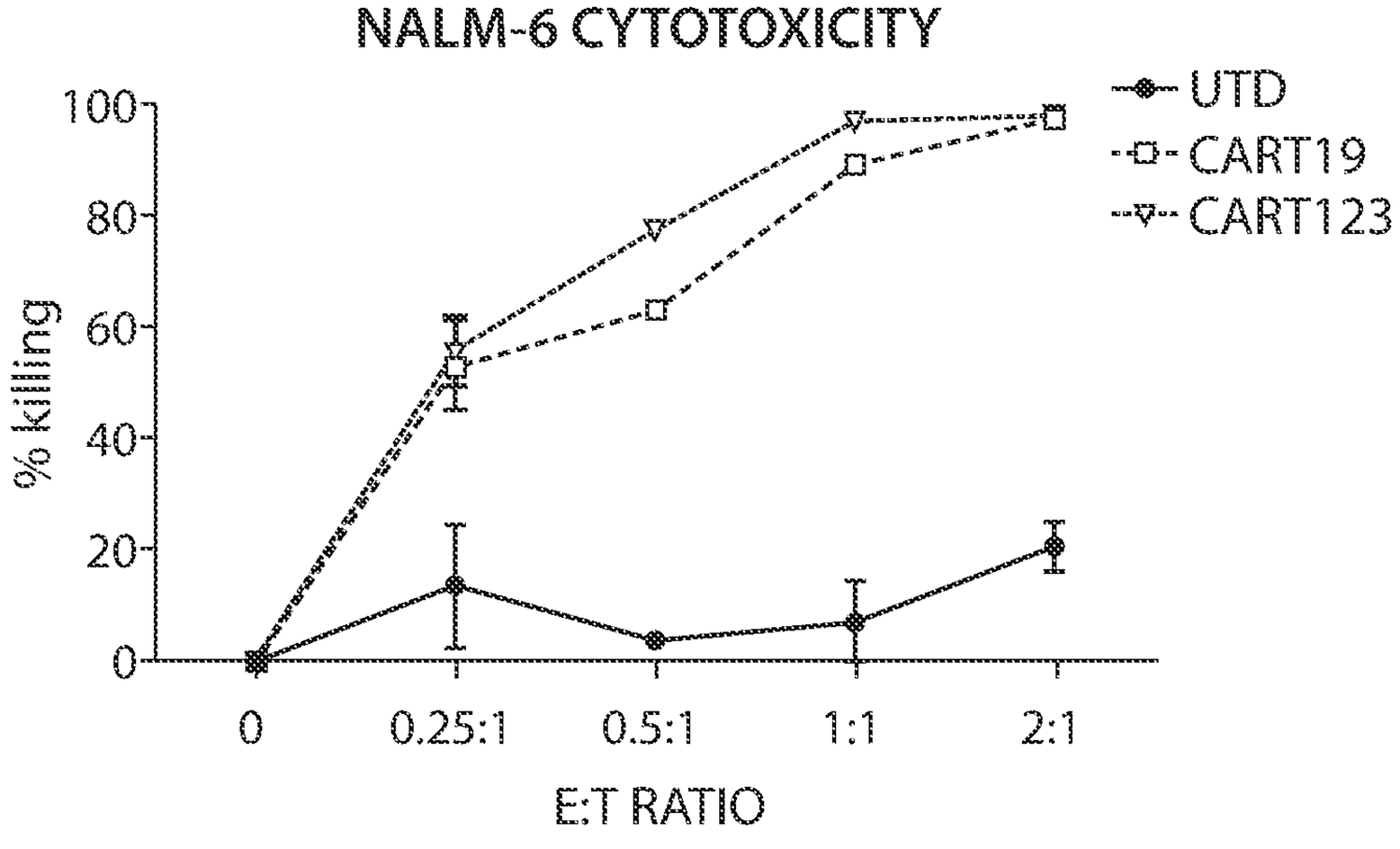


FIG. 50C

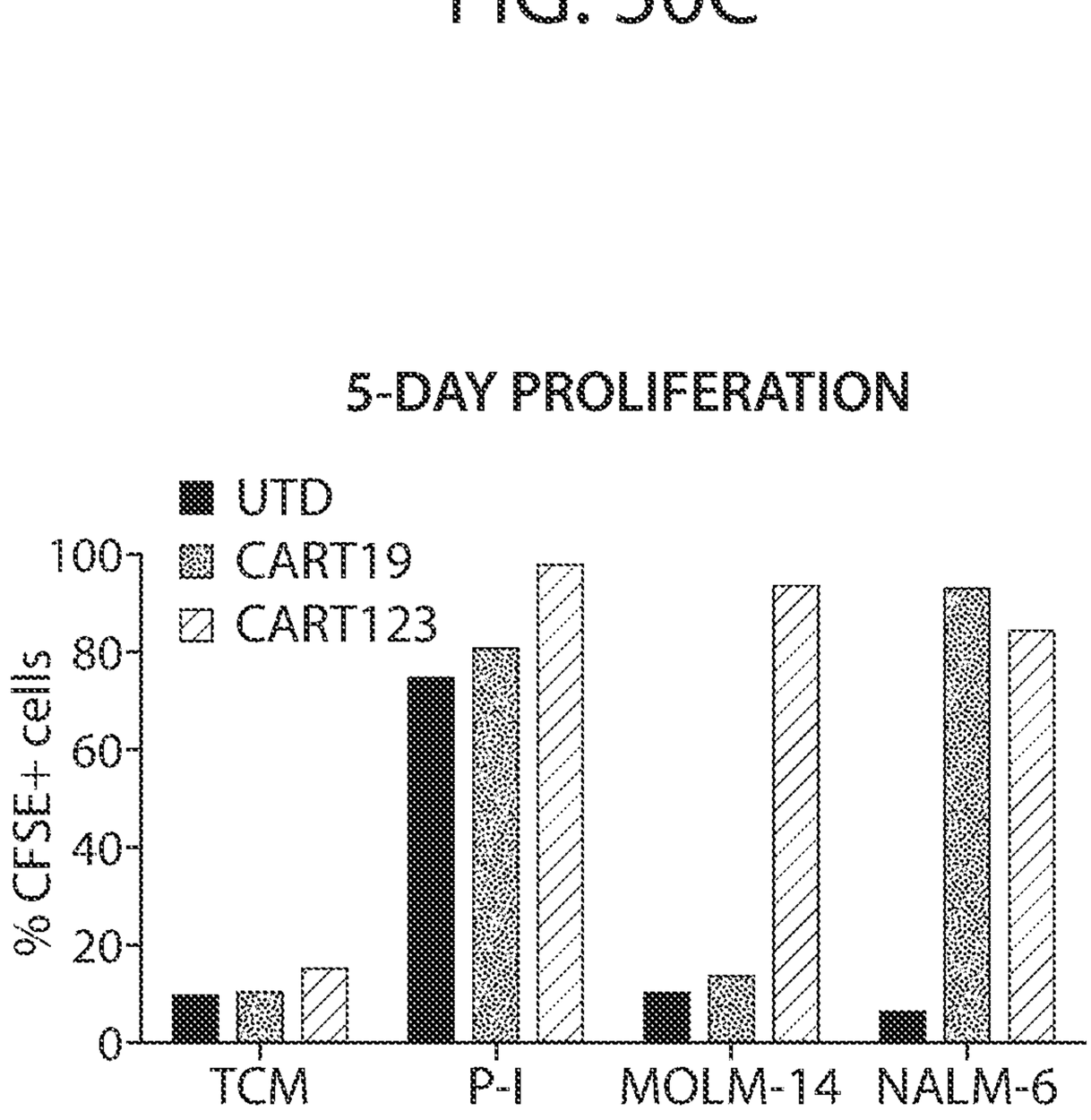


FIG. 50D

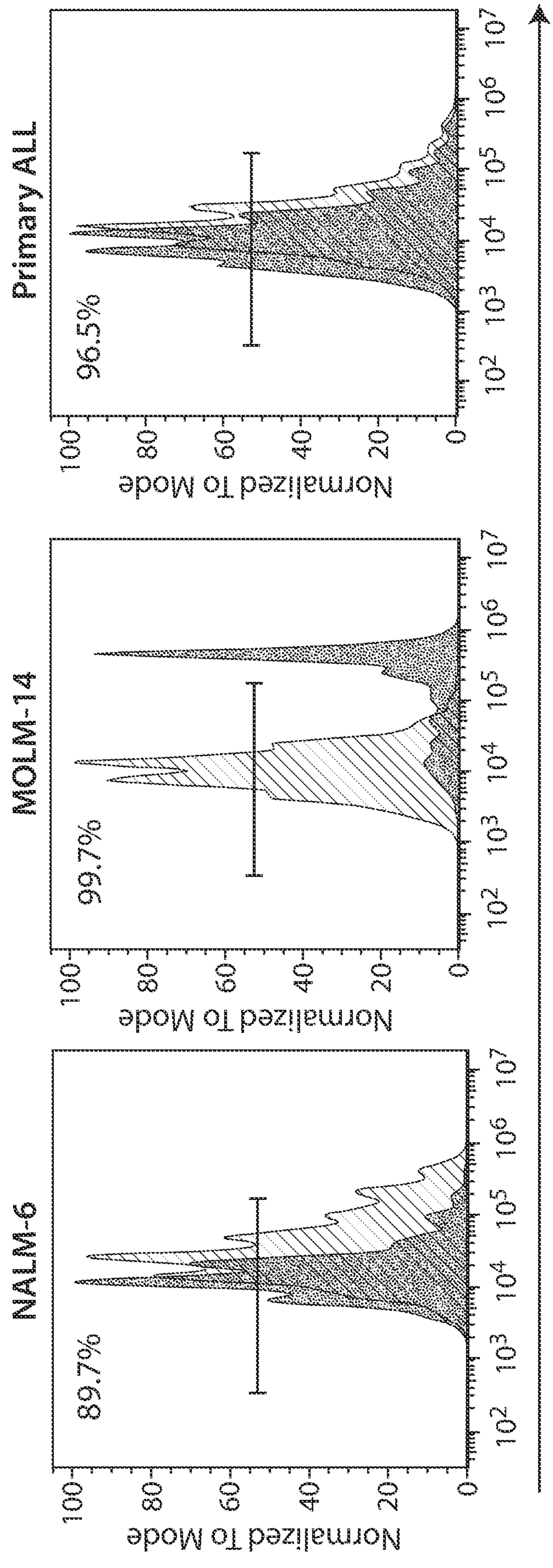

Primary ALL
96.5%
MOLM-14
99.7%
NALM-6
89.7%
Normalized to Mode
100
80
60
40
20
0
10^2
10^3
10^4
10^5
10^6
10^7
CFSE

FIG. 50E

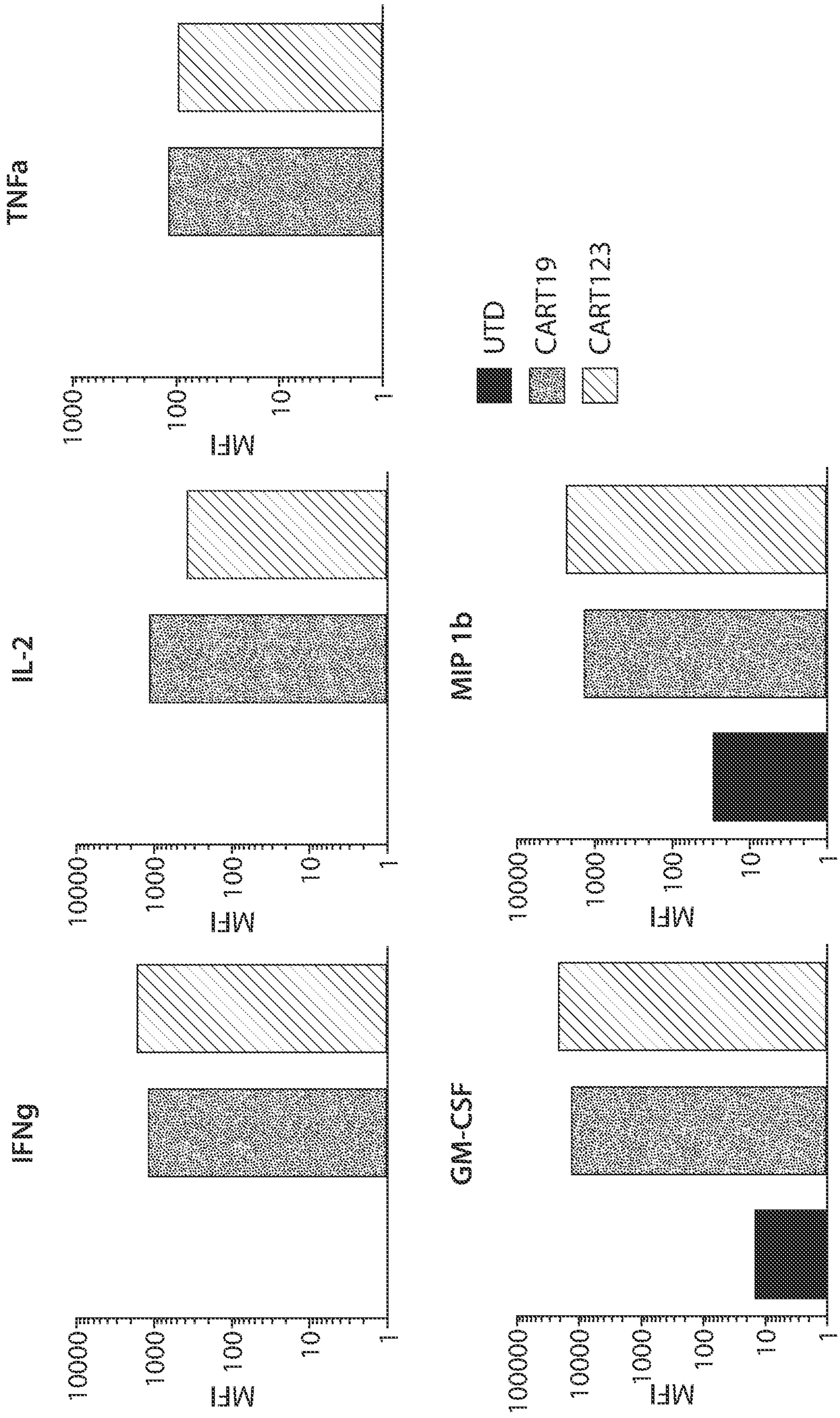

TNFa
MFI
1000
100
10
1
UTD
CART19
CART123
IL-2
MIP 1b
10000
1000
100
10
1
IFNg
GM-CSF
100000

FIG. 5OF

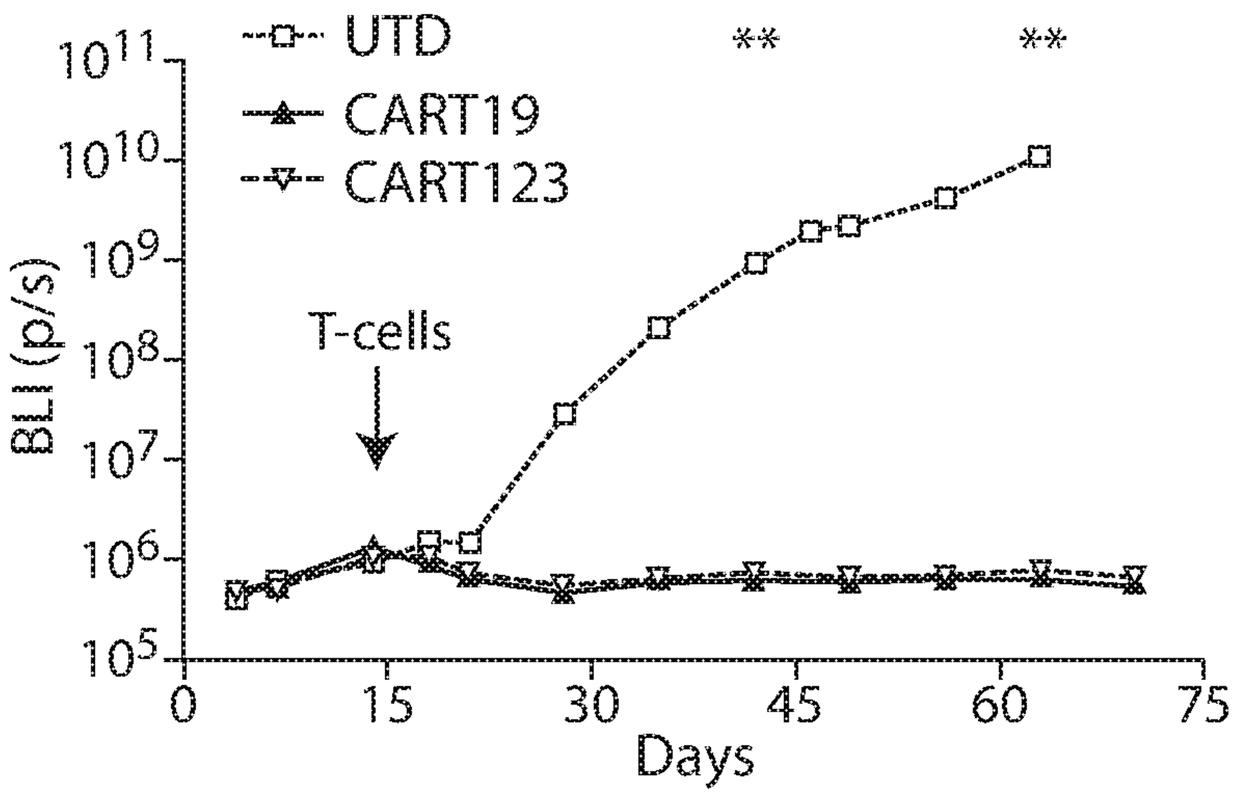


FIG. 51A

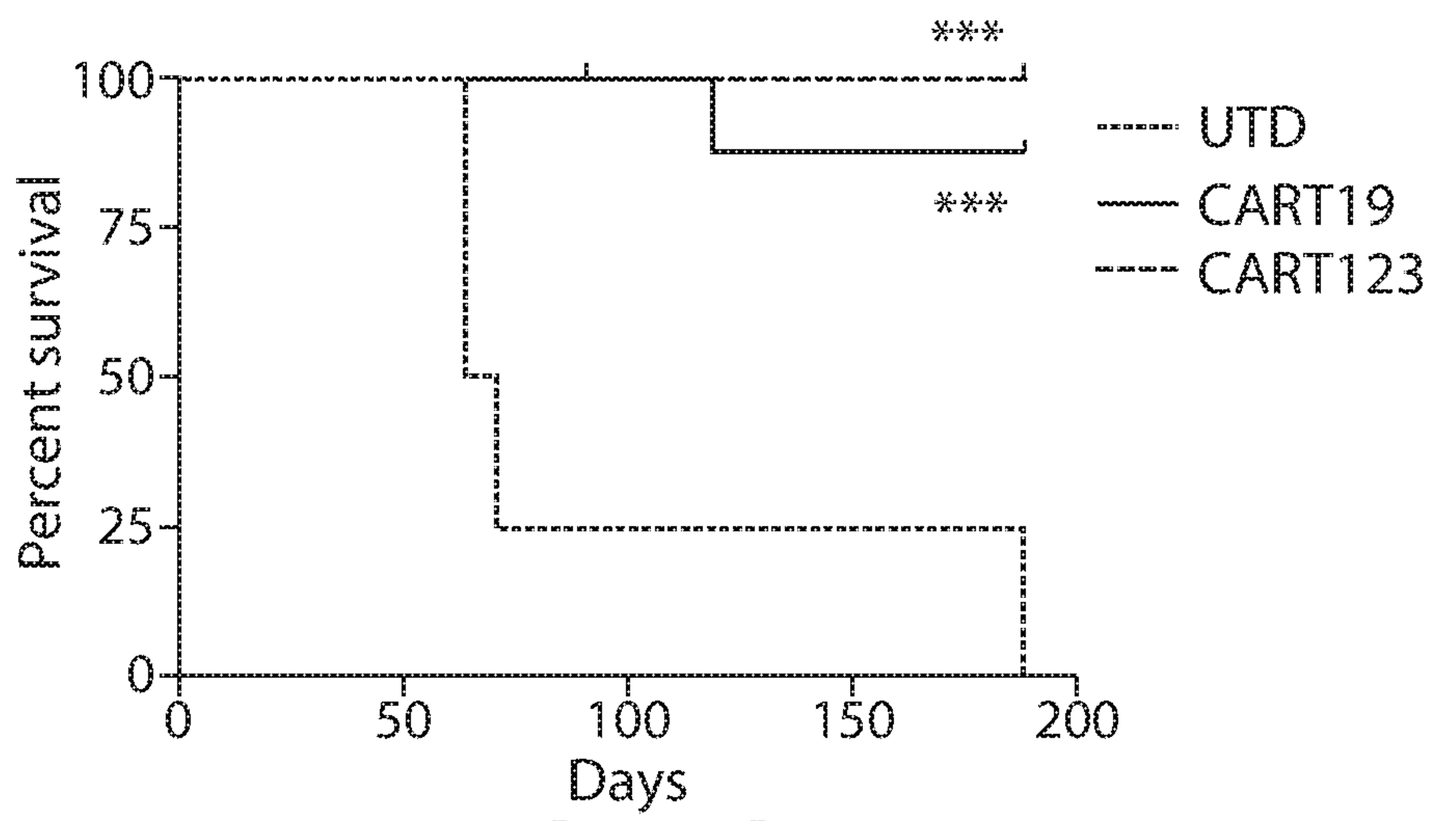


FIG. 51B

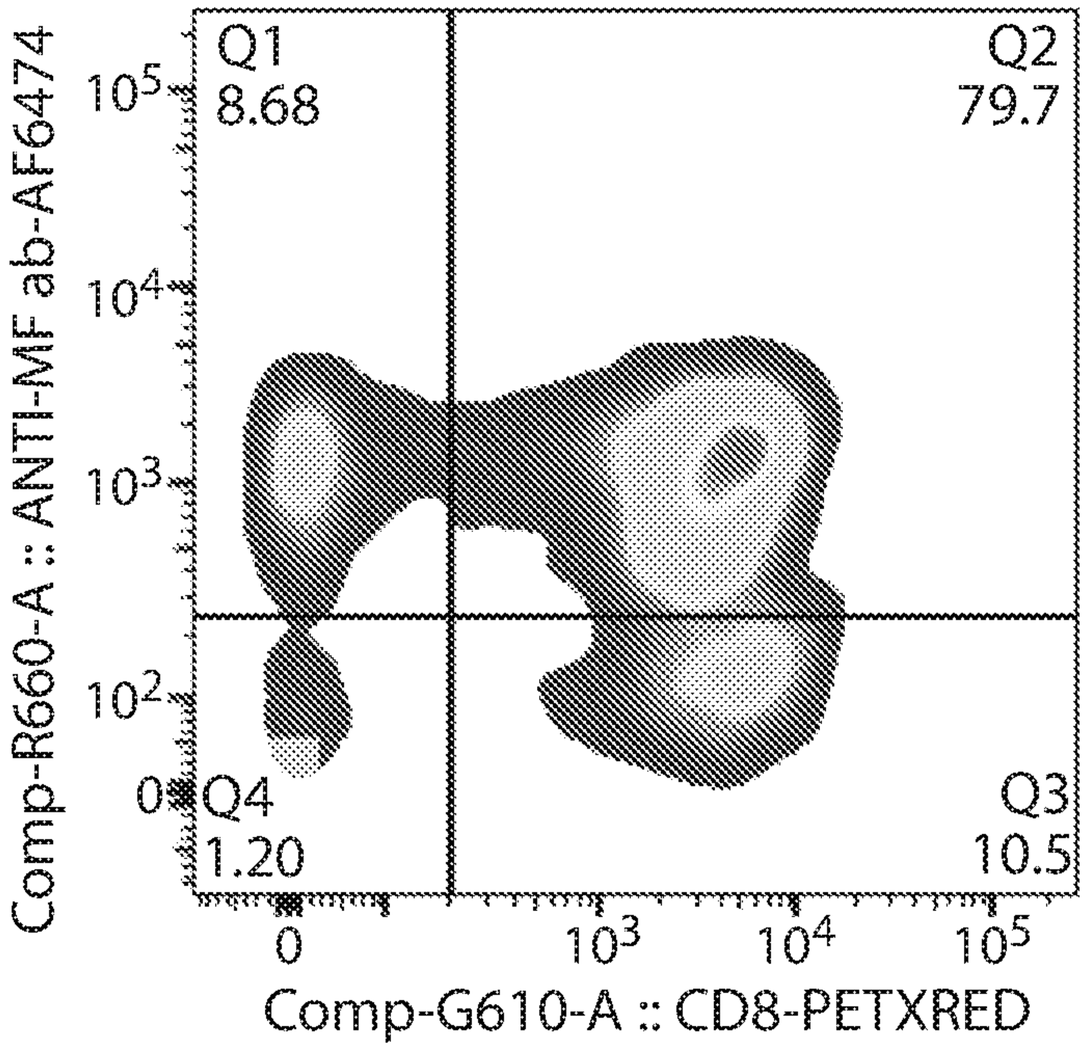


FIG. 51C

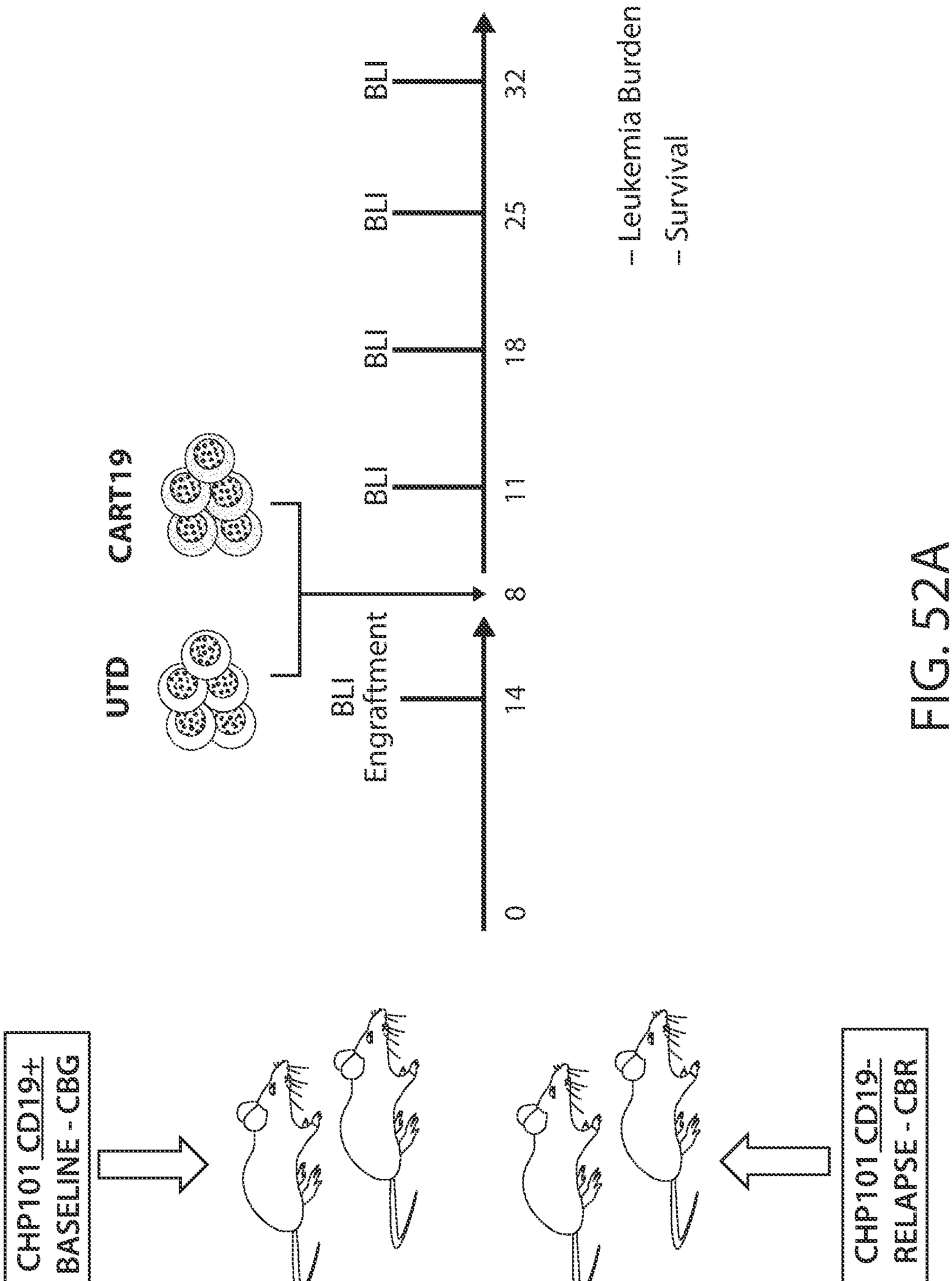

CHP101_CD19+
BASELINE - CBG
CHP101_CD19-
RELAPSE - CBR
UTD
CART19
BLI
Engraftment
0
14
8
11
18
25
BLI
BLI
BLI
BLI
– Survival
– Leukemia Burden

FIG. 52A

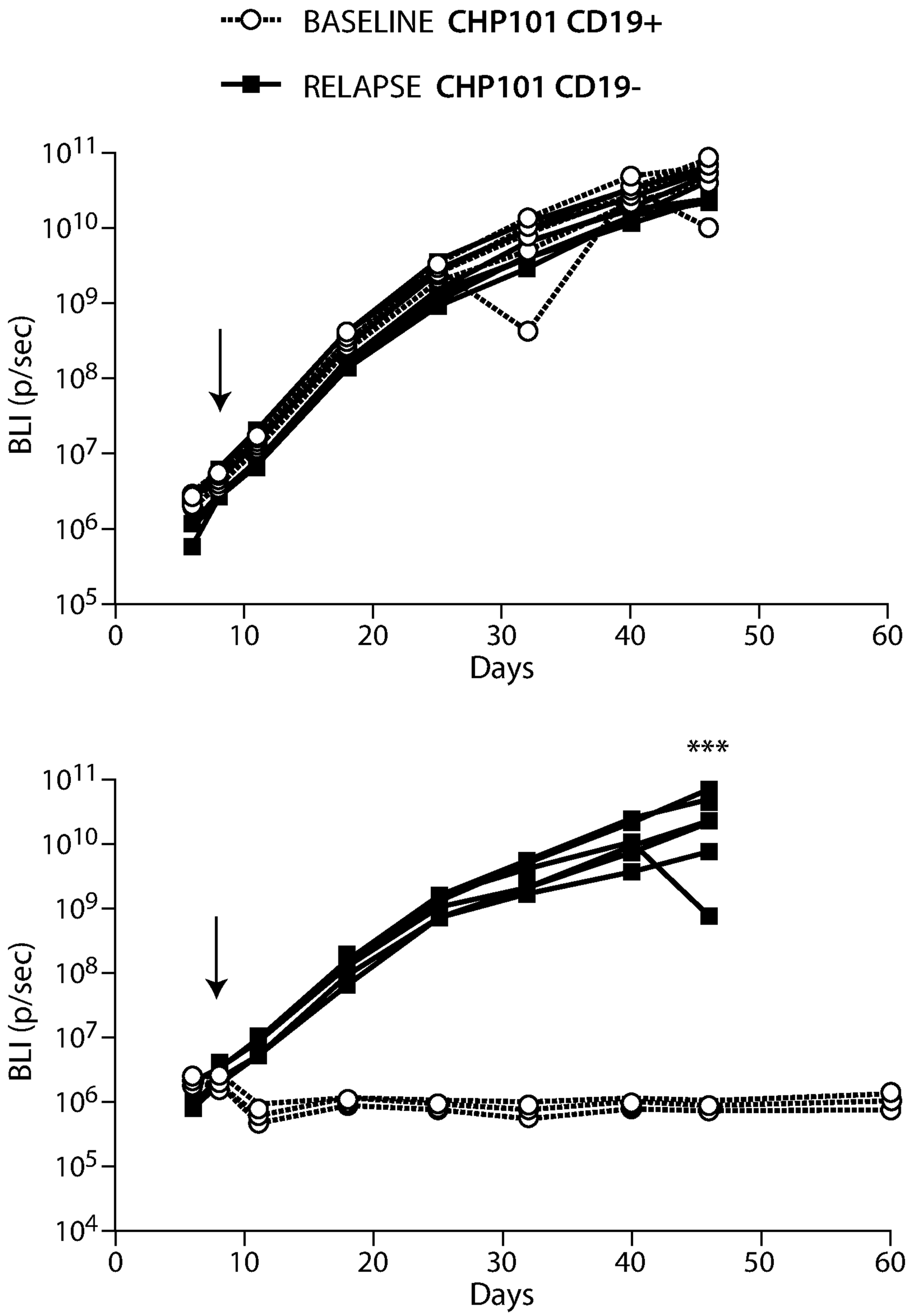

BASELINE CHP101 CD19+
RELAPSE CHP101 CD19-
BLI (p/sec)
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
0
10
20
30
40
50
60
Days
***

FIG. 52B

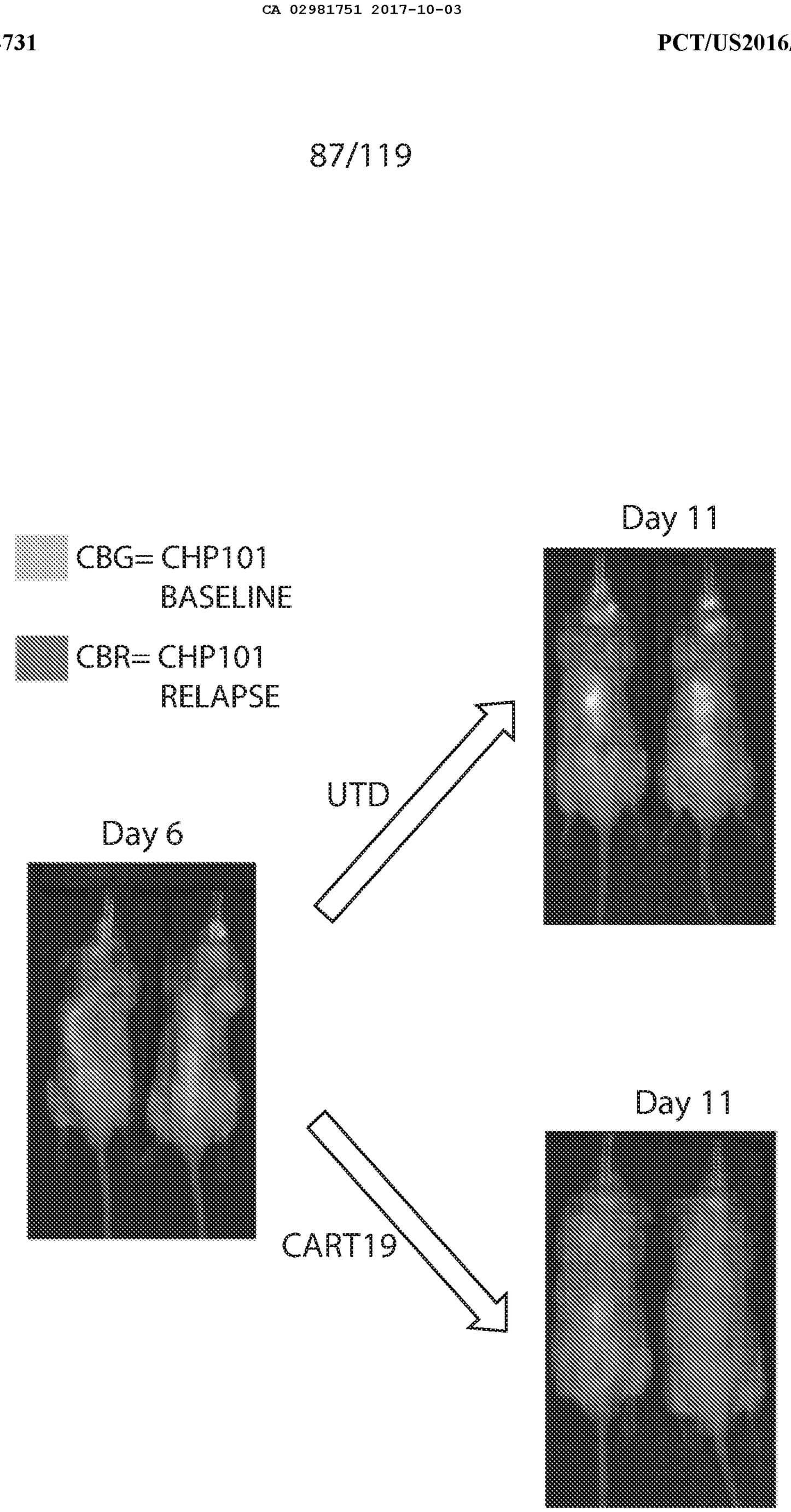

CBG= CHP101
BASELINE
CBR= CHP101
RELAPSE
Day 6
UTD
Day 11
CART19
Day 11

FIG. 52C

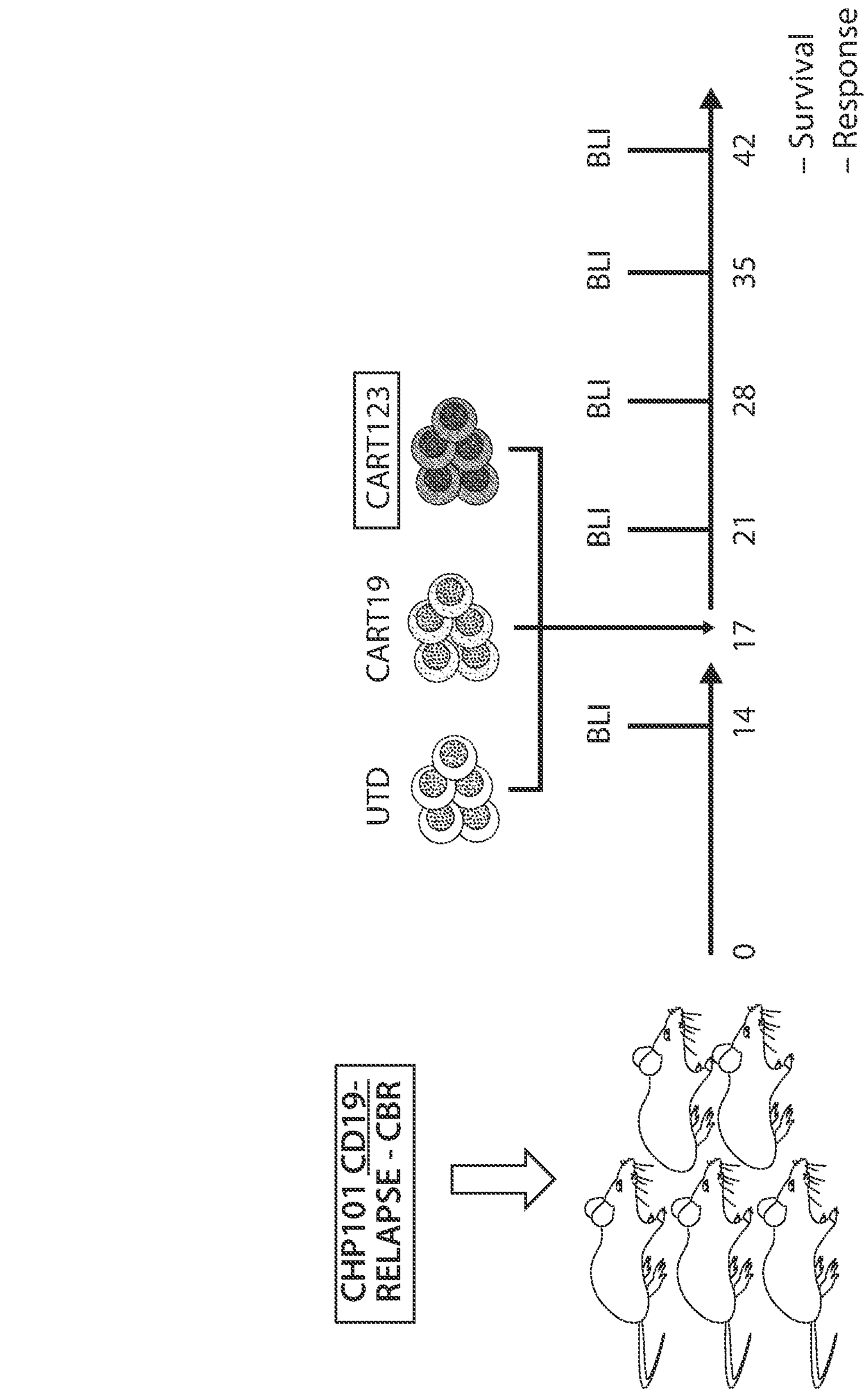

CHP101 CD19-
RELAPSE - CBR
UTD
CART19
CART123
BLI
0
14
17
21
28
35
42
– Survival
– Response

FIG. 52D

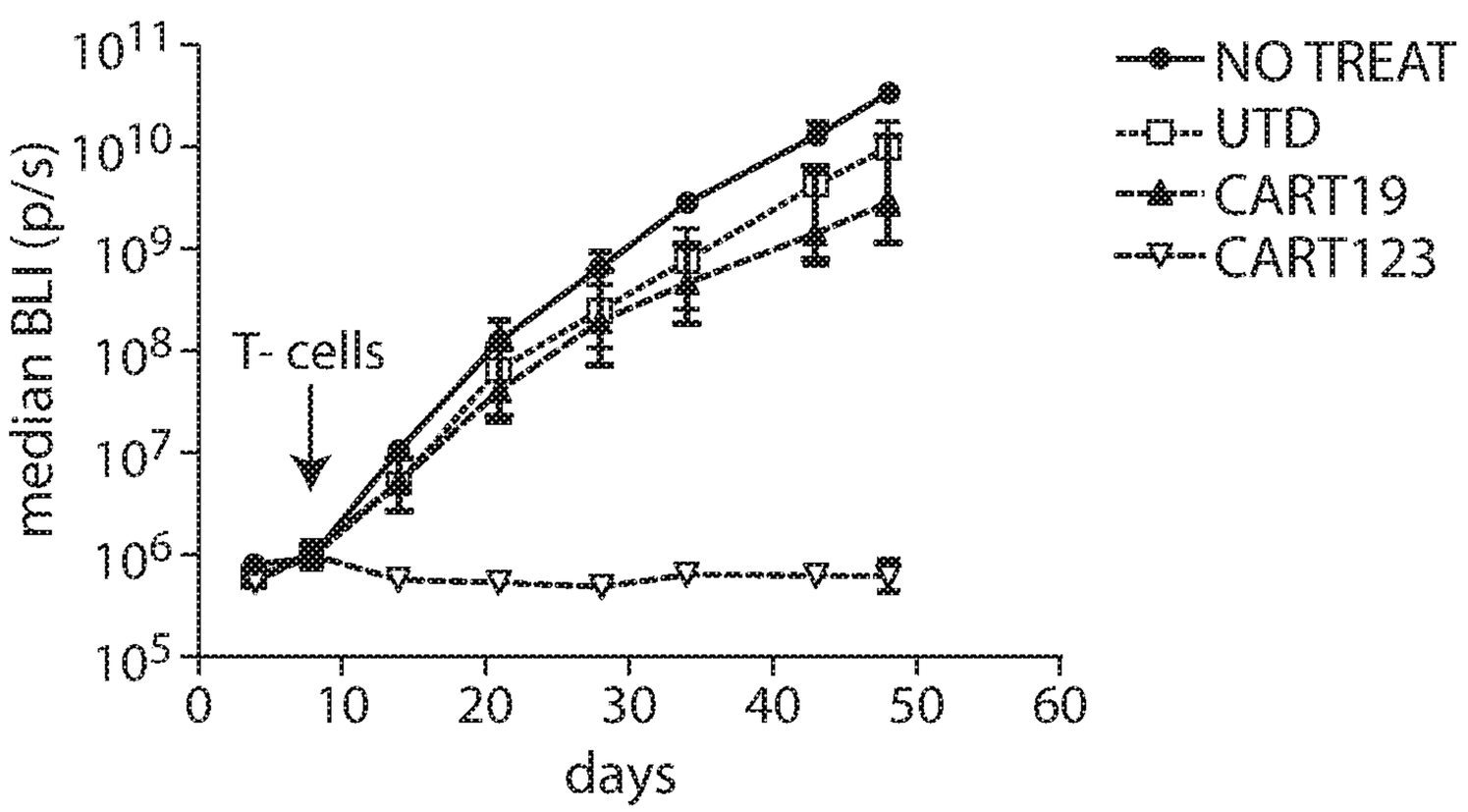


FIG. 52E

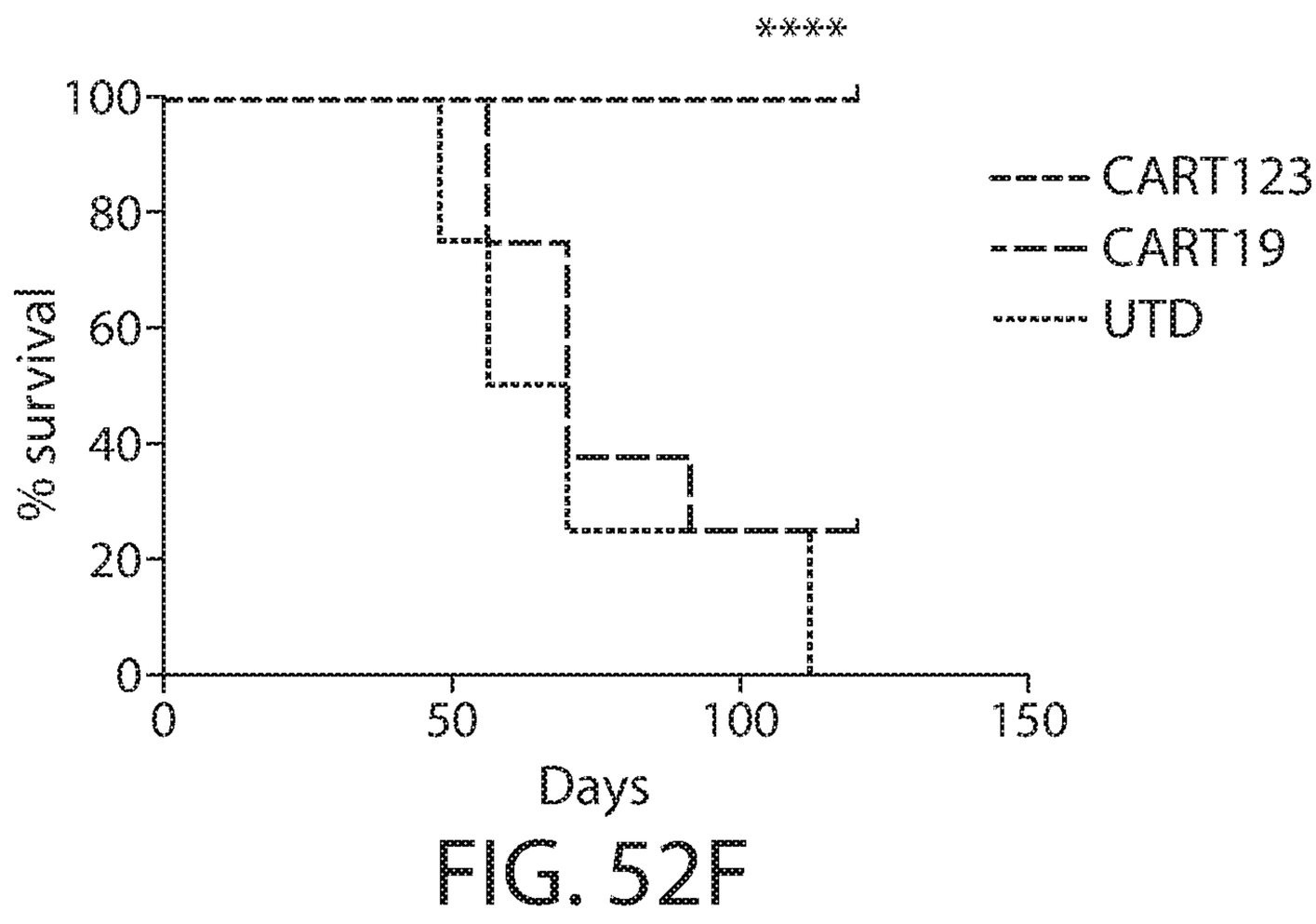


FIG. 52F

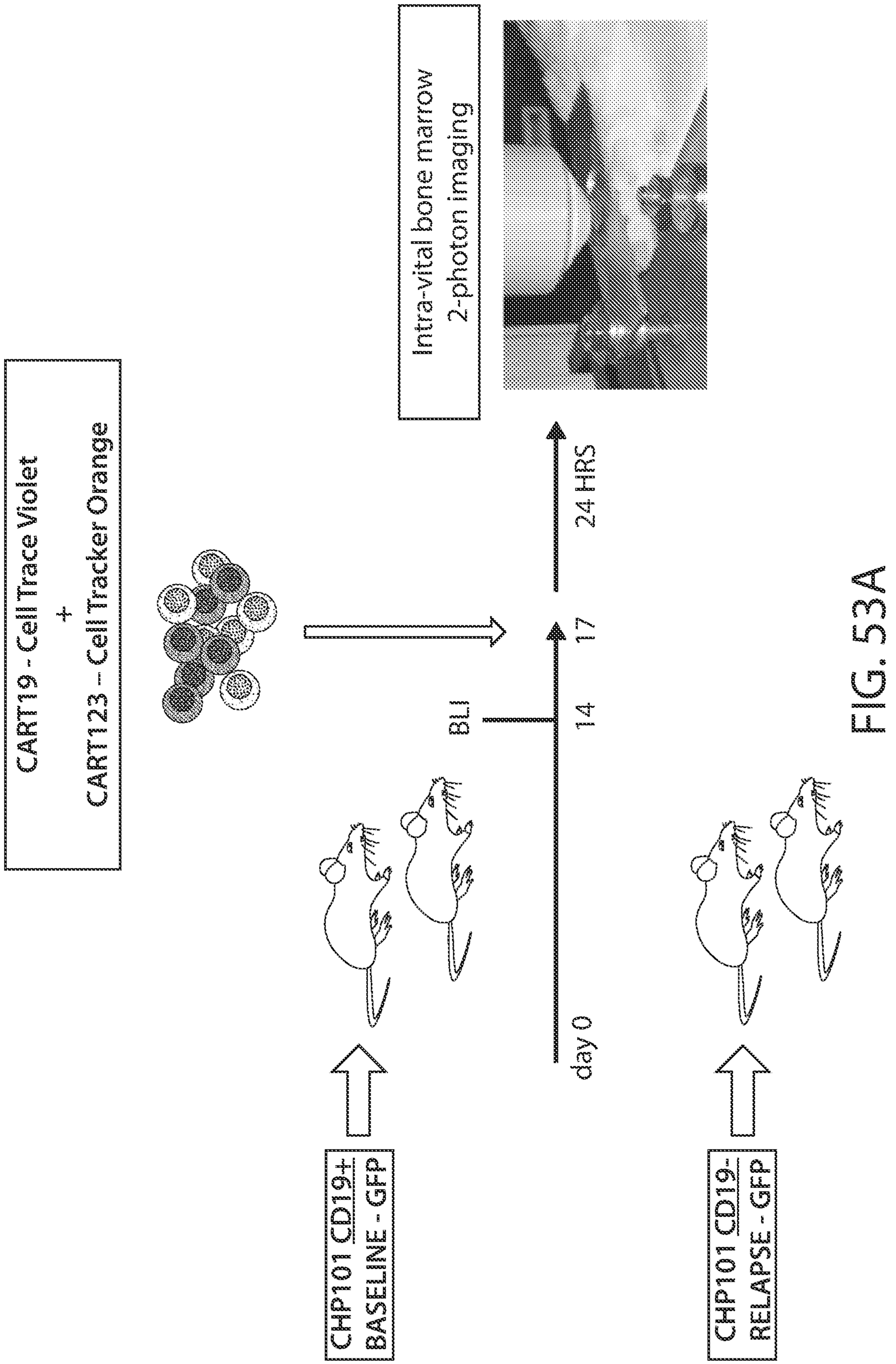

CHP101 CD19+
BASELINE - GFP
CHP101 CD19-
RELAPSE - GFP
day 0
14
BLI
17
24 HRS
CART19 - Cell Trace Violet
+
CART123 – Cell Tracker Orange
Intra-vital bone marrow
2-photon imaging

FIG. 53A

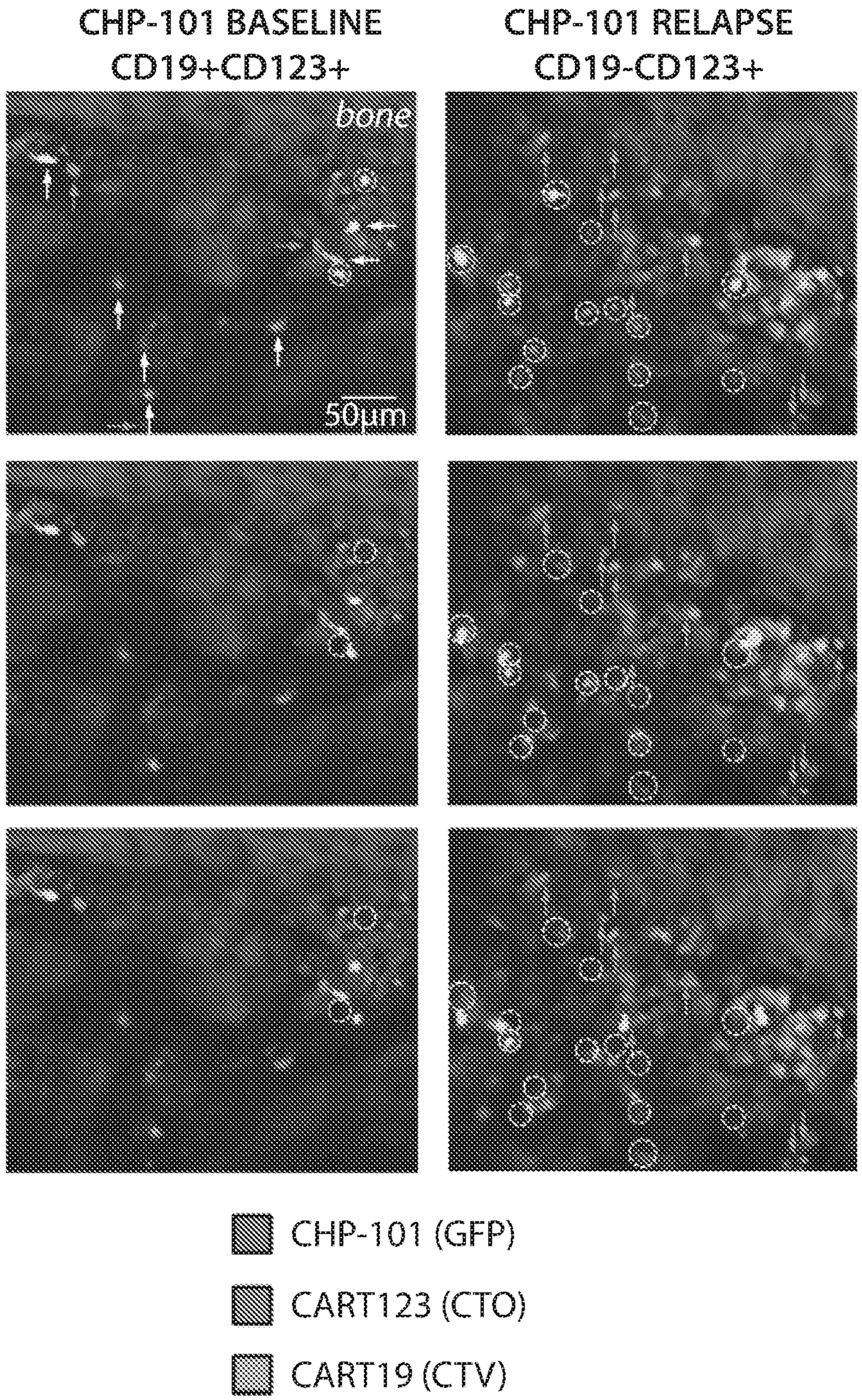

CHP-101 BASELINE
CD19+CD123+
CHP-101 RELAPSE
CD19-CD123+
bone
50µm
CHP-101 (GFP)
CART123 (CTO)
CART19 (CTV)

FIG. 53B

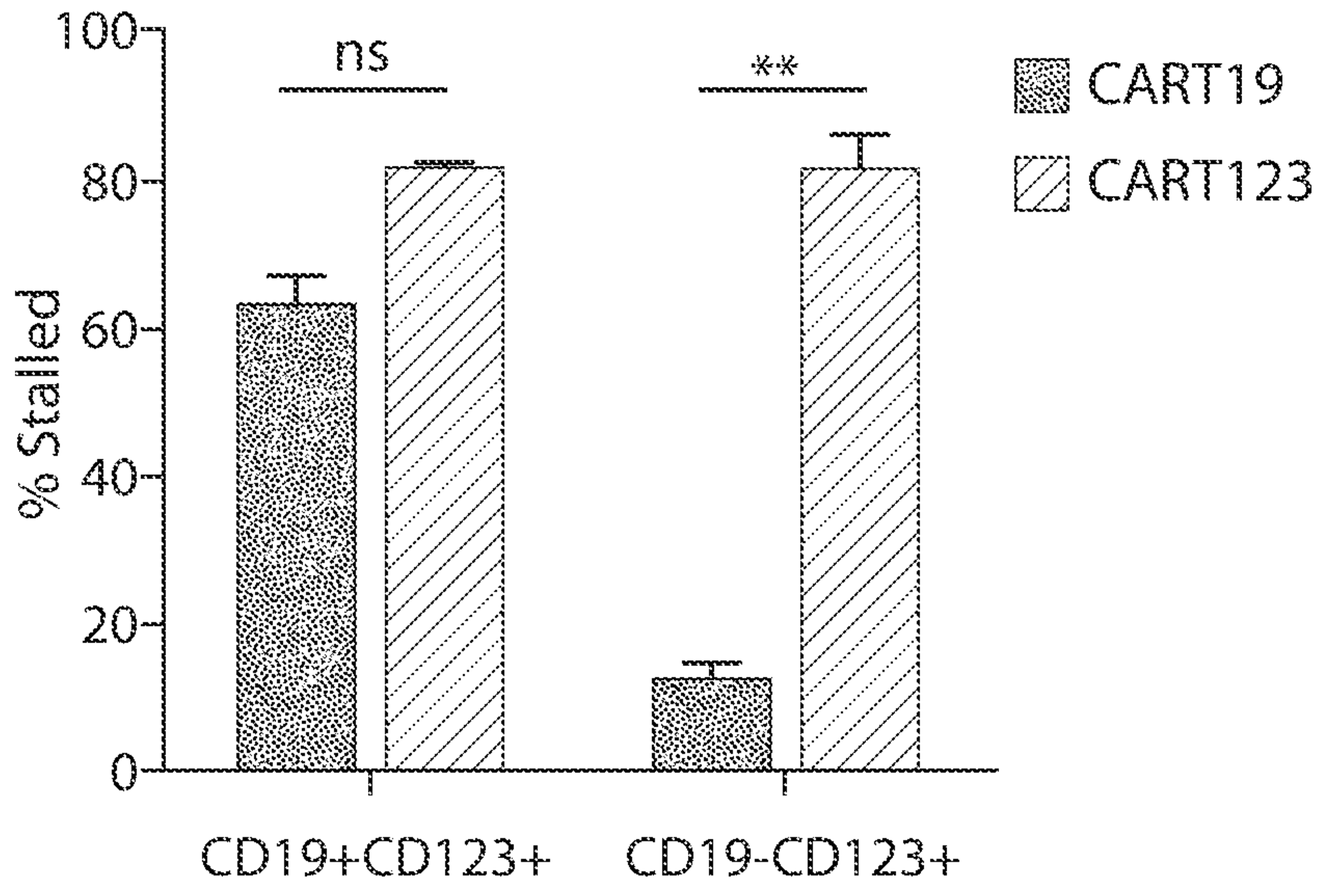

100
80
60
40
20
0
% Stalled
ns
**
CART19
CART123
CD19+CD123+
CD19-CD123+

FIG. 53C

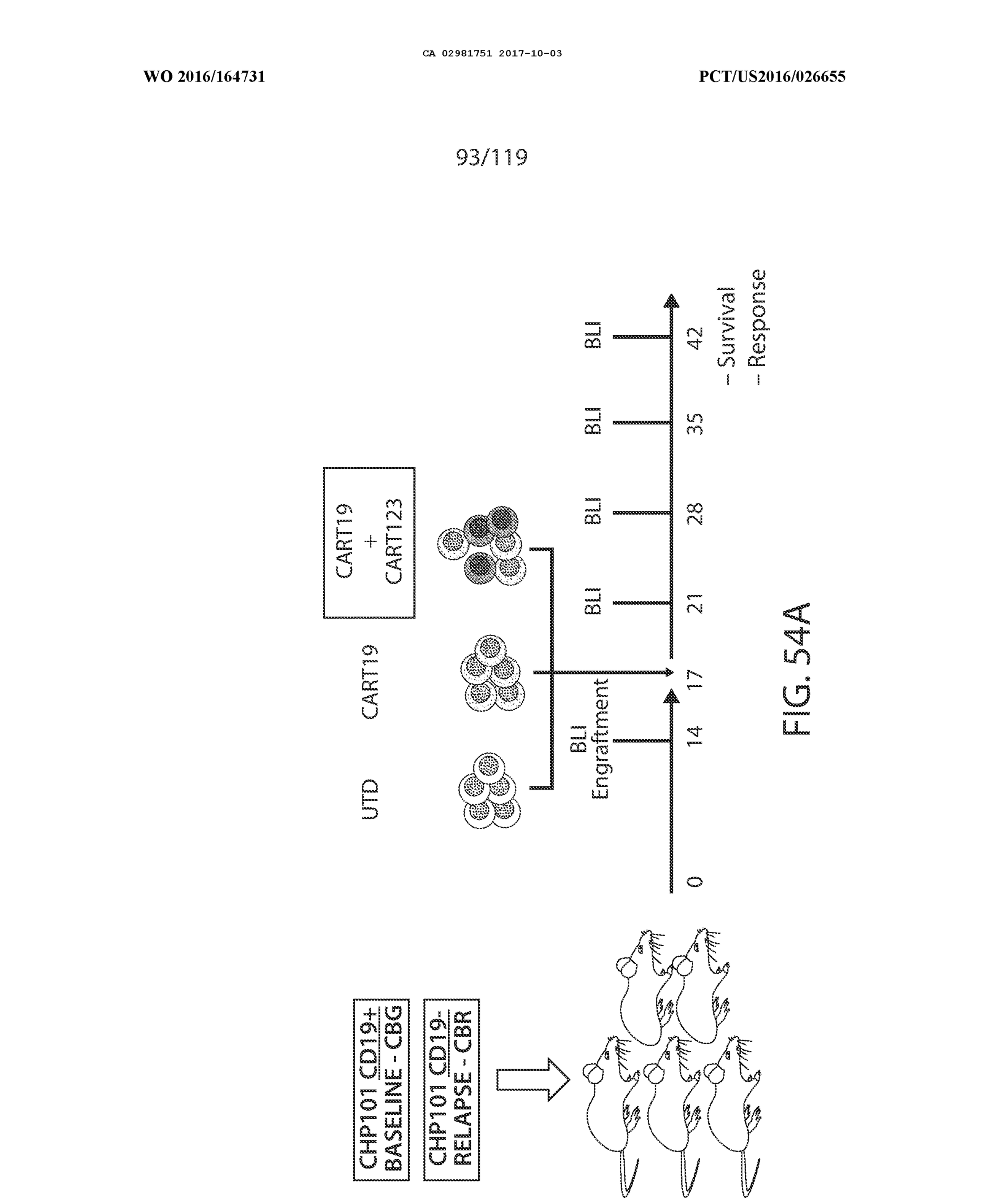

CHP101 CD19+
BASELINE - CBG
CHP101 CD19-
RELAPSE - CBR
CART19
+
CART123
CART19
UTD
BLI
Engraftment
0
14
17
21
28
35
42
BLI
BLI
BLI
BLI
– Survival
– Response

FIG. 54A

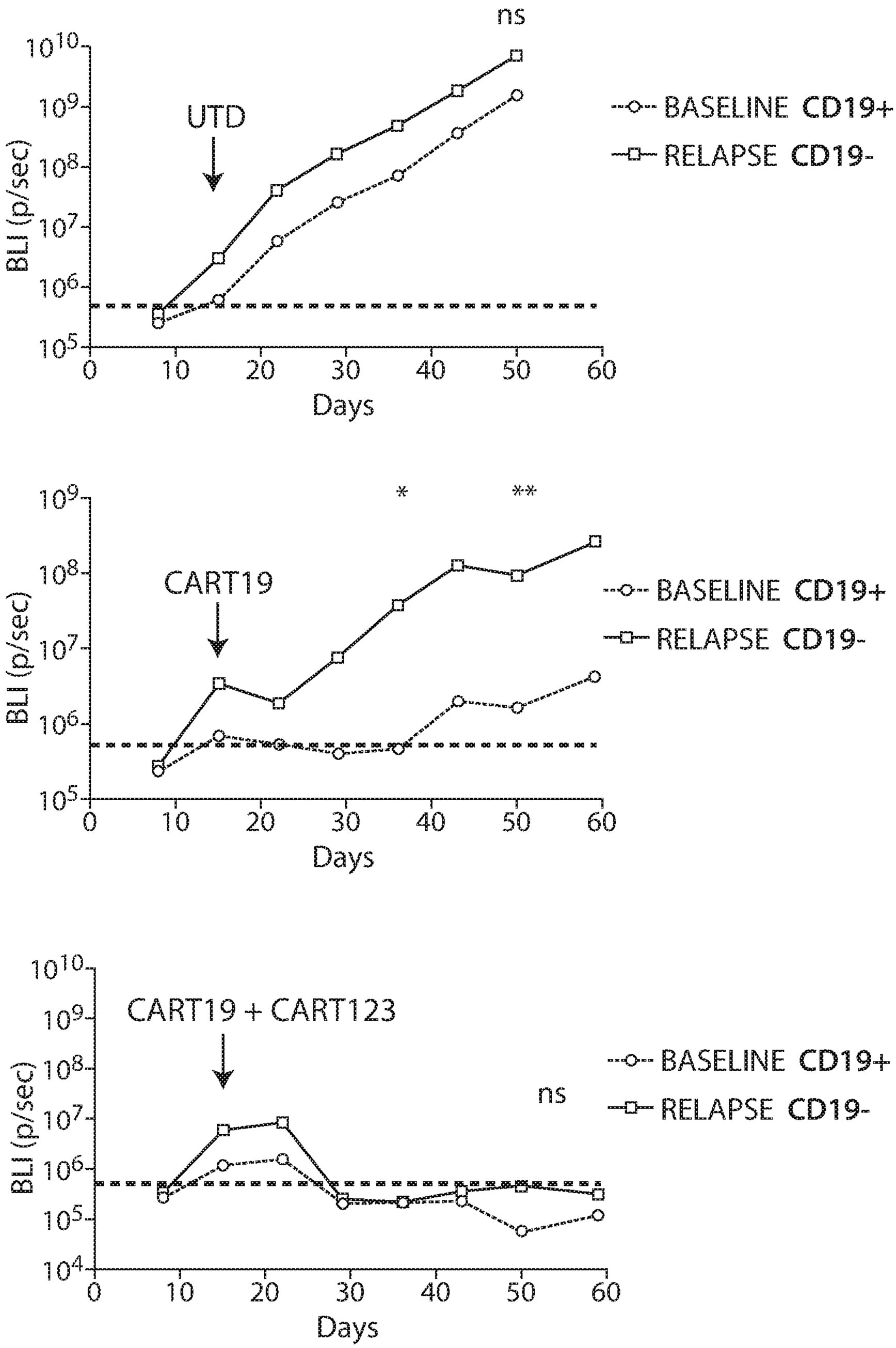

ns
UTD
BLI (p/sec)
Days
BASELINE CD19+
RELAPSE CD19-
*
**
CART19
CART19 + CART123

FIG. 54B

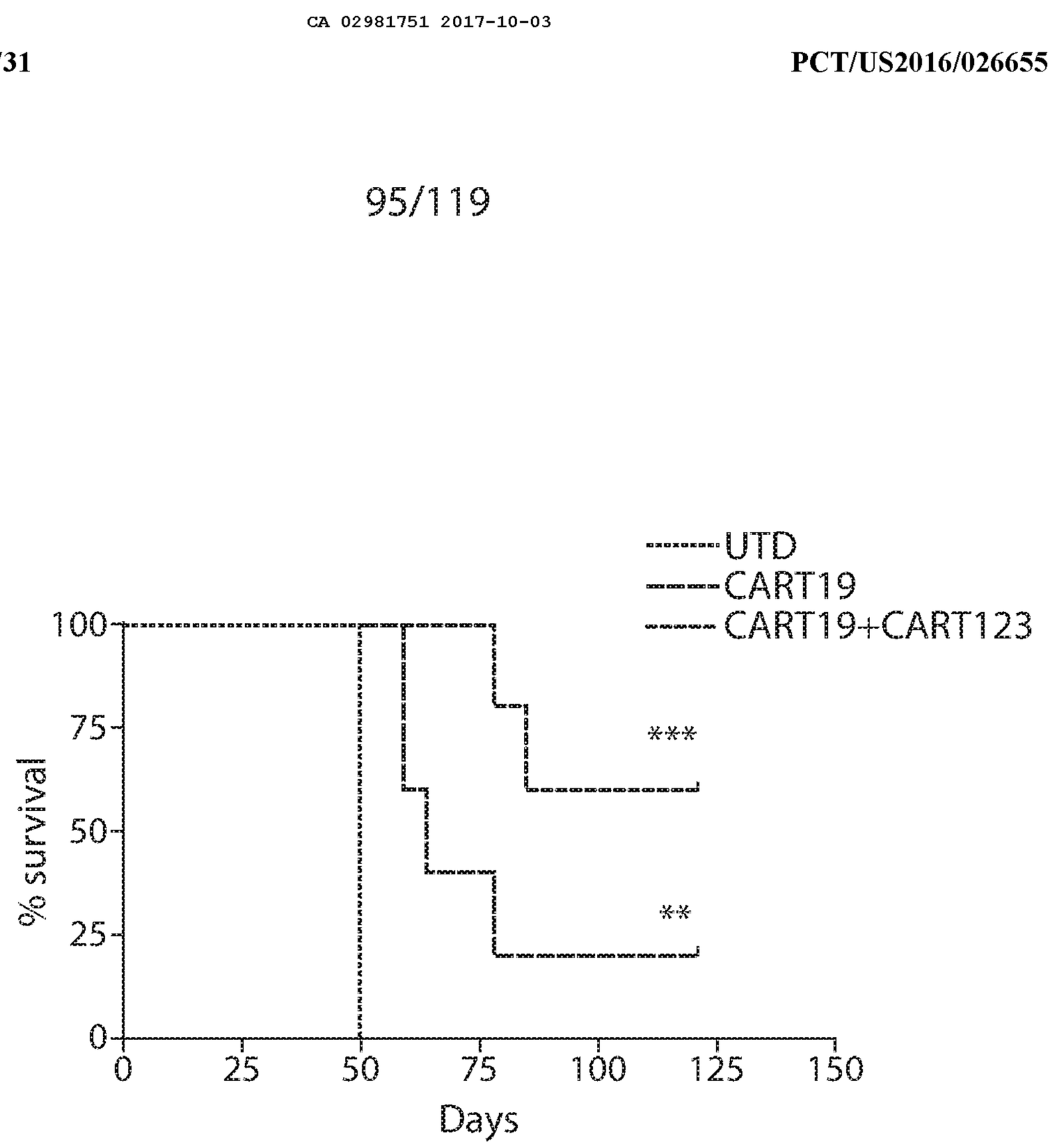

UTD
CART19
CART19+CART123
100
75
50
25
0
% survival
***
**
0
25
50
75
100
125
150
Days

FIG. 54C

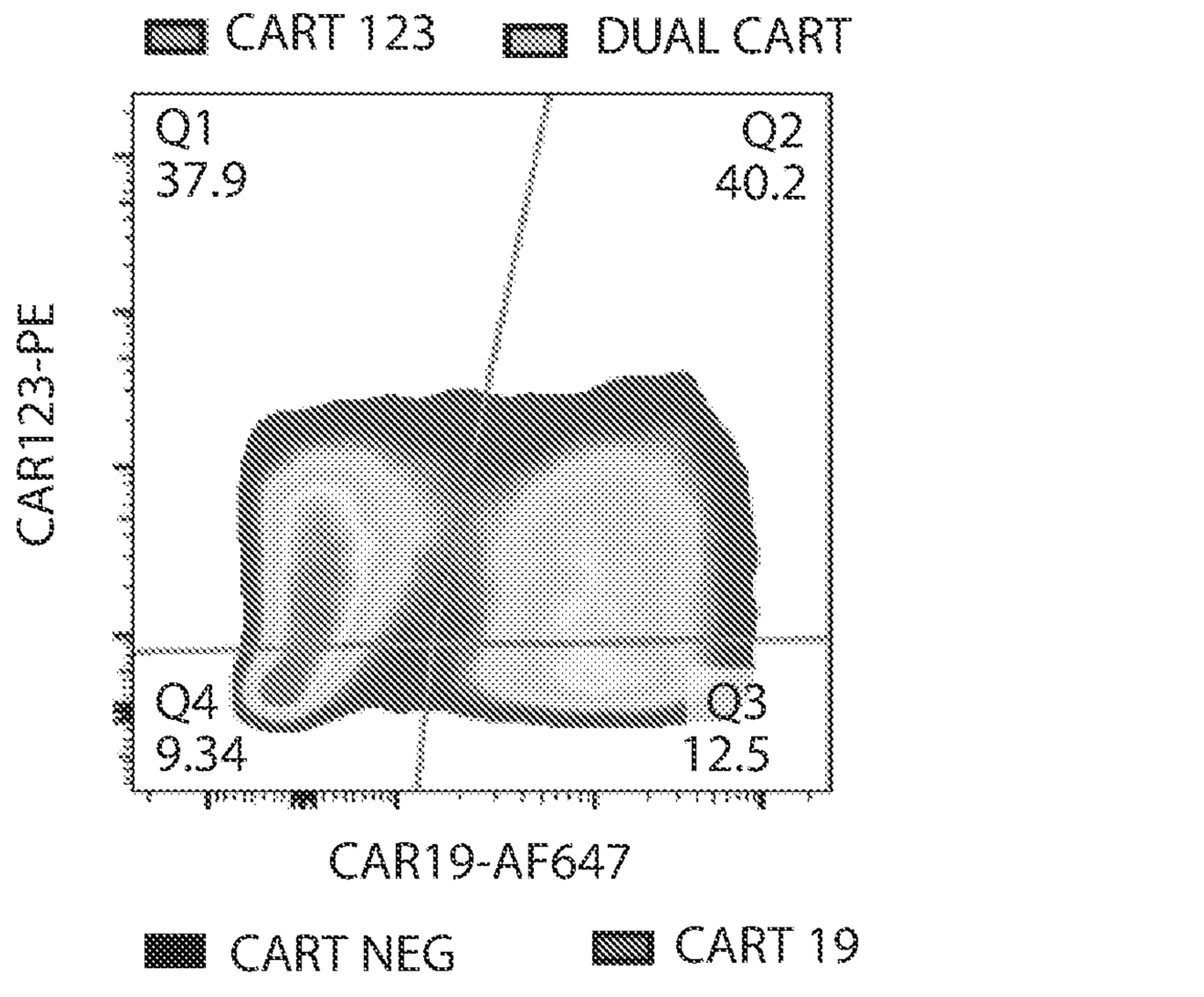


FIG. 55A

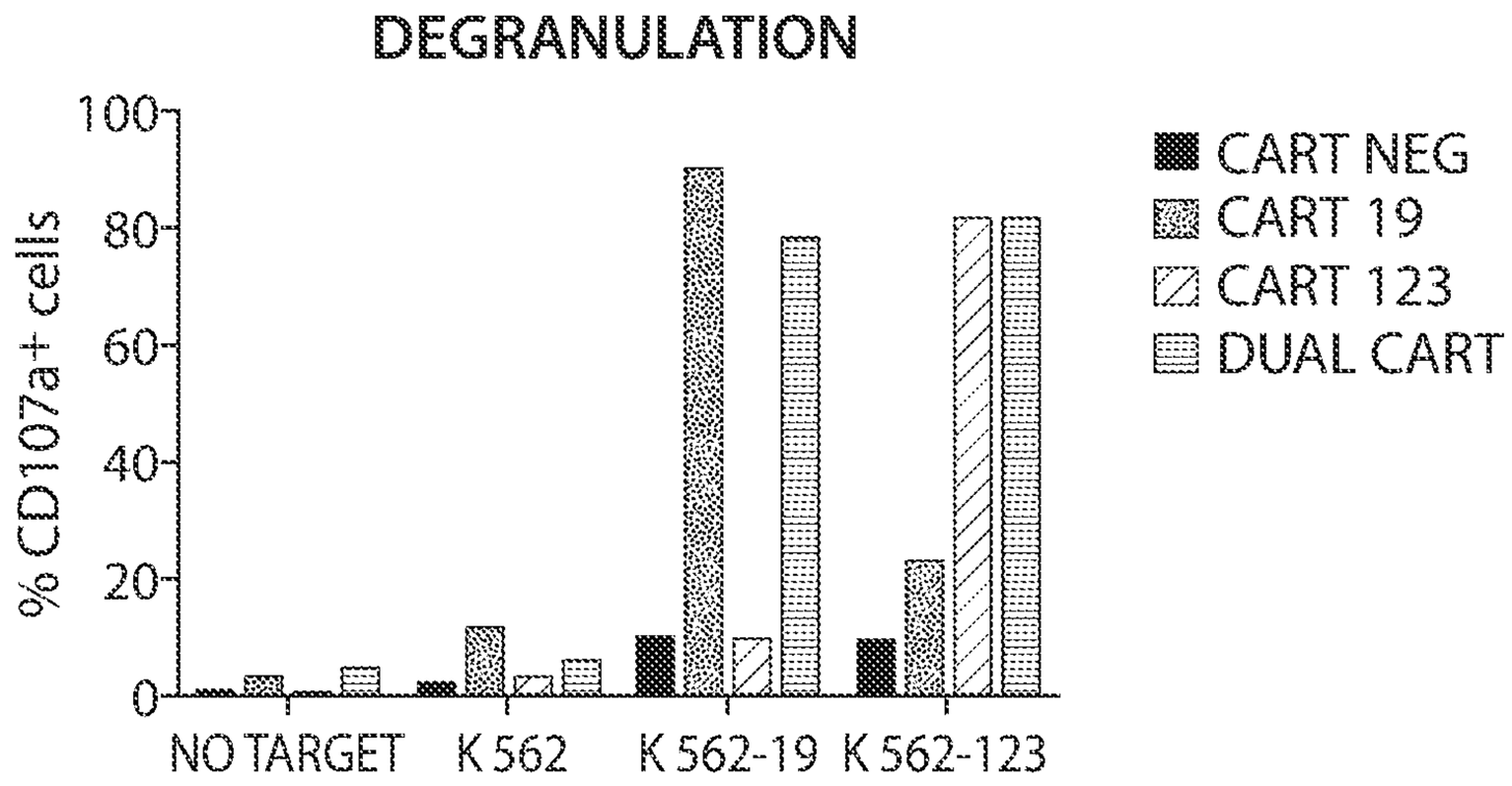


FIG. 55B

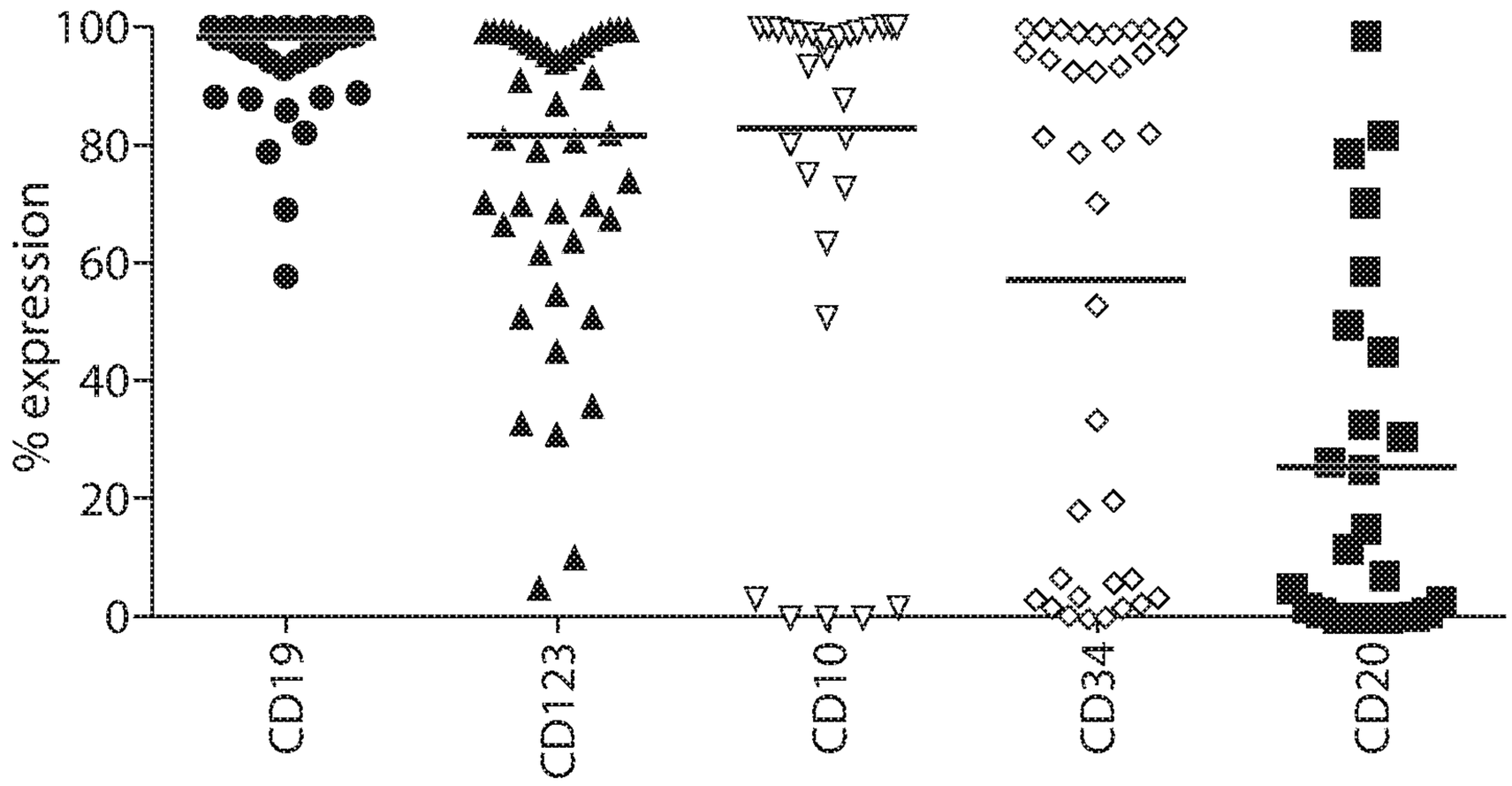


FIG. 56A

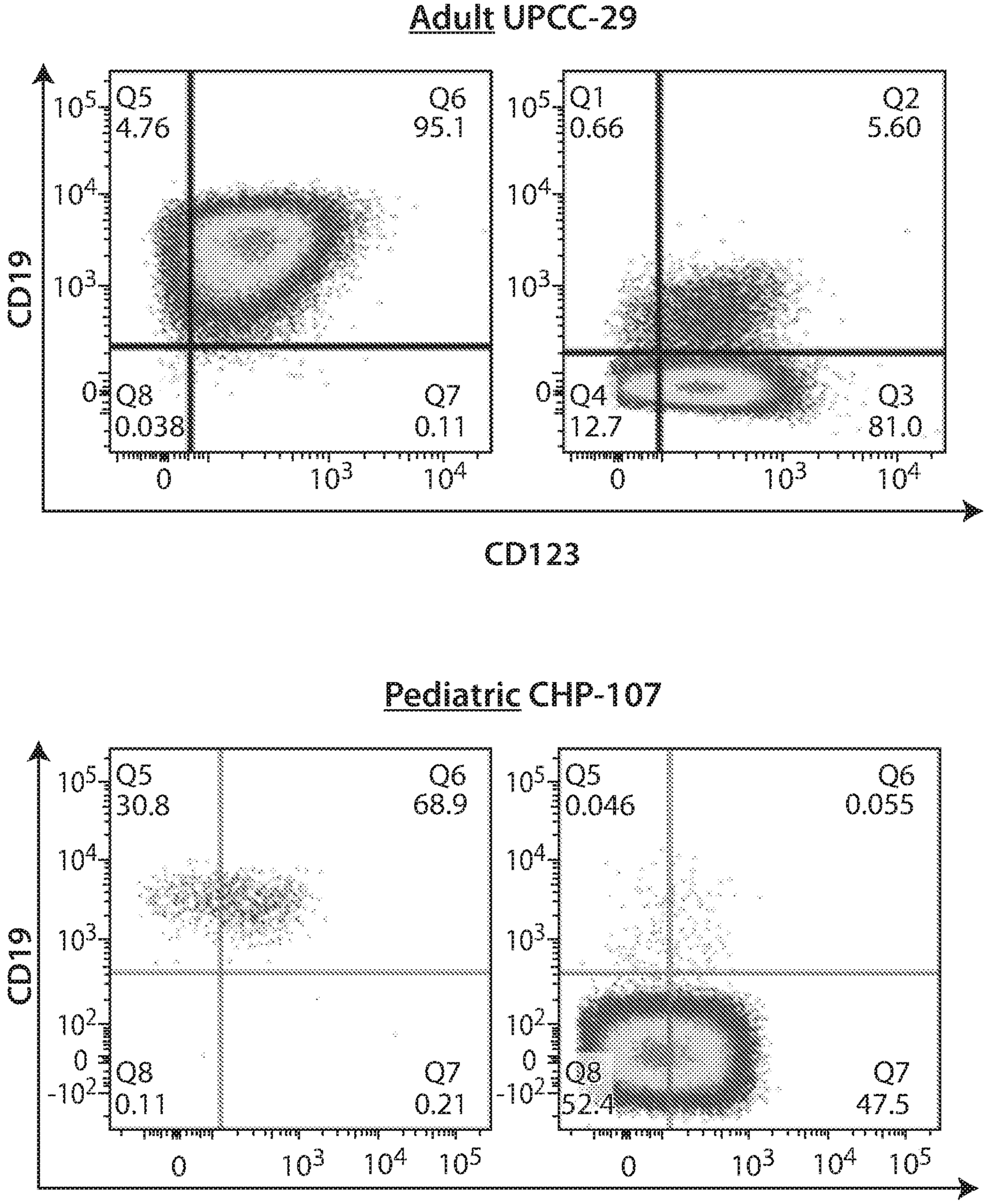

Adult UPCC-29
CD19
Q5
4.76
Q6
95.1
Q8
0.038
Q7
0.11
Q1
0.66
Q2
5.60
Q4
12.7
Q3
81.0
0
10^3
10^4
10^5
CD123
Pediatric CHP-107
CD19
Q5
30.8
Q6
68.9
Q8
0.11
Q7
0.21
Q5
0.046
Q6
0.055
Q8
52.4
Q7
47.5
-10^2
10^2
10^3
10^4
10^5
CD123

FIG. 56B

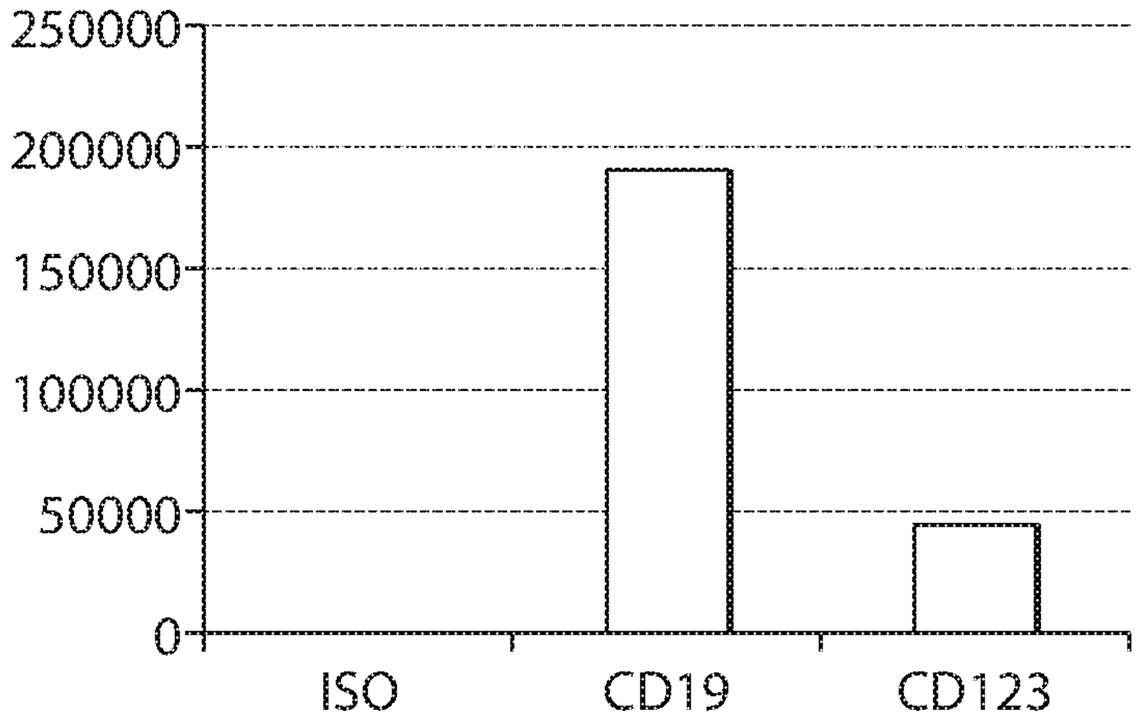


FIG. 57A

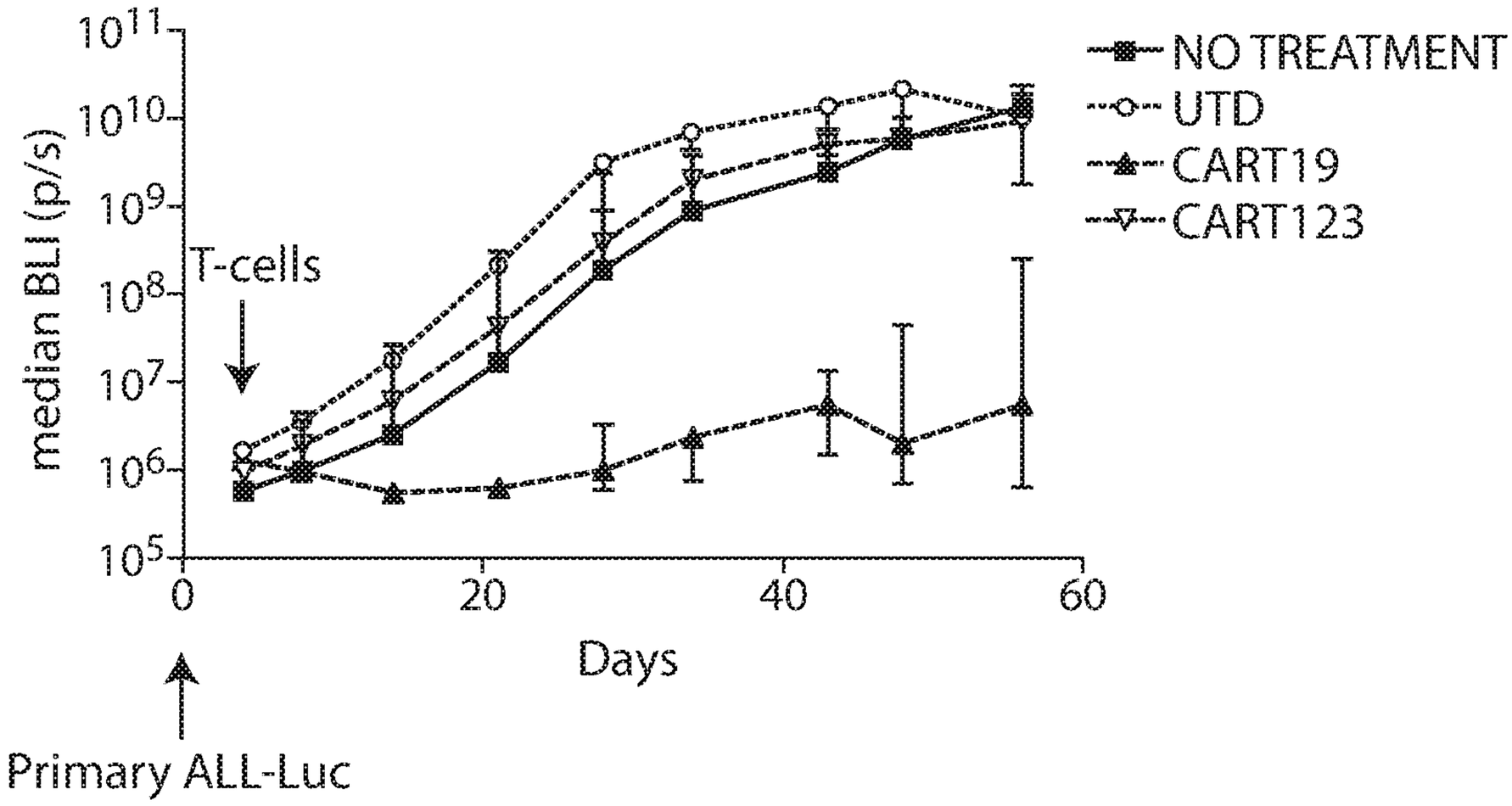


FIG. 57B

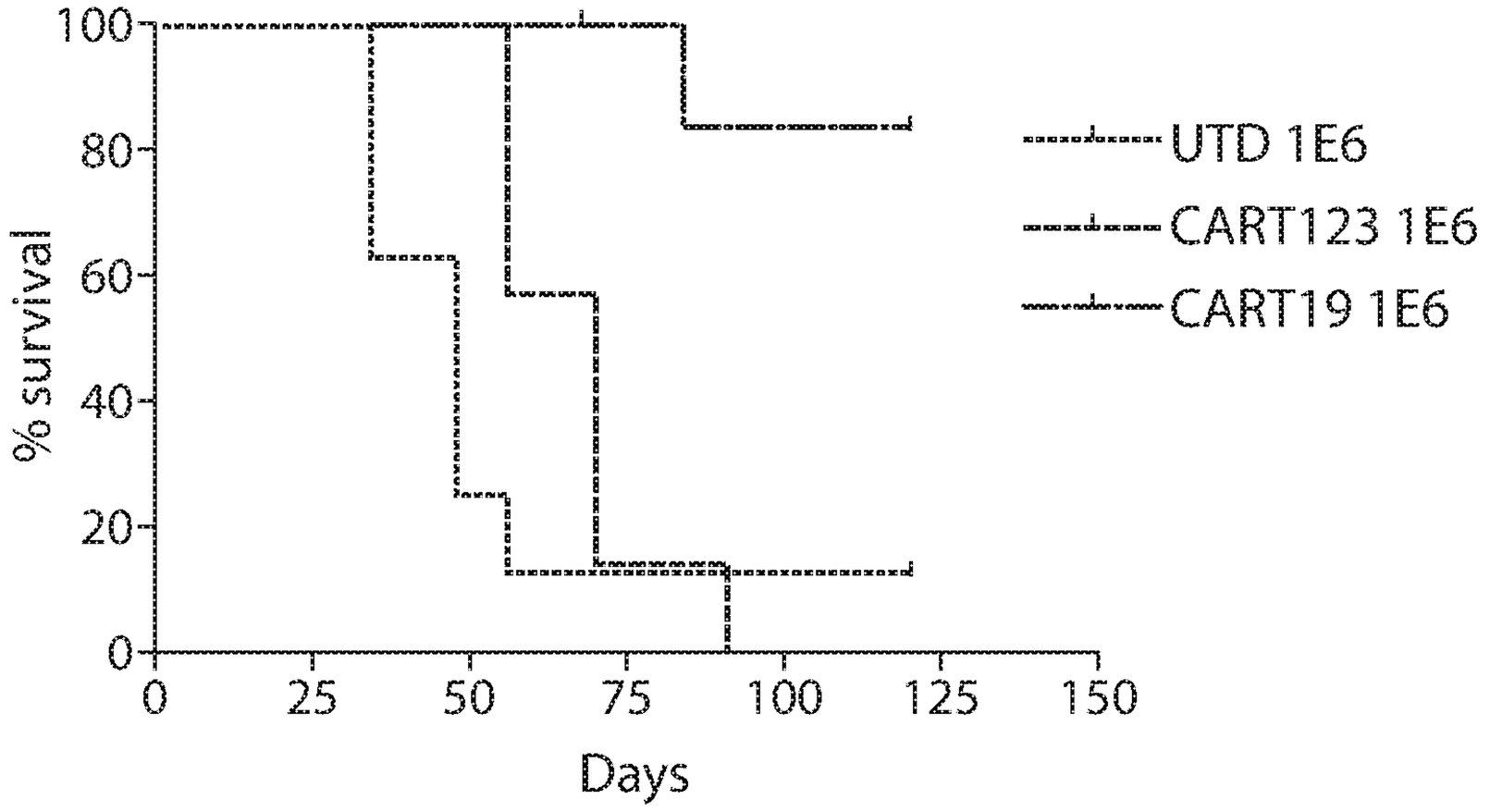


FIG. 57C

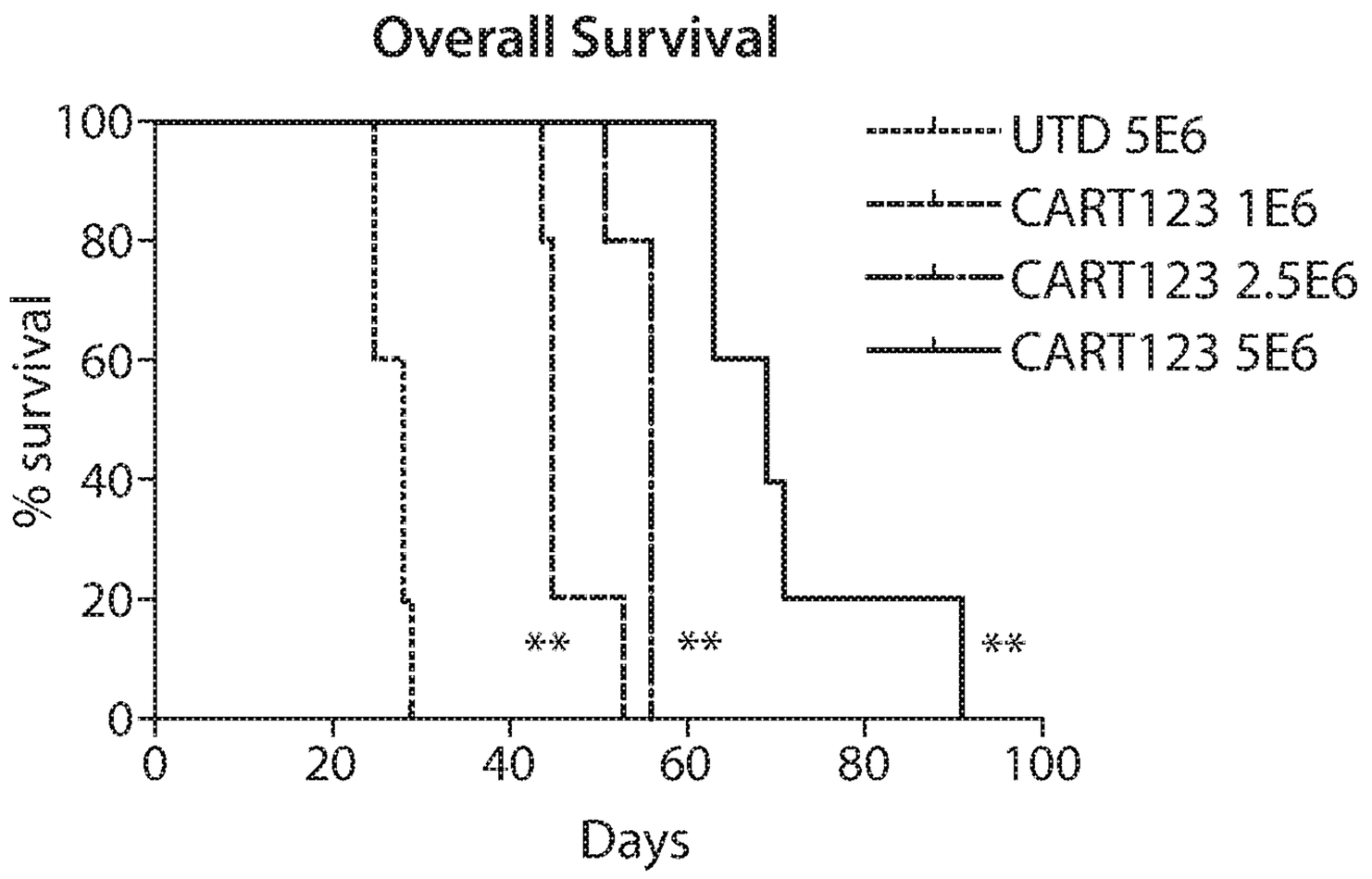


FIG. 57D

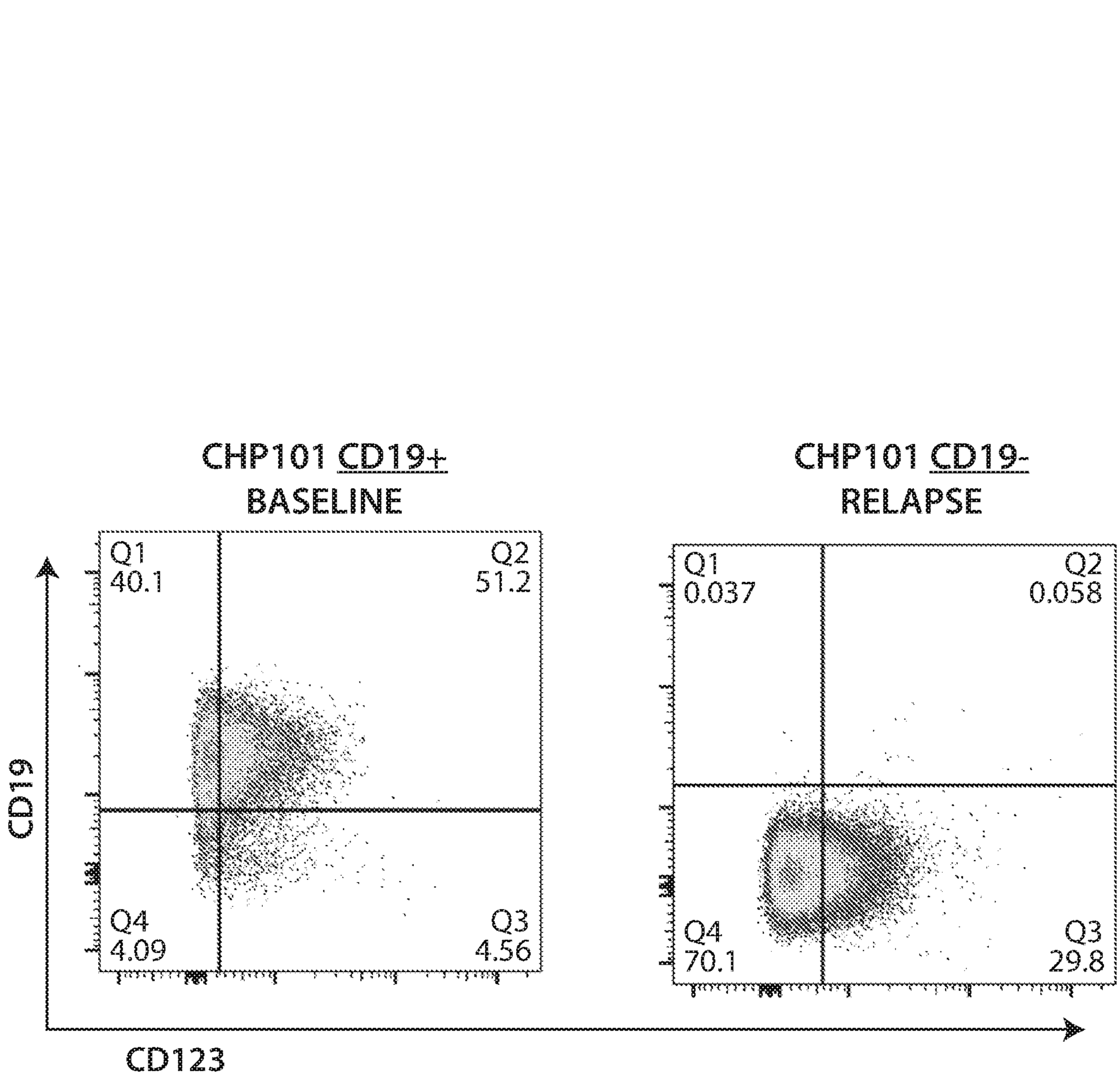

CHP101 CD19+
BASELINE
Q1
40.1
Q2
51.2
Q4
4.09
Q3
4.56
CHP101 CD19-
RELAPSE
Q1
0.037
Q2
0.058
Q4
70.1
Q3
29.8
CD19
CD123

FIG. 58A

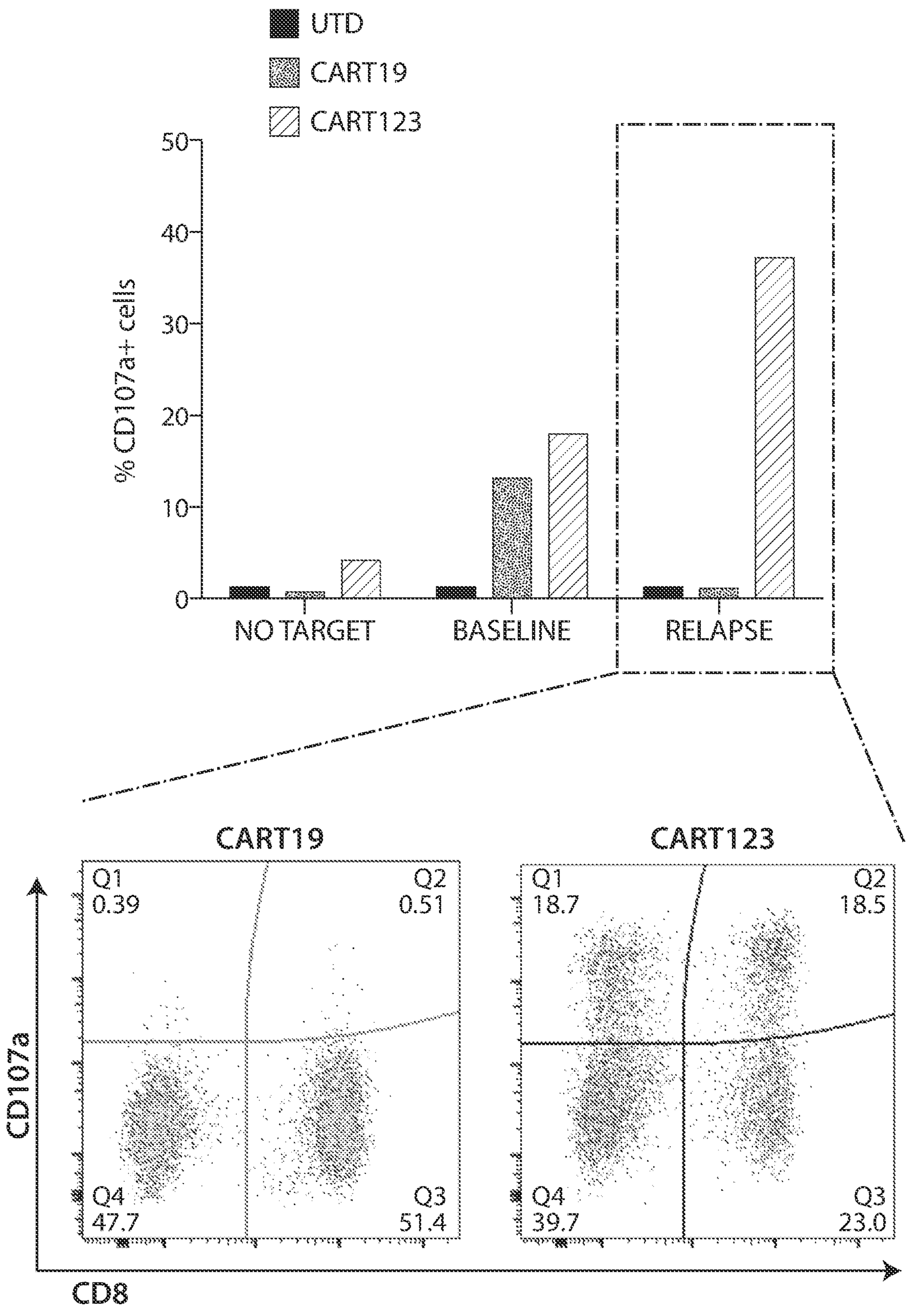

UTD
CART19
CART123
50
40
30
20
10
0
% CD107a+ cells
NO TARGET
BASELINE
RELAPSE
CART19
CART123
Q1
0.39
Q2
0.51
Q4
47.7
Q3
51.4
Q1
18.7
Q2
18.5
Q4
39.7
Q3
23.0
CD107a
CD8

FIG. 58B

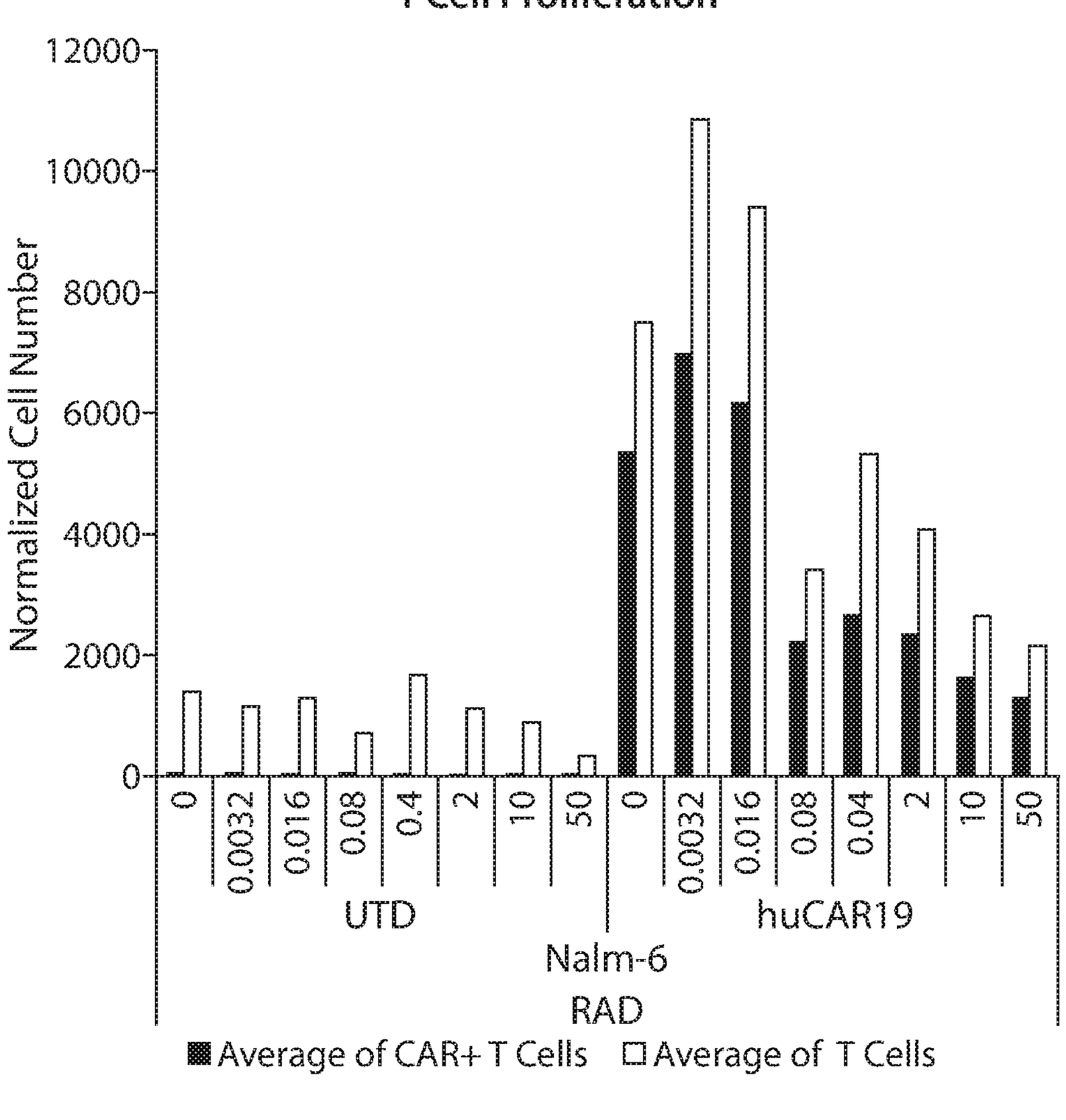

T Cell Proliferation
Normalized Cell Number
12000
10000
8000
6000
4000
2000
0
0
0.0032
0.016
0.08
0.4
2
10
50
0
0.0032
0.016
0.08
0.04
2
10
50
UTD
huCAR19
Nalm-6
RAD
Average of CAR+ T Cells
Average of T Cells

FIG. 59

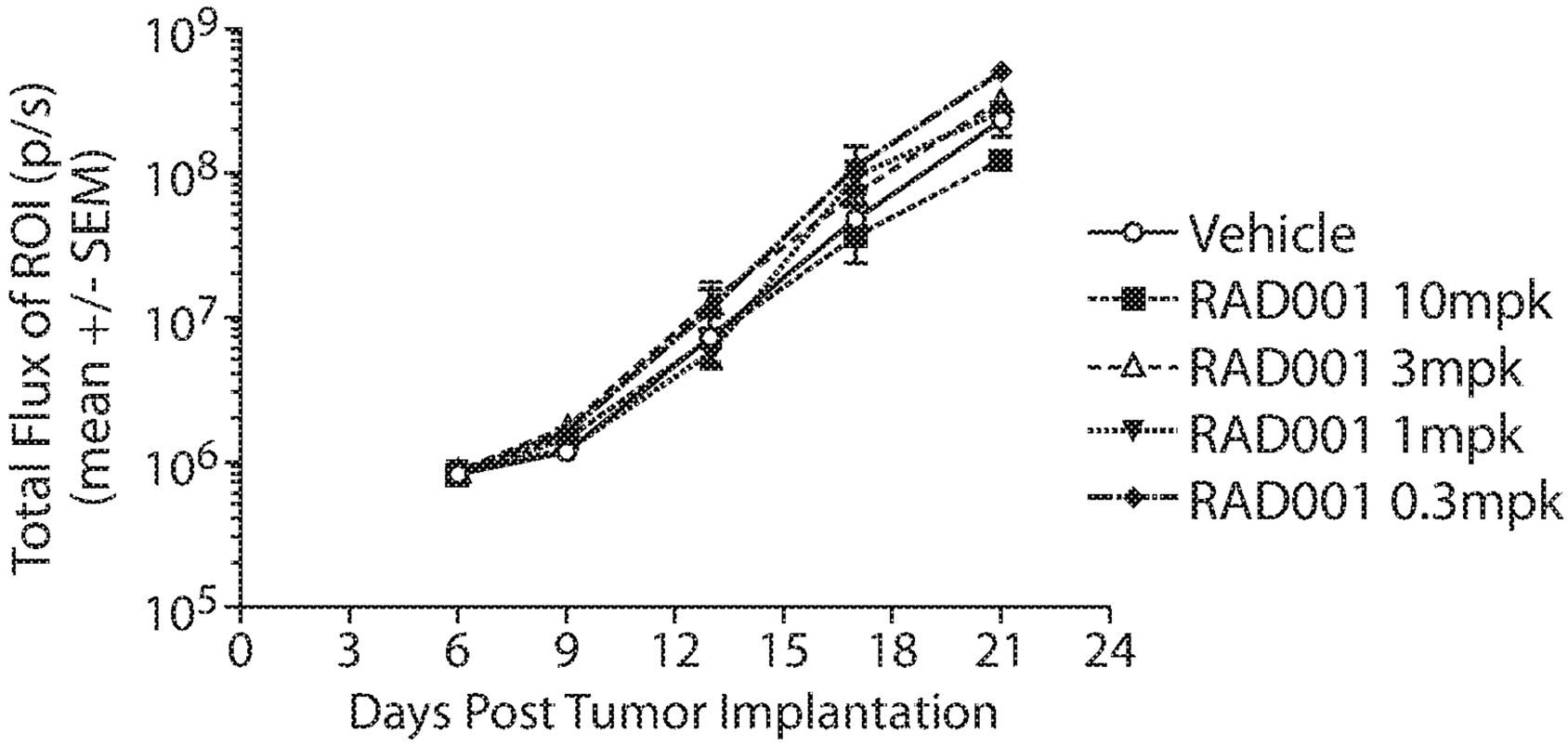

Total Flux of ROI (p/s)
(mean +/- SEM)
10^9
10^8
10^7
10^6
10^5
0
3
6
9
12
15
18
21
24
Days Post Tumor Implantation
Vehicle
RAD001 10mpk
RAD001 3mpk
RAD001 1mpk
RAD001 0.3mpk

FIG. 60

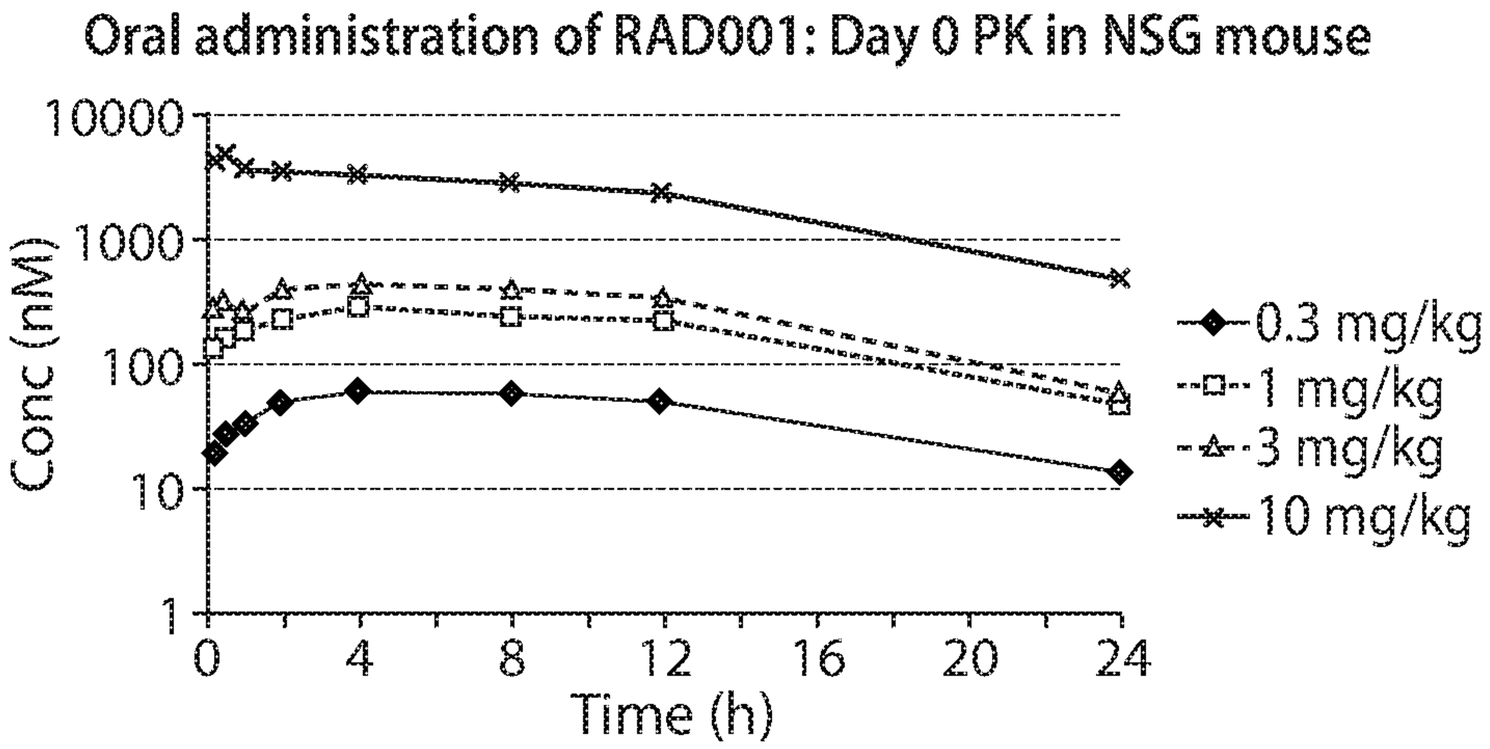


FIG. 61A

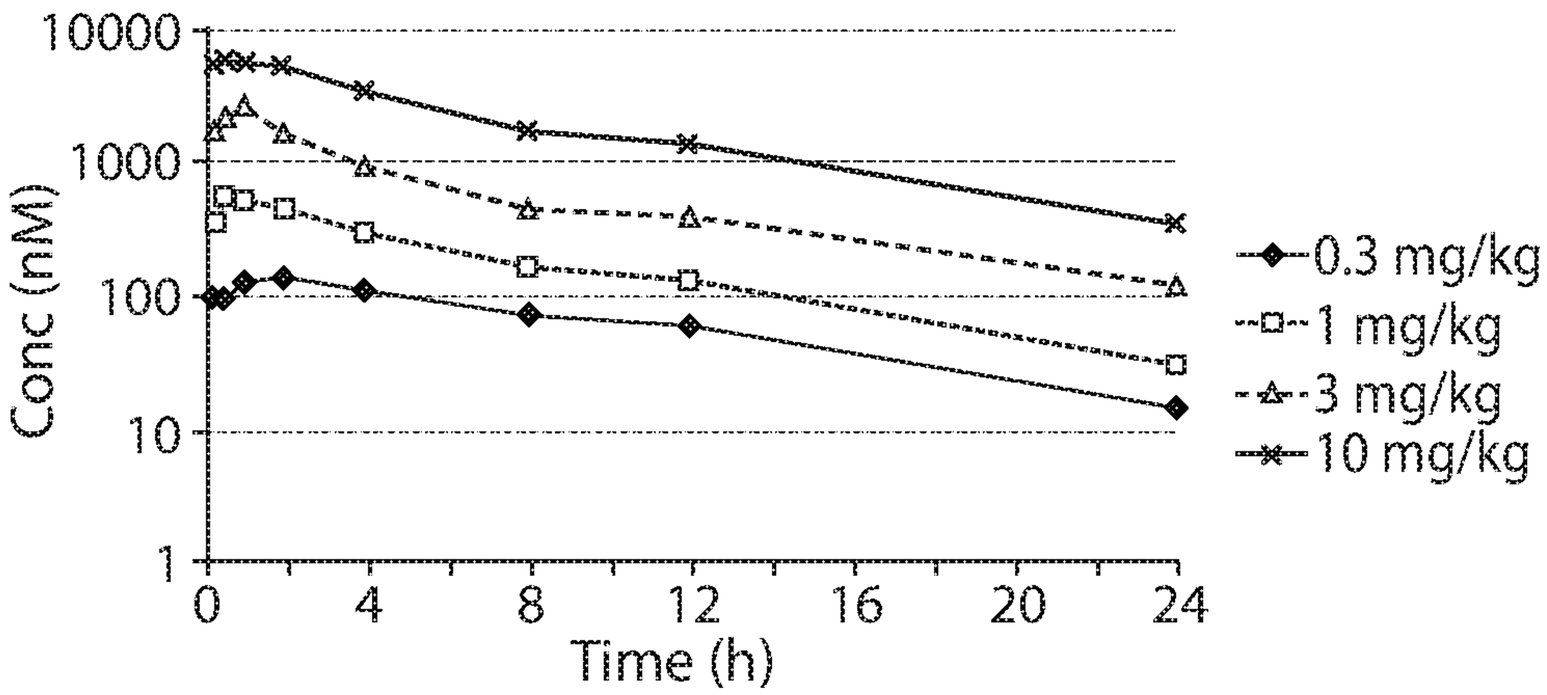


FIG. 61B

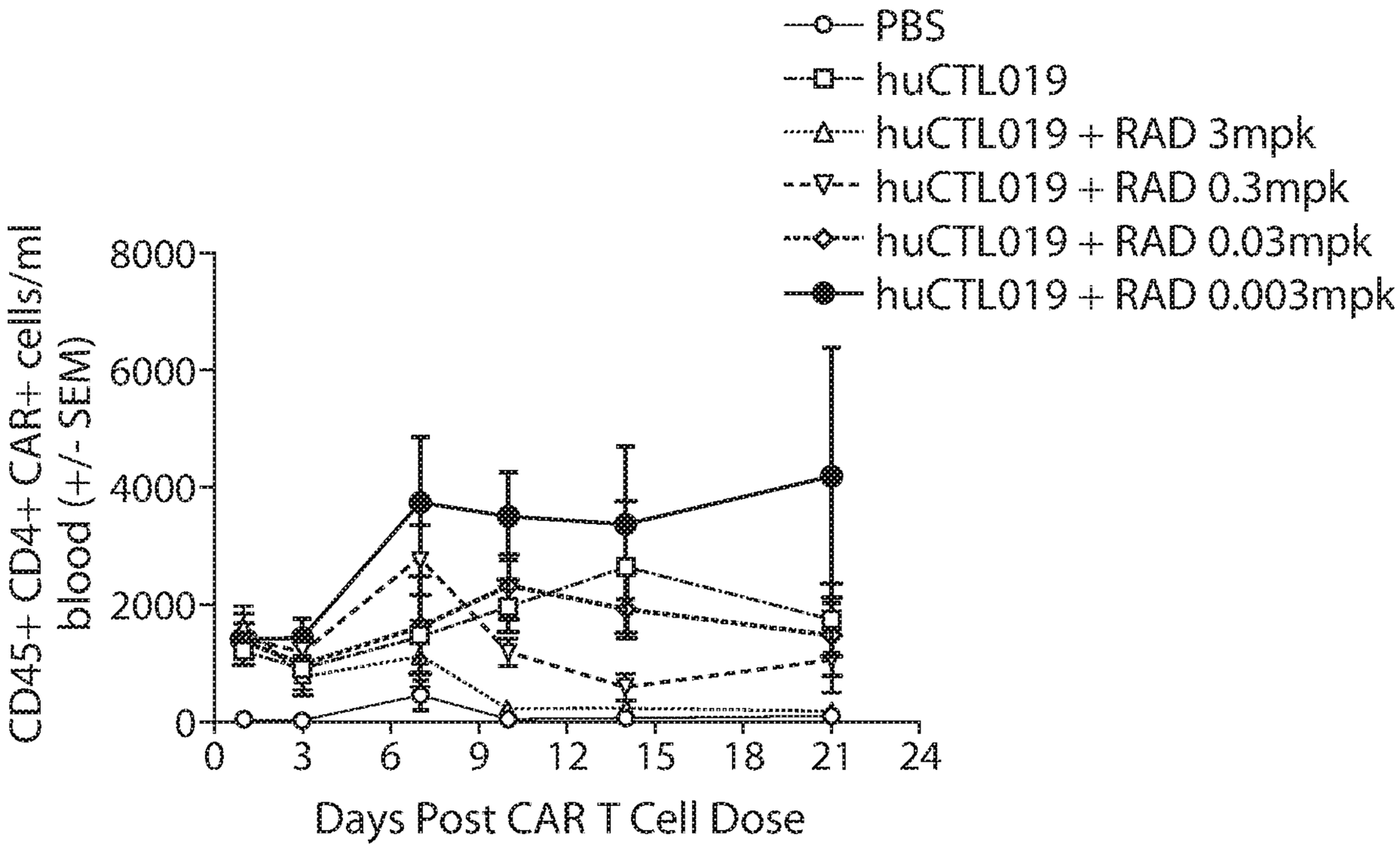


FIG. 62A

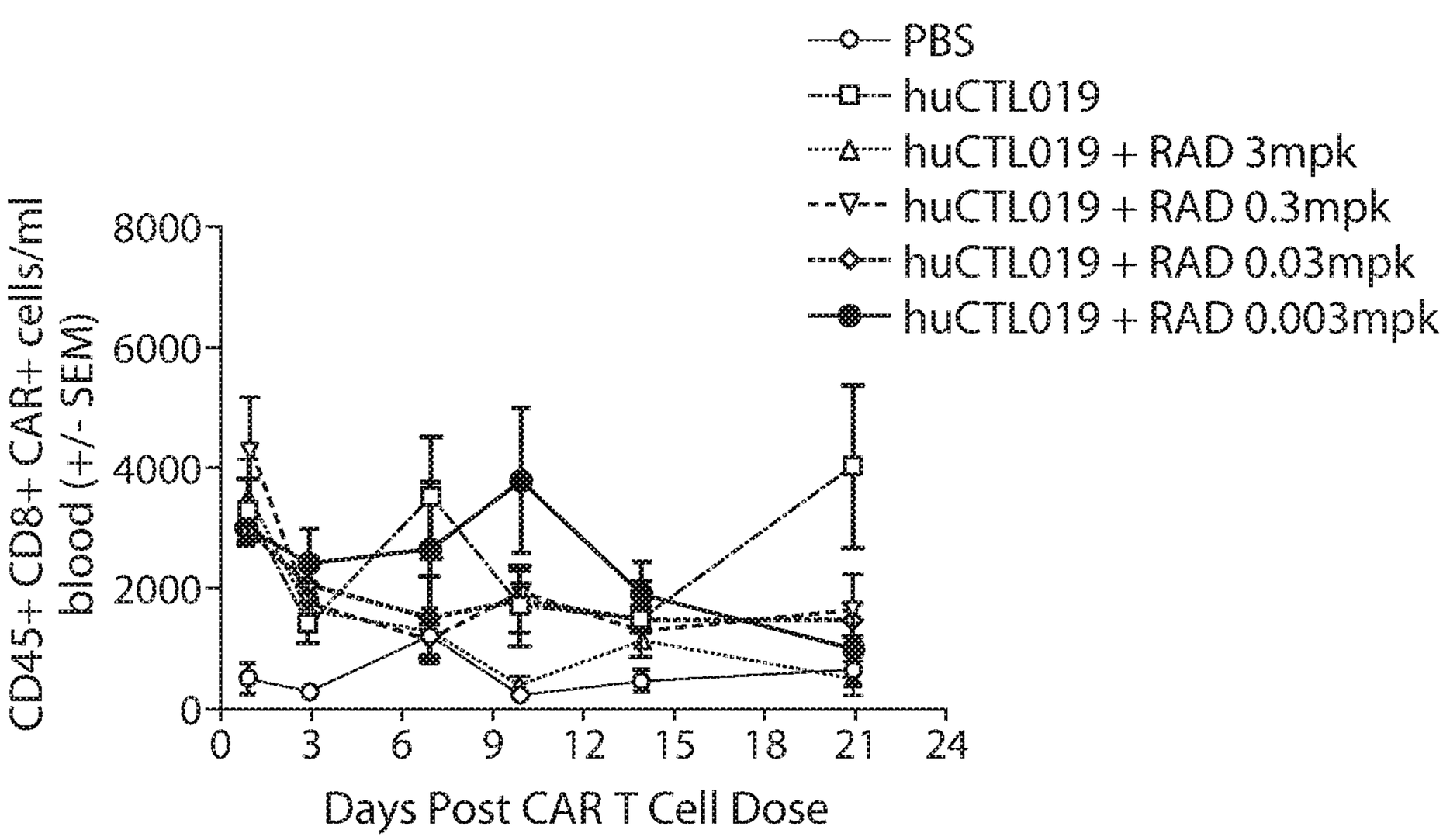


FIG. 62B

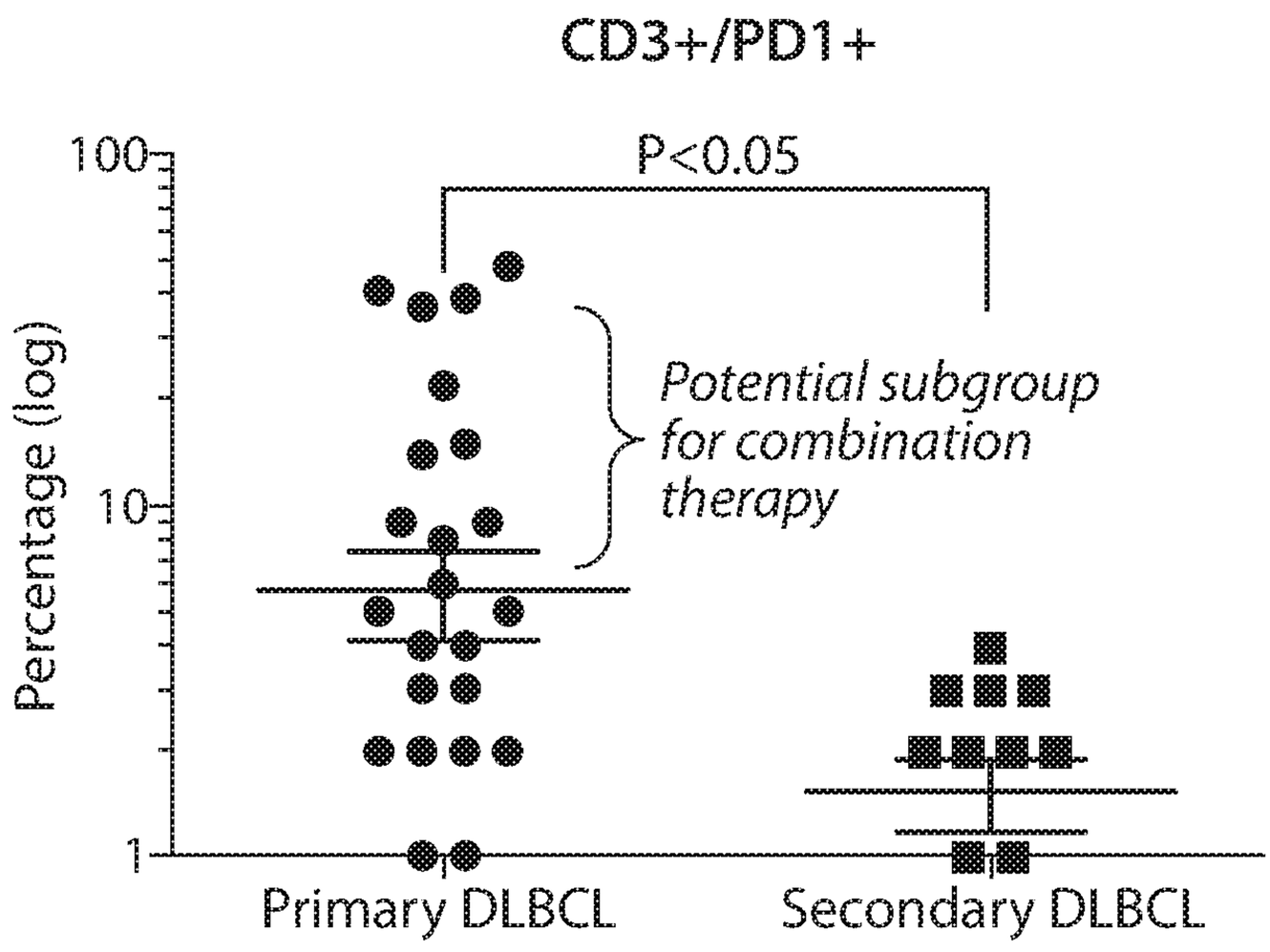

CD3+/PD1+
P<0.05
Potential subgroup for combination therapy
Percentage (log)
100
10
1
Primary DLBCL
Secondary DLBCL

FIG. 63

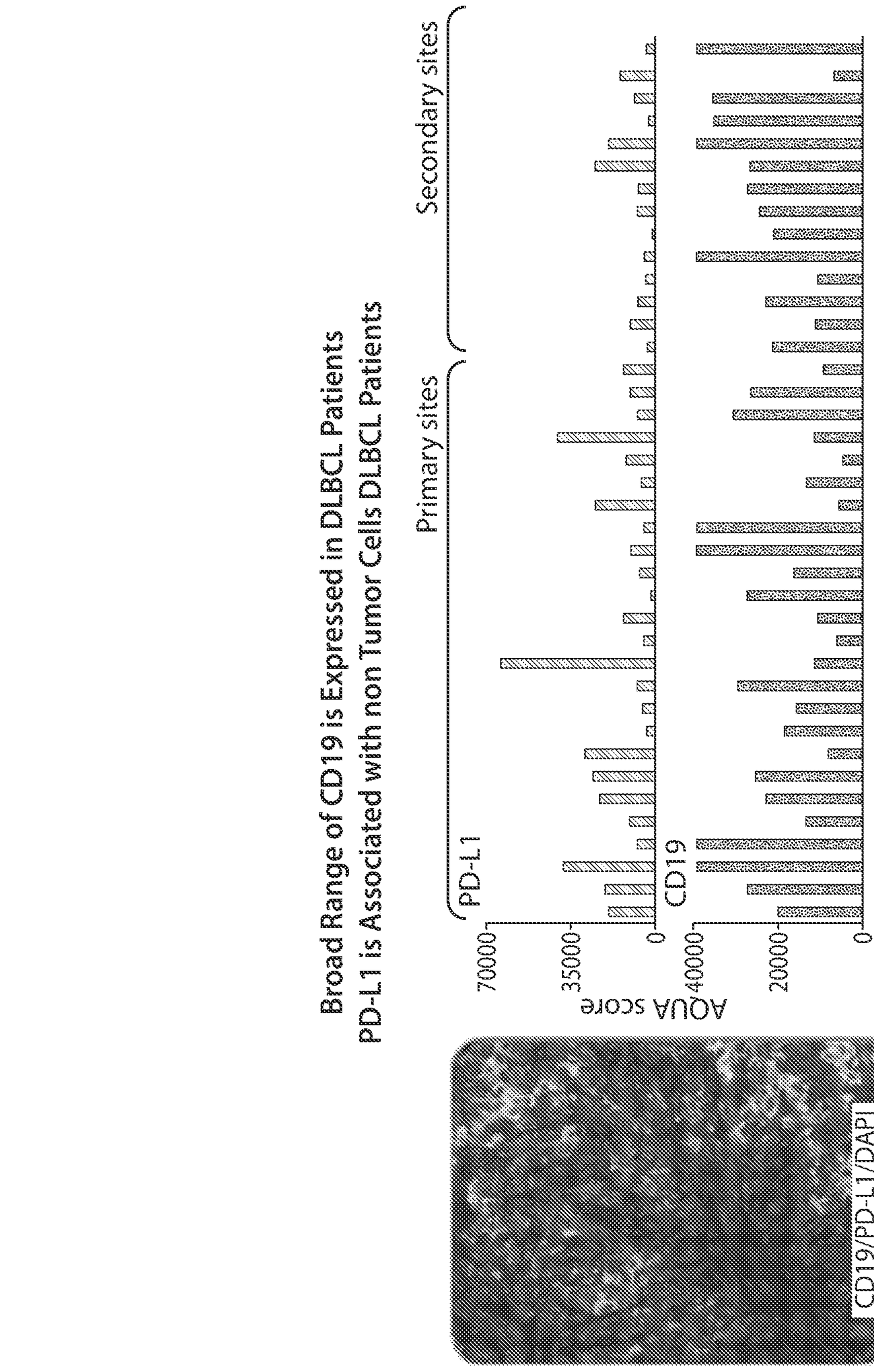

Broad Range of CD19 is Expressed in DLBCL Patients
PD-L1 is Associated with non Tumor Cells DLBCL Patients
Primary sites
Secondary sites
AQUA score
PD-L1
CD19
70000
35000
0
40000
20000
0
CD19/PD-L1/DAPI

FIG. 64

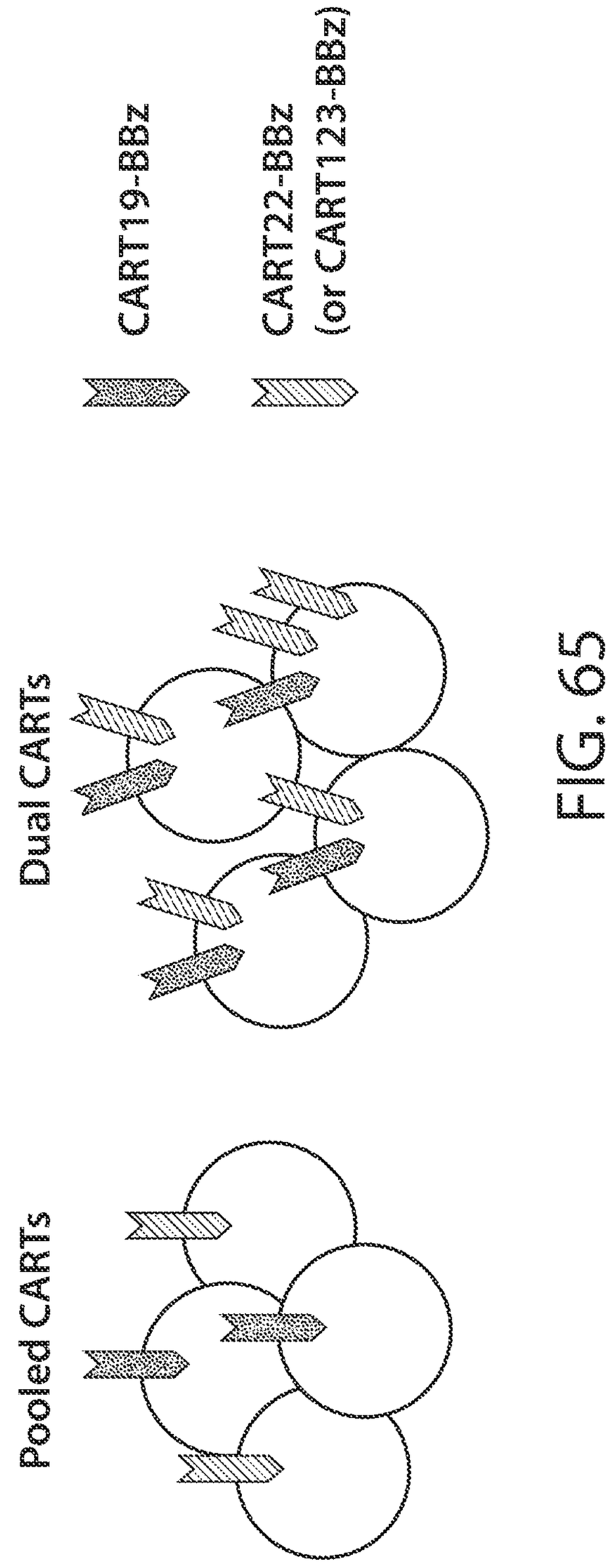

CART19-BBz
CART22-BBz
(or CART123-BBz)
Dual CARTs
Pooled CARTs

FIG. 65

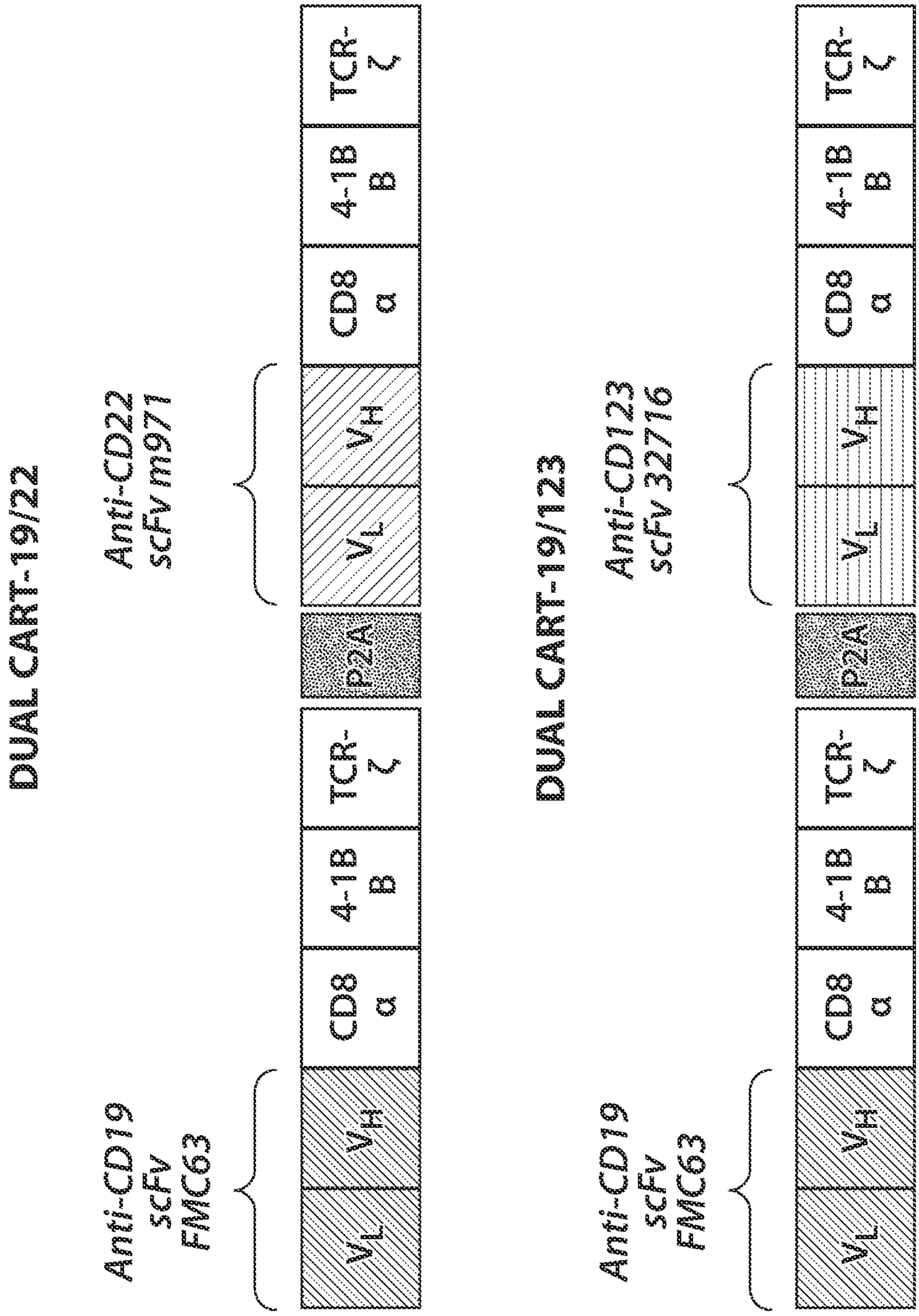

DUAL CART-19/22
Anti-CD22 scFv m971
CD8 a
4-1B B
TCR-ζ
P2A
Anti-CD19 scFv FMC63
VL
VH
DUAL CART-19/123
Anti-CD123 scFv 32716

FIG. 66

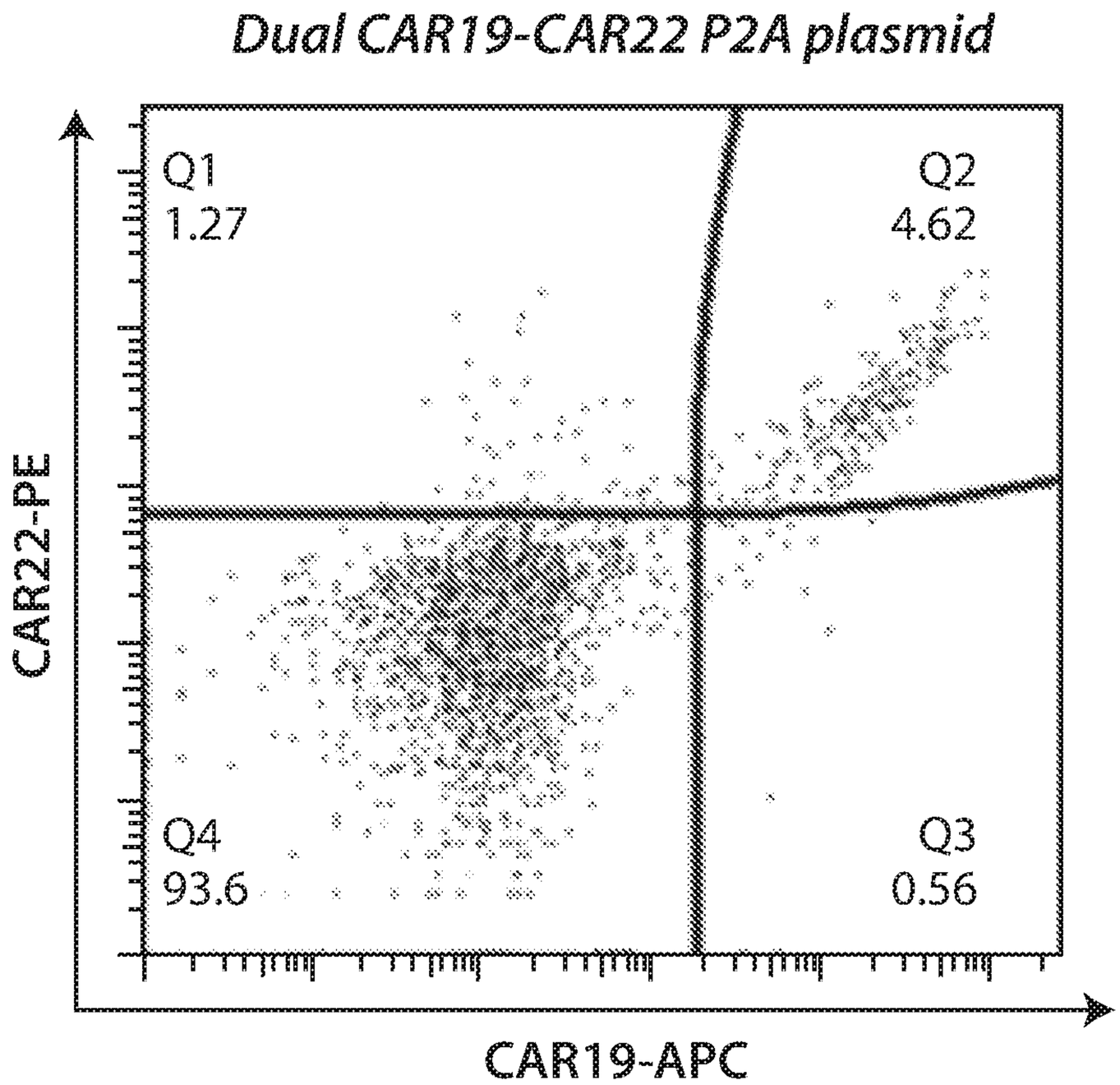

Dual CAR19-CAR22 P2A plasmid
Q1
1.27
Q2
4.62
Q4
93.6
Q3
0.56
CAR22-PE
CAR19-APC

FIG. 67

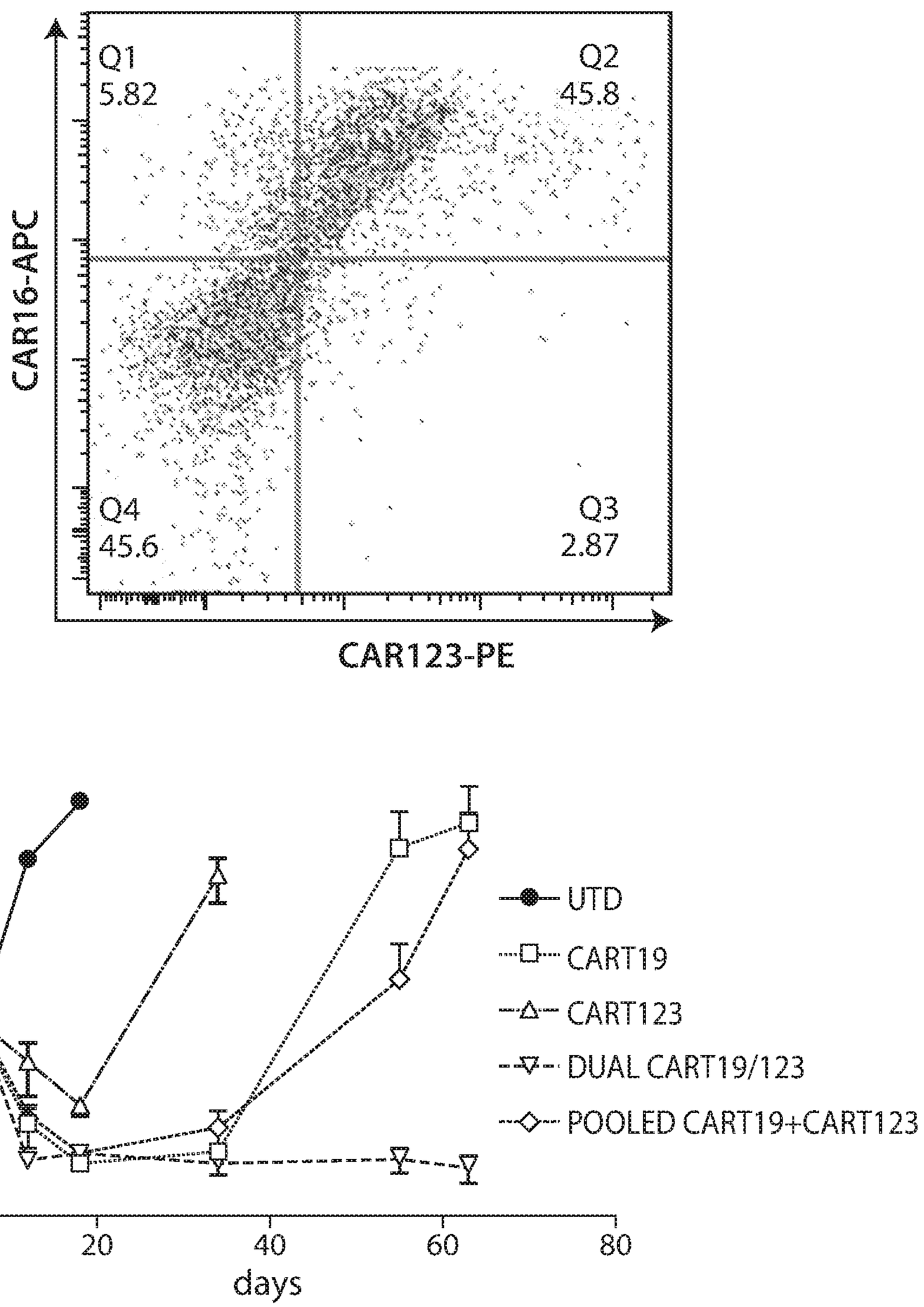
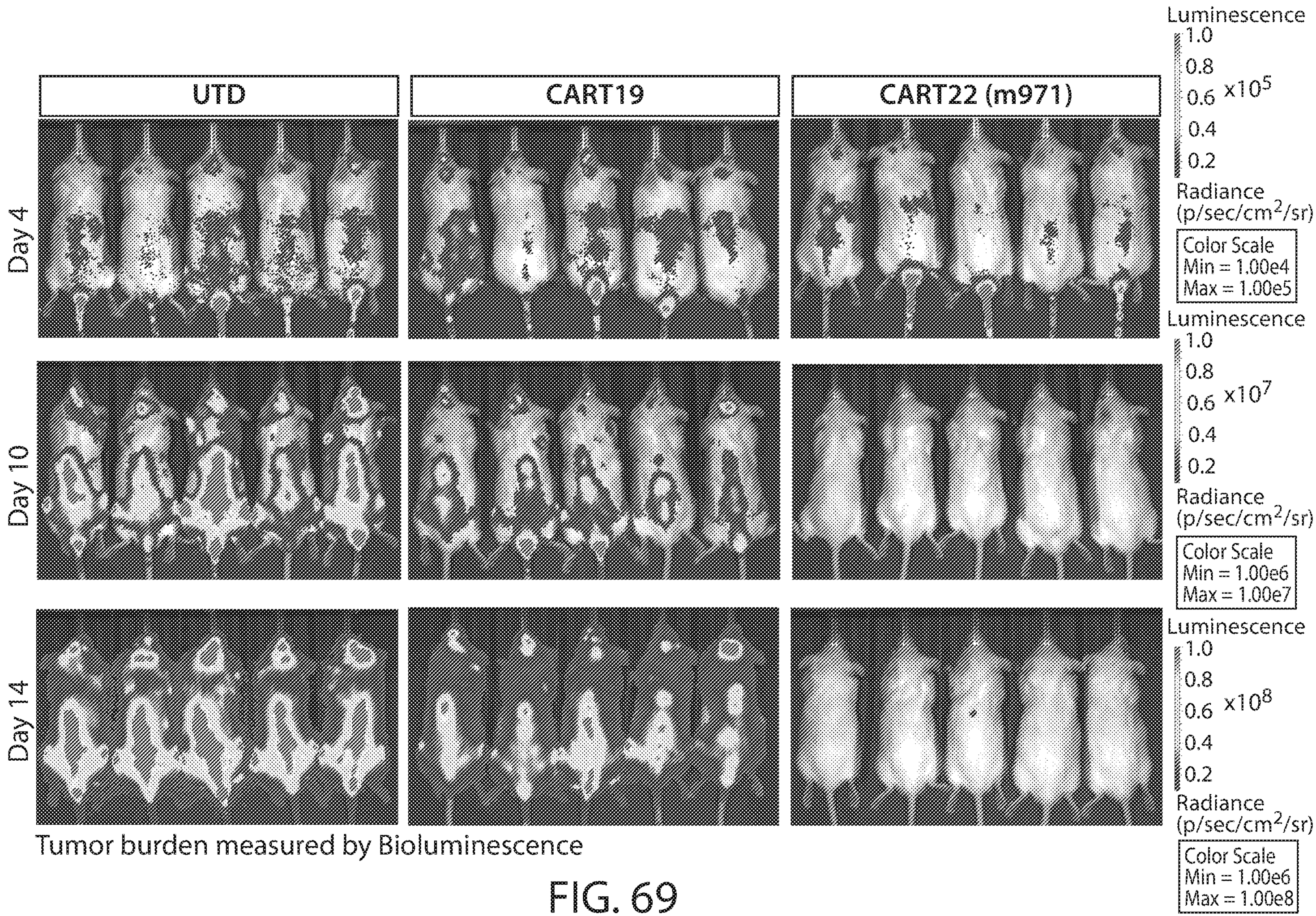

Dual CAR19-CAR123 P2A plasmid
Q1
5.82
Q2
45.8
Q4
45.6
Q3
2.87
CAR16-APC
CAR123-PE
BLI (p/sec)
10^{11}
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
0
20
40
60
80
days
UTD
CART19
CART123
DUAL CART19/123
POOLED CART19+CART123

FIG. 68



Tumor burden measured by Bioluminescence

FIG. 69

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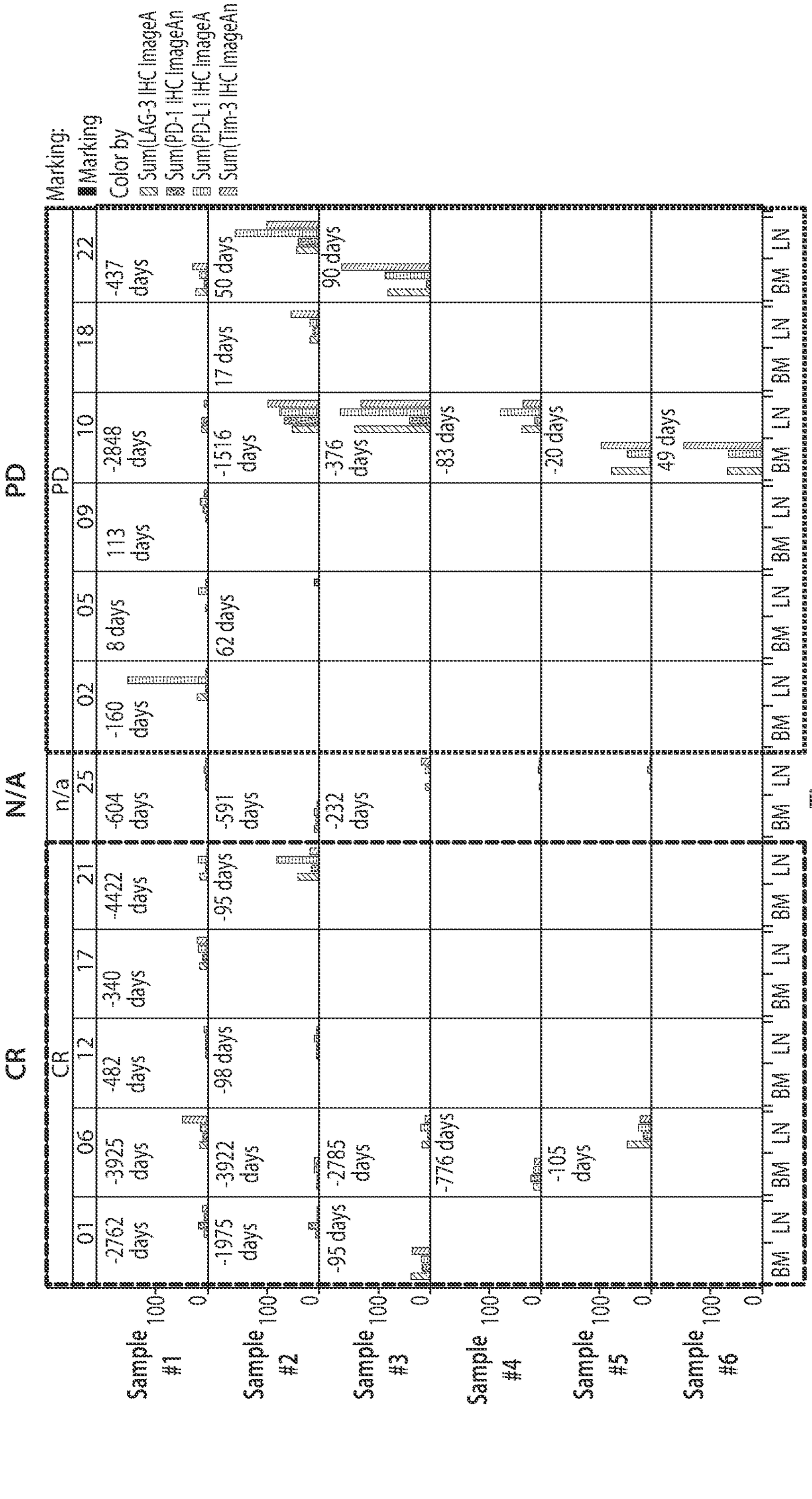
PD
N/A
CR
Marking:
Marking
Color by
Sum(LAG-3 IHC ImageA
Sum(PD-1 IHC ImageAn
Sum(PD-L1 IHC ImageA
Sum(Tim-3 IHC ImageAn
PD
n/a
CR
01
02
05
06
09
10
12
17
18
21
22
25
8 days
113 days
-2848 days
-1516 days
17 days
50 days
-437 days
62 days
-376 days
90 days
-83 days
-20 days
49 days
-2762 days
-160 days
-604 days
-591 days
-4422 days
-95 days
-232 days
-340 days
-482 days
-98 days
-3925 days
-3922 days
-2785 days
-776 days
-105 days
-1975 days
-95 days
BM
LN
Sample #1
Sample #2
Sample #3
Sample #4
Sample #5
Sample #6
100
0
Tissue

FIG. 70

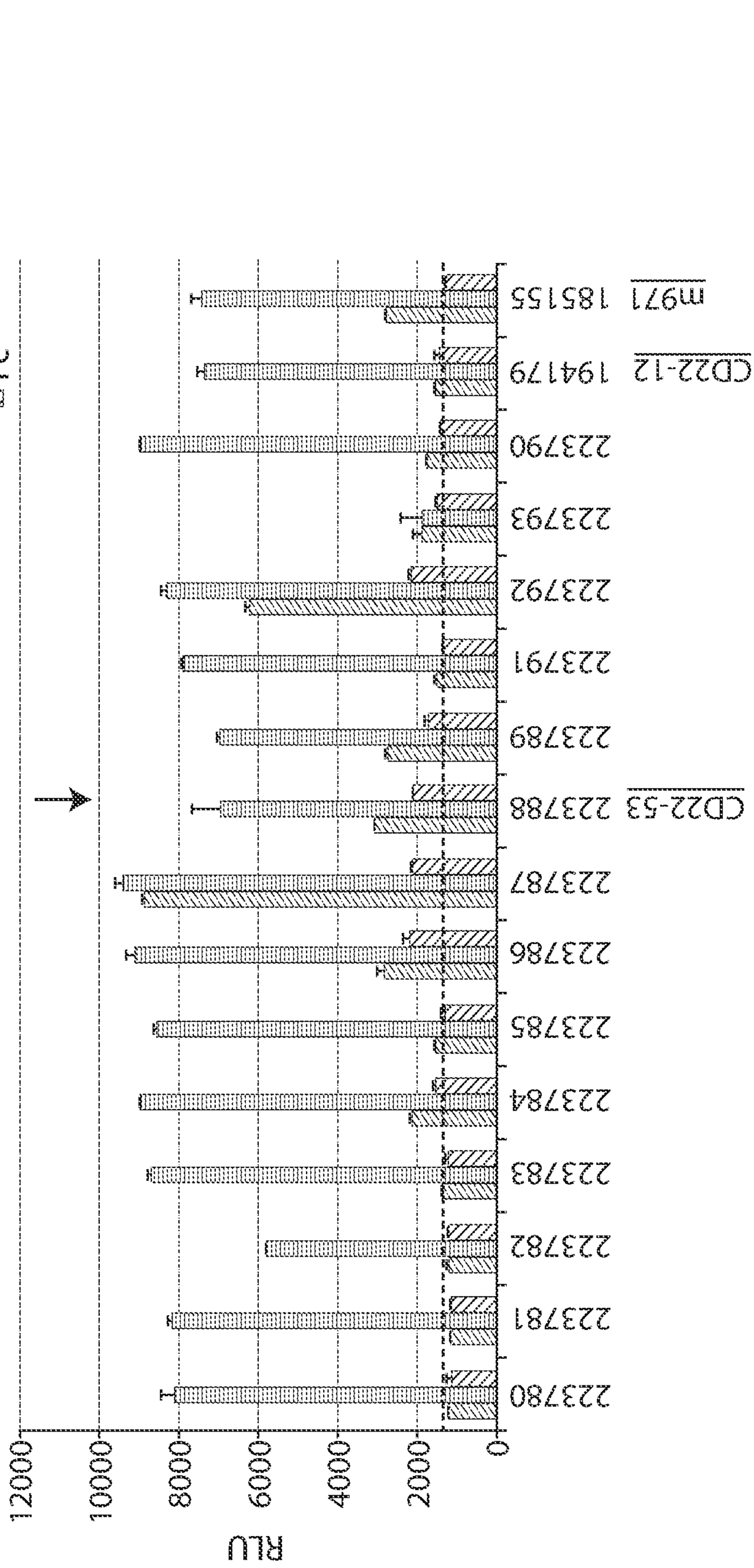
NFAT36-P1-CD22 Binders on anti-Fc antigen coated plate
09012015
CD22-D567 with m971
CD22-D567 w/o m971
Fc
RLU
12000
10000
8000
6000
4000
2000
0
CD22-53
223780
223781
223782
223783
223784
223785
223786
223787
223788
CD22-12
194179
223790
223793
223792
223791
223789
m971
185155

FIG. 71

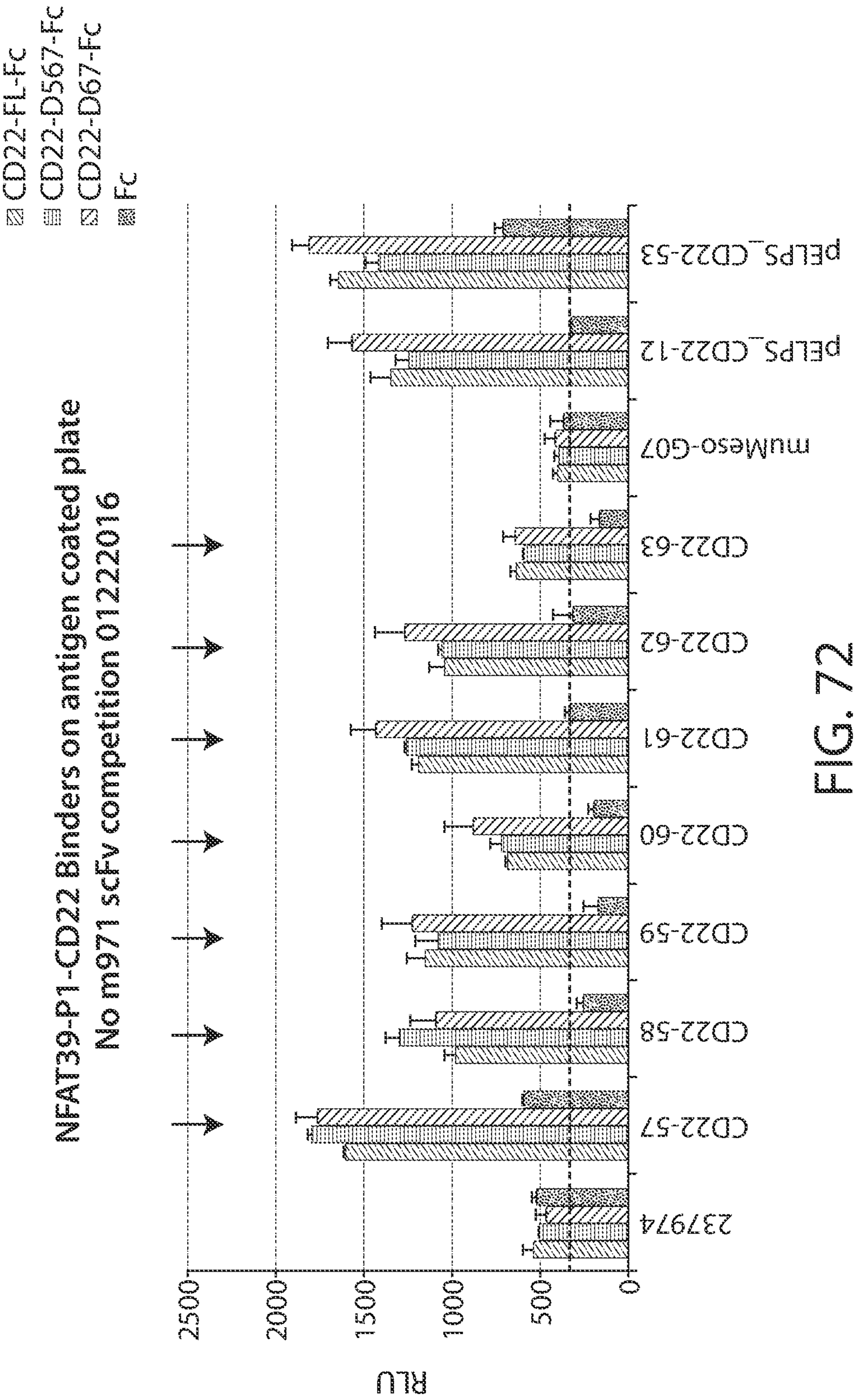
CD22-FL-Fc
CD22-D567-Fc
CD22-D67-Fc
Fc
NFAT39-P1-CD22 Binders on antigen coated plate
No m971 scFv competition 01222016
RLU
2500
2000
1500
1000
500
0
237974
CD22-57
CD22-58
CD22-59
CD22-60
CD22-61
CD22-62
CD22-63
muMeso-G07
pELPS_CD22-12
pELPS_CD22-53

FIG. 72

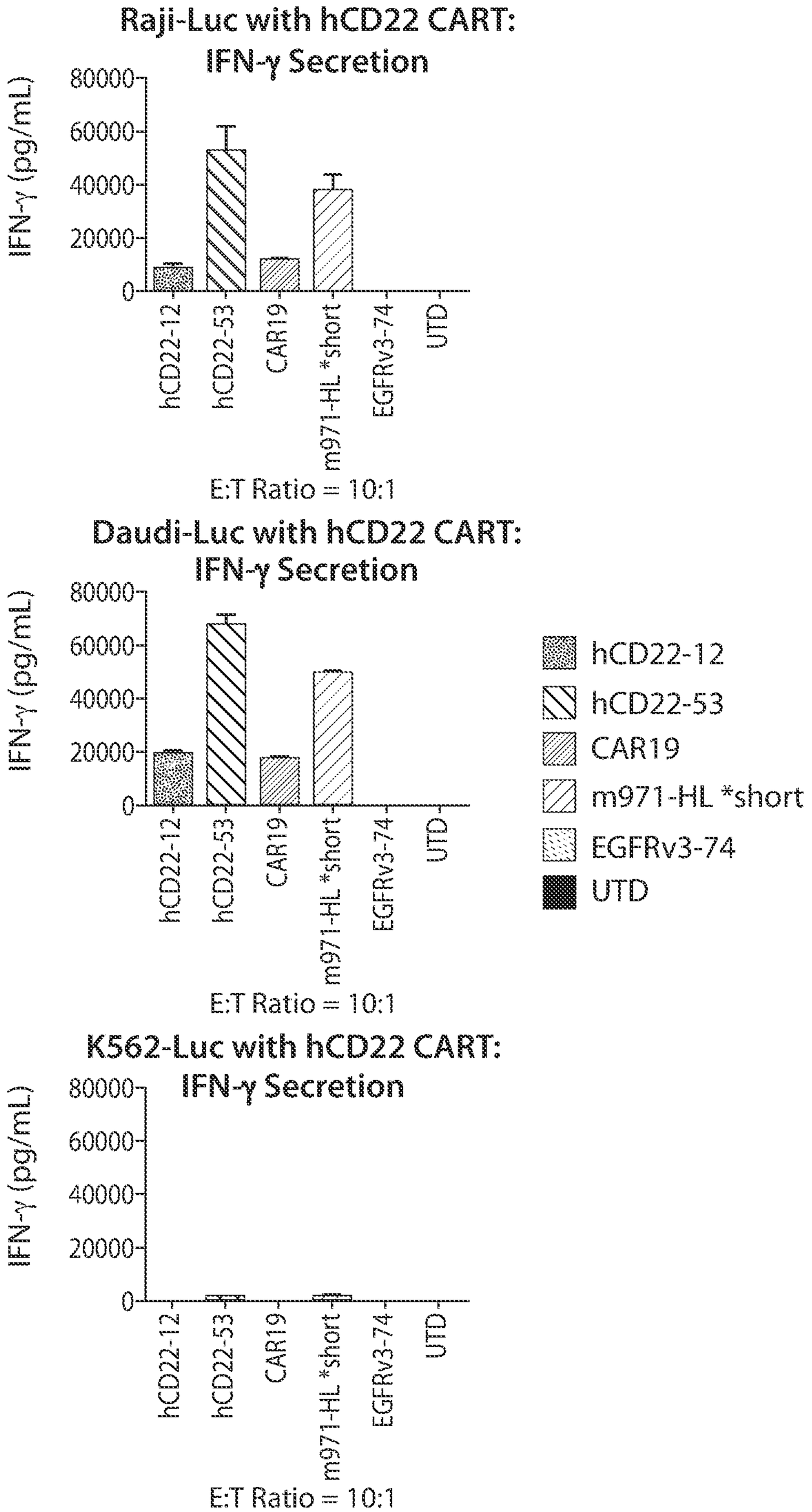
Raji-Luc with hCD22 CART:
IFN-γ Secretion
IFN-γ (pg/mL)
80000
60000
40000
20000
0
hCD22-12
hCD22-53
CAR19
m971-HL *short
EGFRv3-74
UTD
E:T Ratio = 10:1
Daudi-Luc with hCD22 CART:
IFN-γ Secretion
IFN-γ (pg/mL)
80000
60000
40000
20000
0
hCD22-12
hCD22-53
CAR19
m971-HL *short
EGFRv3-74
UTD
E:T Ratio = 10:1
K562-Luc with hCD22 CART:
IFN-γ Secretion
IFN-γ (pg/mL)
80000
60000
40000
20000
0
hCD22-12
hCD22-53
CAR19
m971-HL *short
EGFRv3-74
UTD
E:T Ratio = 10:1

FIG. 73

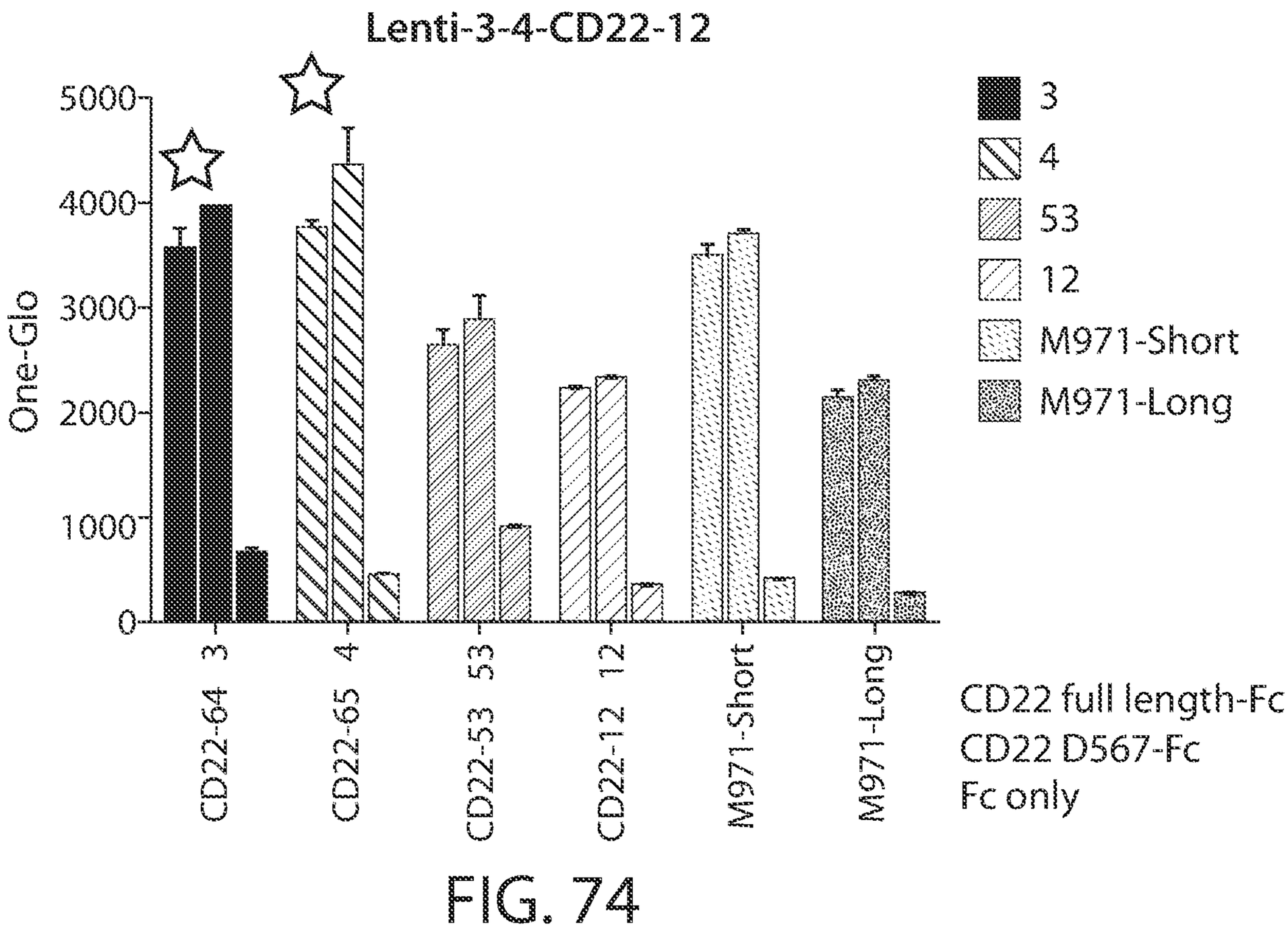
Lenti-3-4-CD22-12
One-Glo
5000
4000
3000
2000
1000
0
CD22-64 3
CD22-65 4
CD22-53 53
CD22-12 12
M971-Short
M971-Long
3
4
53
12
M971-Short
M971-Long
CD22 full length-Fc
CD22 D567-Fc
Fc only

FIG. 74

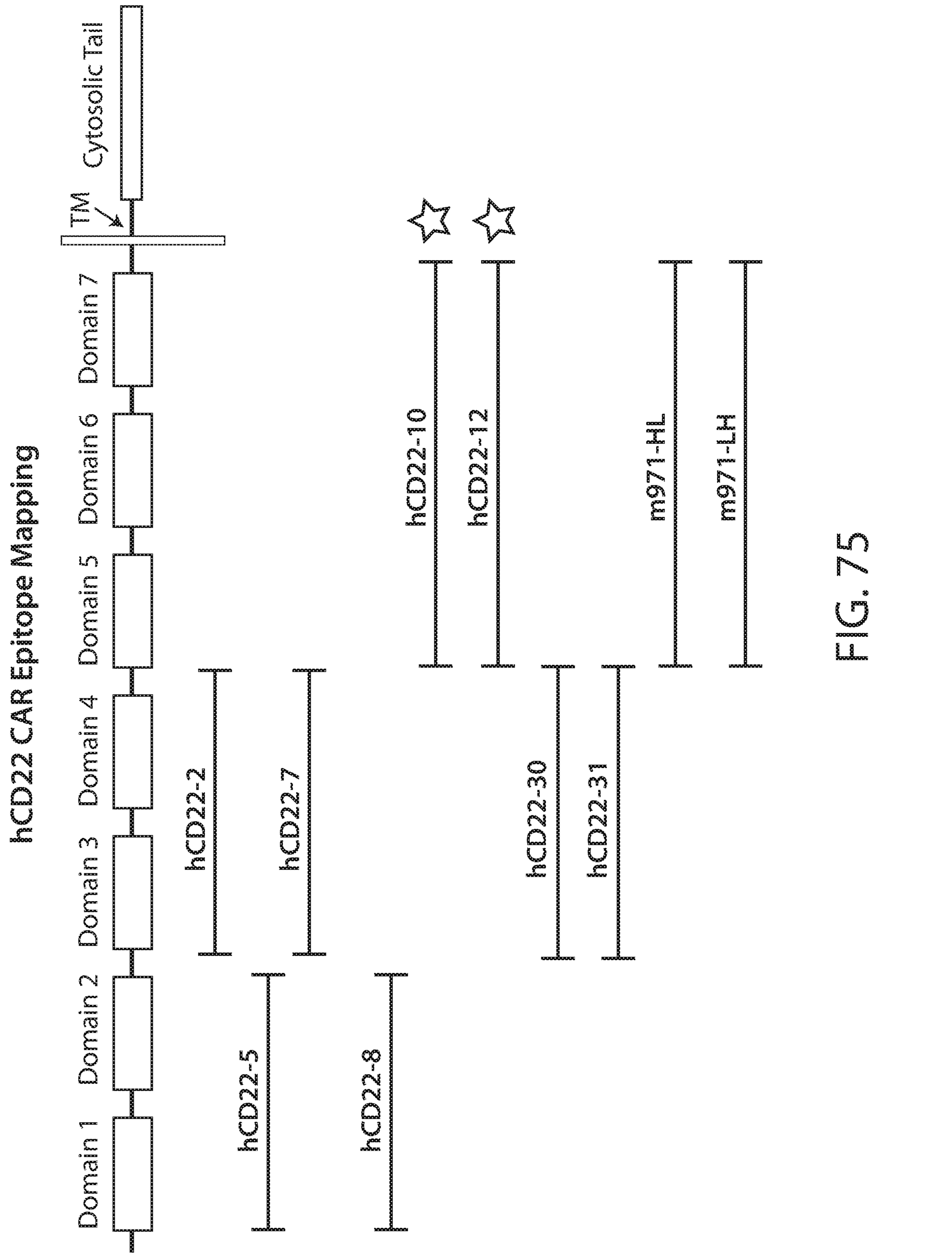
hCD22 CAR Epitope Mapping
Cytosolic Tail
TM
Domain 1
Domain 2
Domain 3
Domain 4
Domain 5
Domain 6
Domain 7
hCD22-10
hCD22-12
hCD22-2
hCD22-7
hCD22-30
hCD22-31
hCD22-5
hCD22-8
m971-HL
m971-LH

FIG. 75