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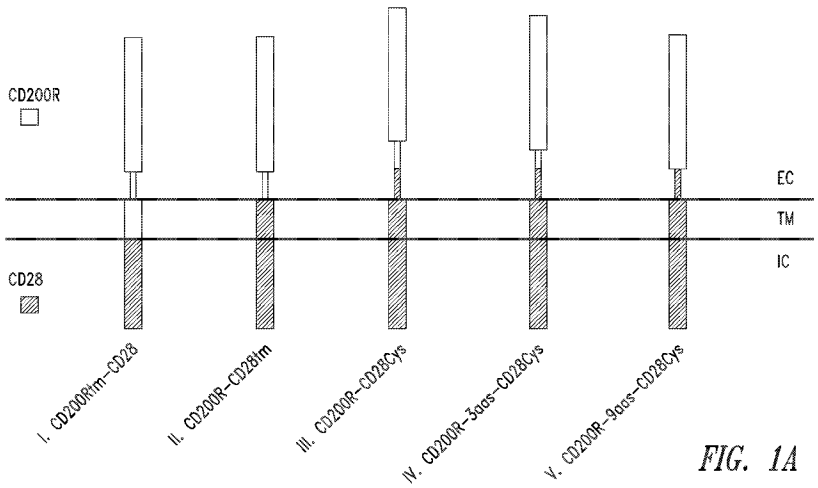

FIG. 1A

(57) Abstract: The present disclosure relates to immunomodulatory fusion proteins containing an extracellular binding domain and an intracellular signaling domain, wherein binding of a target can generate a modulatory signal in a host cell, such as a T cell. The present disclosure also relates to uses of immune cells expressing such immunomodulatory fusion proteins to treat certain diseases, such as cancer or infectious disease.

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IMMUNOMODULATORY FUSION PROTEINS AND USES THEREOF

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing is 360056_433WO_SEQUENCE_LISTING.txt. The text file is 286 KB, was created on March 4, 2016, and is being submitted electronically via EFS-Web.

BACKGROUND

T cell-based immunotherapies began to be developed when tumor-reactive T cells were found among a population of tumor-infiltrating lymphocytes (TILs) (Clark *et al.*, *Cancer Res. 29*:705, 1969). One strategy, known as adoptive T cell transfer, in some contexts involves the isolation of tumor infiltrating lymphocytes pre-selected for tumor-reactivity, clonal expansion of the tumor-reactive T cells induced by anti-CD3 and anti-CD28 antibodies in the presence of IL-2, and finally infusing the expanded cell population back to the tumor-bearing patient (together with chemotherapy and repetitive administration of IL-2) (Dudley *et al.*, *Science 298*:850, 2002). This form of adoptive T cell therapy with tumor infiltrating lymphocytes can be technically cumbersome and leads to complete remission in only a minor fraction of patients with melanoma and is rarely effective in other cancers (Besser *et al.*, *Clin. Cancer Res. 16*:2646, 2010).

Isolation of tumor-reactive T cell clones led to the development of another immunotherapeutic approach – the generation of recombinant T cell receptors (TCRs) specific for particular antigens, which may be introduced into T cells, *e.g.*, using a vector delivery system, to confer specificity for a desired target such as a tumor-associated peptide presented by a major histocompatibility complex (MHC) molecule expressed on a tumor cell (known as human leukocyte antigen (HLA) molecule in humans). Another approach introduces a synthetic receptor, termed a chimeric antigen receptor (CAR), which generally contains an antigen-binding domain, which, *e.g.*, in

the context of anti-tumor therapy can bind to a tumor-specific or associated antigen, linked to one or more intracellular component comprising an effector domains, such as a primary signaling domain such as a TCR signaling domain or in some contexts costimulatory signaling domains. Unlike administration of TILs, the basic procedure for engineered TCR or CAR T cell immunotherapy is generally to genetically modify human T cells with a transgene encoding a tumor targeting moiety, *ex vivo* expansion of the recombinant T cells, and transfusing the expanded recombinant T cells back into patients.

Adoptive T cell therapy using T cells expressing recombinant TCRs has been shown to have a promising clinical benefit, especially in certain B cell cancers. However, effective T cell activation often requires or is enhanced by a concurrent co-stimulatory signal (Chen and Flies, *Nat. Rev. Immunol. 13*: 227-242, 2013). In the tumor microenvironment, co-stimulatory molecules are generally downregulated. As a result, exogenous stimulus via IL-2 is typically needed for T cells that express recombinant TCRs specific for cancer antigens.

Activation of T cells is initiated when the TCR engages a specific peptide presented in MHC on an antigen-presenting cell (APC) (Rossy *et al.*, *Frontiers in Immunol. 3*: 1-12, 2012). The point of interaction of the T cell and the APC becomes the immunological synapse, which is comprised of three concentric supramolecular activation clusters (SMACs), including the central cSMAC, peripheral pSMAC, and the distal dSMAC (Rossy *et al.*, *Frontiers in Immunol. 3*: 1-12, 2012). Within the cSMAC, co-stimulatory receptors can recruit signaling molecules to amplify the TCR signal. Such co-stimulatory receptors can include CD28, and in some contexts form microclusters with the TCR to lower the threshold of activation (Chen and Flies, *Nat. Rev. Immunol. 13*: 227-242, 2013). Access to the cSMAC by transmembrane proteins expressed by T cells may be restricted by the size of the extracellular domain. For example, CD45 has a large ectodomain and is generally excluded from the immunological synapse, thereby preventing its ability to inhibit TCR signaling (James and Vale, *Nature 487*:64-69, 2012).

There remains a need in the immunotherapy field for alternative compositions and methods that provide immunomodulatory signals to host cells for treating various diseases, such as cancer or infections. Presently disclosed embodiments address these needs and provide other related advantages.

BRIEF SUMMARY

In certain aspects, the present disclosure is directed to a fusion protein, comprising an extracellular component that contains a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse.

In some embodiments, a length or spatial distance of a complex formed between the fusion protein and the target or a portion of such fusion protein::target complex (generally the extracellular portion of such complex) is or spans a particular distance, *e.g.*, in some embodiments, is a distance that is less than or less than about a certain distance. In some aspects, a distance of the fusion protein::target complex (or, typically, the extracellular portion thereof) is less than at or about 50 nm, less than at or about 40 nm, less than at or about 30 nm, or less than at or about 20 nm or equal to or less than at or about 15 nm. In some embodiments, it is at or about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nm, such as at or about 14 or 15 nm. In some aspects, the distance is one that is similar to a distance between membranes in an immunological synapse or is a distance that is the same, about the same, or substantially the same, as a distance between the membrane proximal-most portion, *e.g.*, residue, of the extracellular domain of a TCR and the membrane proximal-most portion, *e.g.*, residue, of an MHC (*e.g.*, HLA, such as an MHCI or MHCII) molecule, with respect to a TCR-peptide/MHC complex or the distance spanned by the extracellular portions of such a complex (or spatial distance spanned by the extracellular portion known to be contained within a synapse, such as a complex containing CD8, CD4, CD28, and the respective binding partner or ligand thereof). In some embodiments, spatial distances of

complexes refer to a distance between membranes of two different cells, wherein a first cell and a second cell each express on their surface a binding partner that can form a complex between the membranes when the cells are in proximity to each other. In some aspects, the distance is a distance that is the same, about the same, or substantially the same, as a distance spanned by the extracellular portions of a complex formed between a TCR and cognate interaction with an MHC molecule. In some aspects, such as where a fusion protein comprises a binding domain from a molecule ordinarily capable of entering an immunological synapse or co-localizing with an antigen receptor, the distance is similar to or the same as that spanned by a complex formed between the molecule (having the binding domain used in the fusion protein), and a natural binding partner thereof. In some aspects, such as where the fusion protein comprises a binding domain from a molecule ordinarily not capable of entering an immunological synapse or ordinarily not capable of co-localizing with an antigen receptor, the distance is different than, *e.g.*, less than or substantially less than, that spanned by a complex formed between the molecule (having the binding domain or functional portion thereof used in the fusion protein), and a natural binding partner thereof.

In some embodiments, a binding domain within the extracellular component of a fusion protein of this disclosure contains a target-binding portion of a molecule capable of delivering an inhibitory signal, such as of an inhibitory molecule, *e.g.*, an immunoinhibitory molecule, such as an immunoinhibitory receptor or immune checkpoint molecule. In some aspects, such a molecule is a glycoprotein, checkpoint family member. In certain embodiments, the fusion protein comprising a binding domain from a glycoprotein, checkpoint family member or is not a B7 or B7-binding molecule or is not a CD28-B7-superfamily member (*e.g.*, is not a CD28, CTLA4, ICOS, or other B7 family binding molecule) Exemplary glycoprotein, checkpoint family members include CD200R, SIRPα, CD279 (PD-1), CD2, CD95 (Fas), CTLA4 (CD152), CD223 (LAG3), CD272 (BTLA), A2aR, KIR, TIM3, CD300, or LPA5, or a binding variant of any such molecule. In some embodiments, a binding domain within the extracellular component of a fusion protein of this disclosure comprises a binding partner of any of the foregoing, or a binding variant of any such molecule. In some

aspects of such embodiments, the intracellular portion of a fusion protein includes a signaling domain capable of delivering a stimulatory, such as a costimulatory, signal to a lymphocyte, such as a T cell, such as a costimulatory region of CD28, 41BB, ICOS, or other costimulatory molecule. In some aspects, the intracellular portion of the fusion protein does not include an intracellular signaling domain of the inhibitory molecule, such as of a checkpoint or immunoinhibitory molecule, when the extracellular binding portion is from a checkpoint or immunoinhibitory molecule. In some aspects, a fusion protein does not include a primary signaling domain such as a CD3ζ signaling domain or other domain capable of delivering a primary signal to a T cell.

In certain aspects, the extracellular component or the binding portion thereof contains or is a binding domain of a molecule or ectodomain capable of specifically binding to CD200, such as a binding portion of a CD200R or variant thereof. In some embodiments, the binding domain is or includes a binding region of a molecule or of an ectodomain that is capable of specifically binding to a CD47, such as a SIRP ectodomain or CD47-binding region thereof, such as a SIRPα ectodomain or CD47-binding region thereof. In some embodiments, the binding domain is capable of binding to a PD-L1 or a PD-L2 or a LAG3 molecule. Exemplary targets may be one or more proteins whose expression is increased or upregulated in certain cells or tissues associated with or of a disease or condition to be treated or ameliorated with the fusion proteins and compositions provided herein, such as a tumor cell or tumor microenvironment, or is bound by a receptor generally upregulated on immune cells such as lymphocytes infiltrating a diseased tissue, such as a tumor.

In some embodiments, the extracellular component further includes one or more additional regions or domains, for example, from a molecule other than that from which the binding domain is derived or other than the molecule with which the binding domain shares identity. The one or more additional extracellular domain(s) may include a spacer region, such as one from an immunoglobulin molecule, which may contain all or a portion of a hinge, or constant region domain such as CH2 or CH3 domain, or from another cell surface molecule such as a costimulatory receptor, such as CD28. The additional extracellular domain(s) may include, in some aspects, a

multimerization domain, *e.g.*, a dimerization domain or sequence that may promote homo- or heterodimerization with another molecule, such as multimerization of two or more of the fusion proteins. In some embodiments, such a domain includes a portion of an extracellular domain of a CD28 molecule including at least the transmembrane-proximal-most cysteine, and generally an extracellular portion between such cysteine and the membrane, or modified variant thereof. In some aspects, such a domain includes an amino acid sequence as set forth in SEQ ID NO: 32, or portion thereof, or variant thereof such as having at least 90%, 95%, or 99 % identity thereto. In some aspects, such a domain may be included in order to facilitate or promote multimerization. In some embodiments, a fusion protein contains an extracellular component including a CD200-binding domain, such as an extracellular portion (or portion thereof, such as a binding domain thereof) of a CD200R, such as an extracellular portion of CD200R having an amino acid sequence as set forth in SEQ ID NO: 25 or encoded by a nucleic acid molecule as set forth in SEQ ID NO: 2, or a CD200-binding portion thereof or variant thereof or binding portion thereof. In some aspects of such embodiments, the extracellular portion of the fusion protein further includes a portion of an extracellular region of CD28, such as up to about 9 to about 12 amino acids thereof (*e.g.*, 9 amino acids or 12 amino acids), and in some aspects including a membrane-proximal-most cysteine residue of a CD28 extracellular region. In some such embodiments, the length of the CD200R portion of the extracellular region is reduced in length corresponding to the number of additional residues in the CD28-derived portion, such as by about 9 to about 12 amino acids (*e.g.*, 9 amino acids or 12 amino acids), or by a sufficient number of amino acids that the distance spanned by the extracellular portion of a complex between the fusion protein and a CD200 molecule is similar to, substantially similar to, or the same as that spanned by the extracellular portion of a complex between a human CD200R, *e.g.*, a CD200R, and CD200; or that spanned by the extracellular portion of a complex between a TCR in cognate interaction with an MHC molecule (*e.g.*, MHC I or MHCII) in binding to a cognate peptide-MHC complex; or that of an immunological synapse. In some aspects, the fusion protein further includes a transmembrane domain, such as a CD28

transmembrane, such as a transmembrane domain encoded by the sequence set forth as SEQ ID NO: 4 or portion thereof, or a modified version thereof, such as a variant modified to contain additional charged regions or residues or hydrophilic residues to facilitate intermolecular interactions. In some embodiments, the protein further includes a CD28 intracellular signaling domain, such as a costimulatory domain of CD28, such as one that is capable of recruiting one or more adapter molecules to a CD28 in response to ligation. In some aspects, the CD28 intracellular domain includes or is a sequence encoded by the nucleotide sequence of SEQ ID NO: 5 or a portion or functional variant thereof.

In some embodiments, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD200R, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some embodiments, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD200R, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28 and an intracellular signaling domain of a CD137 (4-1BB).

In some embodiments, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD200R, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD137 (4-1BB).

In some embodiments, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a SIRPα, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some embodiments, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD279 (PD-1), (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some embodiments, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD95 (Fas), (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some embodiments, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a TIM3, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some embodiments, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein:target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a LAG3, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In some embodiments, the present disclosure is directed to a fusion protein comprising an extracellular component comprised of a binding domain that specifically

binds a target, an intracellular component comprised of an intracellular signaling domain, and a hydrophobic component connecting the extracellular and intracellular components, provided that the length of a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse, wherein (a) the extracellular component comprises an extracellular portion of a CD2, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

In certain aspects, the present disclosure is directed to a nucleic acid molecule encoding a fusion protein as described herein.

In certain aspects, the present disclosure is directed to a vector comprising a nucleic molecule that encodes a fusion protein as described herein.

In certain other aspects, the present disclosure is directed to a host cell comprising a fusion protein, nucleic acid, or vector as described herein.

In certain other aspects, a method of increasing the activity of an immune cell is provided, comprising administering to a subject in need of increased immune cell activity an effective amount of a host cell as described herein.

In other aspects, the present disclosure is directed to a method of enhancing or prolonging an immune response, comprising administering to a subject in need of enhanced or prolonged immune cell activity an effective amount of a host cell as described herein.

In still other aspects, the present disclosure provides a method of stimulating an antigen-specific T cell response, comprising administering to a subject in need of increased immune cell activity an effective amount of a host cell as described herein.

In other aspects, the present disclosure is directed to a method of inhibiting an immunosuppressive signaling pathway, comprising administering to a subject in need thereof an effective amount of a host cell as described herein.

In other aspects, the present disclosure is directed to a method of treating cancer, comprising administering to a subject having cancer a therapeutically effective amount of a host cell as described herein.

In other aspects, the present disclosure is directed to a method of inhibiting immune resistance of cancer cells, comprising administering to a subject in need thereof an effective amount of a host cell as described herein.

In still other aspects, the present disclosure provides a method for treating a tumor, comprising administering to a subject having a tumor a therapeutically effective amount of a host cell as described herein, wherein the administered host cell is capable of proliferating in an immunosuppressive tumor microenvironment.

A method of treating an infection, comprising administering to a subject having the infection a therapeutically effective amount of a host cell as described herein, is also provided by the present disclosure.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show CD200R-CD28 constructs expressed at high levels on primary murine $CD8^+$ T cells. (A) Schematic representation of exemplary CD200R-CD28 constructs. Construct "I" contains CD200R extracellular ("EC") and transmembrane ("TM") domains and a CD28 intracellular ("IC") signaling domain (CD200Rtm-CD28). Construct "II" contains the extracellular domain of CD200R and the transmembrane and intracellular domains of CD28 (CD200R-CD28tm). Constructs "III-V" also incorporate a portion of the extracellular domain of CD28 to the transmembrane-proximal cysteine to promote multimerization and enhance CD28 signaling. To account for any extra extracellular amino acids (*e.g.*, from one to about 50 amino acids; such as exemplary murine constructs disclosed here contain an extra nine (9) amino acids and exemplary human constructs disclosed here contain twelve (12) amino acids), some constructs have a truncated portion of an extracellular or intracellular domain (*e.g.*, a CD200R that preserves an N linked glycosylation site). For example, construct IV has a truncated portion of CD200R that is truncated by 3 amino acids. Construct V has a truncated portion of CD200R that is truncated 9 amino acids. Constructs "I", "II", and "V" maintain the short spatial distance between the cells (*e.g.*,

between a T cell and an antigen presenting cell) and may co-localize with the TCR within the cSMAC and deliver a strong co-stimulatory signal. (B) Transgenic expression of murine CD200R-CD28 constructs on TCR_{gag} T cells as detected by anti-CD200R antibody. The control vector contains green fluorescent protein (GFP).

Figures 2A to 2G show that CD200R-CD28 constructs promote proliferation, accumulation, and effector function in response to $CD200^+$ tumor target cells *in vitro*, and accumulate in the immunological synapse. Splenocytes from naive TCR_{gag} mice were stimulated *in vitro* with anti-CD3, anti-CD28, and recombinant human IL-2 (100 U/ml) and transduced with retroviral supernatant for 2 days. Cells were restimulated every 7 days with irradiated FBL and splenocytes and cultured with rhIL-2 (50 U/mL) for up to three stimulations. T cells were used for assays 5-7 days after the last stimulation. (A) Proliferation of CD200R-CD28 and GFP control TCR_{gag} T cells as measured by CellTrace Violet dilution. T cells were stimulated with $CD200^-$ FBL (upper panels) or $CD200^+$ FBL (lower panels) for 3 days. (B) Preferential expansion/survival of transduced TCR_{gag} T cells during co-culture with non-transduced TCR_{gag} T cells during weekly cycles of stimulation with irradiated $CD200^+$ FBL and splenocytes. (C) Enrichment of transduced T cells. Repeated restimulation with irradiated $CD200^+$ tumor cells enriched the cells transduced with CD200R-9aas-CD28Cys compared to wild-type T cells transduced with an empty GFP control vector. (D) Increased CD200R and CD200 signal intensity at T cell:FBL synapse. Lipid rafts are increased at the immunological synapse (I). CD200R-9aas-CD28Cys fusion proteins co-localized with lipid rafts, indicating that the fusion proteins concentrate within the immunological synapse (III, IV). (E) CD200R-$CD28^+$ $CD8^+$ T cells display enhanced ability to lyse $CD200^+$ FBL cells *in vitro*. Target tumor cells were labeled with different dilutions of the fluorescent dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), as indicated. Effector TCR_{gag} T cells transduced with the indicated CD200R-CD28 fusion protein or an empty vector control were incubated at the indicated effector to target ratio with a 1:1 mix of $CD200^+$ FBL ($CFSE^{hi}$) and non-specific EL4 ($CFSE^{lo}$) control targets for 5 hours. The percentage of FBL of the sum of FBL and control tumor cells was determined by flow cytometry. The percentage lysis

was determined by dividing the percent of FBL incubated with T cells by the percent of FBL incubated without T cells. (F) Target tumor cells for CFSE assay in (G). Target tumor cells were labeled with different dilutions of the fluorescent dyes CellTrace Violet (CTV) or CFSE. A 1:1:1 mix of EL4 cells (CTV+), $CD200^+$ FBL ($CFSE^{hi}$) and non-specific EL4 ($CFSE^{lo}$) control targets was generated. (G) CFSE cytotoxicity assay. TCR_{gag} T cells were transduced with CD200R-CD28 receptor or GFP control vector. Effector TCR_{gag} T cells were incubated at the indicated effector to target ratio with a 1:1 mix of $CD200^-$ FBL or $CD200^+$ FBL and non-specific EL4 control targets for 4 hours. The percentage of FBL of the sum of FBL and control tumor cells was determined by flow cytometry. The percentage lysis was determined by dividing the percent of FBL incubated with T cells by the percent of FBL incubated without T cells.

Figures 3A to 3D show that T cells transduced with CD200R-9aas-CD28Cys preferentially accumulate in response to tumor challenge *in vivo* and express surface proteins consistent with an effector phenotype after injection into Cytoxan-treated, FBL-bearing mice. Transduced TCR_{gag} T cells were generated as described in Example 2. (A) Experimental schematic. C57BL/6 mice were injected with 4×10^6 $CD200^+$ FBL cells. Five days later, CD200R-9aas-CD28Cys (Thy1.1 homozygous) and eGFP control (Thy1.1 heterozygous) TCR_{gag} T cells were co-injected into Cytoxan-treated FBL-bearing B6 mice at 4×10^6 cells/mouse. IL-2 was administered every 2 days (2×10^4 U/dose). On day 8 post-T cell transfer, mice were euthanized and spleens and inguinal lymph nodes harvested. (B) CD200R-9aas-CD28Cys TCR_{gag} T cells accumulate in the spleen in response to FBL. (LN=lymph node; Spl=spleen). (C) Comparison of surface proteins 3 days post-transfer for T cells transduced to express CD200R-9aas-CD28Cys, T cells transduced with an empty vector, and endogenous T cells. CD200R-9aas-CD28Cys TCR_{gag} T cells expressed reduced CD62L compared to control TCR_{gag} T cells, suggesting an effector T cell phenotype. (D) Comparison of surface proteins 15 days post-transfer for cells transduced to express CD200R -9aas-$CD28Cys^+$ T cells, T cells transduced with an empty vector, and endogenous T cells. CD200R-9aas-CD28Cys TCR_{gag} T cells express similar levels of cell surface proteins compared to control TCR_{gag} T cells.

Figures 4A to 4D show that adoptive immunotherapy with CD200R-CD28-transduced T cells can eradicate disseminated leukemia. (A) Experiment schematic. C57BL/6 mice were injected with 4 x 10^6 $CD200^+$ FBL cells. Five days later, CD200R-CD28tm, CD200R-CD28Cys, CD200R-9aas-CD28Cys, or eGFP TCR_{gag} T cells were injected i.p. into Cy-treated FBL-bearing mice at 10^5 cells/mouse. IL-2 was administered every 2 days (2 x 10^4 U/dose) in a cohort of mice as indicated. (B) Representative example of expression of cell surface proteins in CD200R-CD28tm transduced T cells and non-transduced T cells on day of injection with IL-2, as determined by flow cytometry. (C) Survival of mice treated in the presence of IL-2 injections. (D) Survival of mice treated in the absence of IL-2 injections. Transfer of CD200R-9aas-CD28Cys TCR_{gag} T cells significantly improved survival in the absence of IL-2 injections ($p < 0.05$, log-rank Mantel-Cox test).

Figures 5A to 5C show that T cells expressing CD200R-9aas-CD28Cys do not induce detectable autoimmune liver damage or infiltrate normal tissues. (A) Experiment schematic. Cytoxan-treated Alb/Gag mice were injected with 4 x 10^6 $CD200^+$ FBL cells. Five days later, CD200R-9aas-CD28Cys, and eGFP TCR_{gag} T cells were injected i.p. into the Cytoxan-treated FBL-bearing mice at 10^5 cells/mouse. IL-2 was administered every 2 days (2 x 10^4 U/dose) in a cohort of mice as indicated. Three and 7 days post-transfer, liver damage was assessed by quantification of serum levels of liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). (B) AST and ALT levels measured at 3 and 7 days post-transfer for mice receiving no T cells, control T cells expressing GFP, or T cells expressing CD200R-9aas-CD28Cys did not vary by treatment. (C) Assessment of T cell infiltration of normal tissue. Limited presence of T cells in liver tissue was observed using antibodies specific to the T cell marker CD3 (left panel), with no significant difference between recipients of CD200R-9aas-CD28Cys TCR_{gag} or control TCR_{gag} T cells (right panel).

Figures 6A to 6D show that 4-1BB co-stimulatory signaling domains promote accumulation and effector function of transduced T cells *in vitro* and promote survival of tumor-bearing recipients of transduced T cell in response to $CD200^+$ tumor target cells. (A) Schematic representation of CD200R-CD28 ("V"), -4-1BB ("VI"), and –

CD28-4-1BB ("VII") constructs. (B) Expansion of transduced TCR_{gag} T cells relative to non-transduced TCR_{gag} T cells after weekly stimulation with irradiated $CD200^+$ FBL and splenocytes. CD200R-4-1BB and CD200R-CD28-4-1BB also promote accumulation of transduced T cells *in vitro*. (C) CD200R-9aas-4-1BB^+ $CD8^+$ T cells displayed an enhanced ability to lyse $CD200^+$ FBL cells *in vitro* relative to controls, using a standard CFSE-based cytotoxicity assay. The percentage of FBL of the sum of FBL and control tumor cells was determined by flow cytometry. The percentage lysis was determined by dividing the percent of FBL incubated with T cells by the percent of FBL incubated without T cells. (D) CD200R-41BB-transduced T cells also promote survival relative to controls. C57BL/6 mice were injected with 4 x 10^6 $CD200^+$ FBL cells. Five days later, CD200R-9aas-CD28, CD200R-9aas-4-1BB, CD200R-9aas-CD28-4-1BB, or eGFP TCR_{gag} T cells were injected i.p. into Cytoxan-treated FBL-bearing mice at 10^5 cells/mouse.

Figures 7A to 7D show that human primary T cells transduced to express a WT1-specific TCR and a CD200Rtm-CD28 fusion protein exhibit enhanced proliferation to target cells that express CD200 and increased cytokine production in response to tumor cells that express CD200. (A) Expression of the $WT1_{126}$–specific TCR, C4, and CD200Rtm-CD28. (B) Expression of CD200 in T2 and K562 cells. T2 cells exhibit low-level endogenous CD200 expression. (C) Proliferation of T cells as indicated by CFSE. Cells that proliferate in response to antigen show reduced CFSE fluorescence intensity. T cells transduced with both C4 and the IFP show enhanced proliferation to target cells expressing low levels of CD200 relative to T cells transduced with C4 only. (D) Cytokine production in response to exposure to CD200dim tumor cells, as measured by flow cytometry. Relative to control T cells transduced with the TCR C4 alone, T cells transduced with both C4 and the IFP CD200Rtm-CD28 show increased cytokine production.

Figures 8A to 8E show that fusion proteins comprising SIRPα extracellular components and CD28 co-stimulatory signaling domains promote accumulation and proliferation of transduced T cells *in vitro*. (A) Schematic representation of exemplary SIRPα-CD28 constructs. Construct "I" contains SIRPα extracellular ("EC") and

transmembrane ("TM") domains and a CD28 intracellular ("IC") signaling domain (SIRPαtm-CD28). Construct "II" contains the extracellular domain of SIRPα and the transmembrane and intracellular domains of CD28 (SIRPα-CD28tm). Constructs "III-VI" also incorporate a portion of the extracellular domain of CD28 to the transmembrane-proximal cysteine to promote multimerization and enhance CD28 signaling. To account for the extra extracellular amino acids (*e.g.*, extra nine (9) amino acids for murine constructs, or twelve (12) amino acids for human constructs), some constructs have a truncated portion of an extracellular or intracellular domain (*e.g.*, a SIRPα that preserves an N linked glycosylation site). Construct IV has a truncated portion of SIRPα that is truncated 6 amino acids to preserve an N linked glycosylation site. Construct V has a truncated portion of SIRPα that is truncated 9 amino acids. Construct VI has a truncated portion of SIRPα that is truncated 23 amino acids. Constructs "I", "II", and "V" maintain the short spatial distance between the cells (*e.g.*, between a T cell and an antigen presenting cell) and may co-localize with the TCR within the cSMAC and deliver a strong co-stimulatory signal. (B) Expansion of transduced TCR_{gag} T cells relative to non-transduced TCR_{gag} T cells after weekly stimulation with irradiated $SIRP\alpha^+$ FBL and splenocytes. SIRPα-CD28 constructs promote accumulation of transduced T cells *in vitro*, with SIRPα-9aas-CD28Cys exhibiting enhanced accumulation. (C) Proliferation of T cells transduced with SIRPα-CD28 constructs in a CellTrace Violet (CTV) dilution proliferation assay. T cells expressing SIRPα-CD28 constructs engineered to maintain T cell-tumor cell distance exhibited enhanced proliferation relative to nontransduced T cells. (D) $CD47^+$ tumor cells were killed after co-culture with SIRPα-$CD28^+$ T cells transduced to express SIRPαtm-CD28 or SIRPα-9aas-CD28Cys constructs. In contrast, tumor cells were not eradicated when cultured with T cells receiving empty vector, or a truncated SIRPα lacking its intracellular domain. (E) Results of an IncuCyte assay used to quantify killing of $CD47^+$ tumor cells. $CD47^+$ FBL tumor cells were transduced with mCherry. Loss of red signal indicates killing of tumor cells. Killing of tumor cells was tested at the effector:target ratios of 10:1, 2:1, and 0.4:1. SIRPα-$CD28^+$ T cells killed $CD47^+$ tumor cells, even at the lowest effector-to-target ratio tested.

Figures 9A and 9B show that fusion proteins comprising PD-1 extracellular components and CD28 co-stimulatory signaling domains promote cytokine production *in vitro*. (A) Schematic representation of exemplary PD-1-CD28 constructs. Construct "I" contains PD-1 extracellular ("EC") and transmembrane ("TM") domains and a CD28 intracellular ("IC") signaling domain (PD1tm-CD28). Construct "II" contains the extracellular domain of PD-1 and the transmembrane and intracellular domains of CD28 (PD1-CD28tm). Constructs "III-VII" also incorporate a portion of the extracellular domain of CD28 adjacent to the transmembrane-proximal cysteine to promote multimerization and enhance CD28 signaling. To account for the extra extracellular amino acids (*e.g.*, extra nine (9) amino acids for murine constructs, or twelve (12) amino acids for human constructs), constructs IV-VII have a truncated portion of PD-1. Construct IV has a truncated portion of PD-1 that is truncated 9 amino acids. Construct V has a truncated portion of PD-1 that is truncated 12 amino acids. Construct VI has a truncated portion of PD-1 that is truncated 15 amino acids. Construct VII has a truncated portion of PD-1 that is truncated 21 amino acids. Constructs "I", "II", and "V" maintain the short spatial distance between the cells (*e.g.*, between a T cell and an antigen presenting cell) and may co-localize with the TCR within the cSMAC and deliver a strong co-stimulatory signal. (B) PD1-CD28^{+} T cells exhibited increased cytokine production in response to stimulation for 5 hours in the presence of Brefeldin A with FBL cells that endogenously express the PD-1 ligands, PD-L1 and PD-L2. Stimulated T cells were assessed for intracellular expression of the effector cytokines, IFNγ and TNFα, by flow cytometry.

Figure 10 shows co-expression of the TCR C4 and a PD-1 IFP (PD1-12aas-CD28Cys, PD1-15aas-CD28Cys, or PD1-21aas-CD28Cys). T cells transduced with C4 and PD1-12aas-CD28Cys or PD1-15aas-CD28Cys exhibited high transduction efficiencies and expression of both proteins.

Figures 11A to 11C show that fusion proteins comprising Fas extracellular components and CD28 co-stimulatory signaling domains accumulate *in vitro* upon stimulation with irradiated FBL cells. (A) Schematic representation of exemplary Fas-CD28 constructs. Construct "I" contains Fas extracellular ("EC") and transmembrane

("TM") domains and a CD28 intracellular ("IC") signaling domain (Fastm-CD28). Construct "II" contains the extracellular domain of Fas and the transmembrane and intracellular domains of CD28 (Fas-CD28tm). Constructs "III" and "IV" also incorporate a portion of the extracellular domain of CD28 adjacent to the transmembrane-proximal cysteine to promote multimerization and enhance CD28 signaling. To account for the extra extracellular amino acids (*e.g.*, extra nine (9) amino acids for murine constructs, or twelve (12) amino acids for human constructs), construct IV has a truncated portion of Fas, wherein the Fas extracellular domain is truncated 9 amino acids. Constructs "I", "II", and "IV" maintain the short spatial distance between the cells (*e.g.*, between a T cell and an antigen presenting cell) and may co-localize with the TCR within the cSMAC and deliver a strong co-stimulatory signal. (B) Accumulation of TCR_{gag} T cells transduced with Fas constructs over multiple stimulations with irradiated FBL cells. All of the constructs promoted accumulation of T cells relative to control T cells. (C) Expression of Fas-CD28 constructs but not full-length (FL) Fas promoted survival or expansion of T cells upon multiple stimulations *in vitro*.

Figures 12A and 12B show the structure and expression of fusion proteins comprising LAG3 extracellular components and CD28 co-stimulatory signaling domains. (A) Schematic representation of exemplary LAG3-CD28 constructs. Construct "I" contains LAG3 extracellular ("EC") and transmembrane ("TM") domains and a CD28 intracellular ("IC") signaling domain (LAG3tm-CD28). Construct "II" contains the extracellular domain of LAG3 and the transmembrane and intracellular domains of CD28 (LAG3-CD28tm). Constructs "III" and "IV" also incorporate a portion of the extracellular domain of CD28 adjacent to the transmembrane-proximal cysteine to promote multimerization and enhance CD28 signaling. To account for the extra extracellular amino acids (*e.g.*, extra nine (9) amino acids for murine constructs, or twelve (12) amino acids for human constructs), construct IV has a truncated portion of LAG3, wherein the LAG3 extracellular domain is truncated 9 amino acids. Constructs "I", "II", and "IV" maintain the short spatial distance between the cells (*e.g.*, between a T cell and an antigen presenting cell) and may co-localize with the TCR

within the cSMAC and deliver a strong co-stimulatory signal. (B) Expression of LAG3-CD28 constructs by murine $CD8^+$ T cells, as determined by anti-LAG3 antibody staining and flow cytometry. T cells transduced to express LAG3-CD28 constructs (LAG3tm-CD28; LAG3-CD28tm; LAG3-CD28Cys; LAG3-9aas-CD28Cys) exhibited expression of the constructs, in contrast with control T cells that received empty vector.

Figures 13A and 13B show the structure and expression of fusion proteins comprising TIM3 extracellular components and CD28 co-stimulatory signaling domains. (A) Schematic representation of exemplary TIM3-CD28 constructs. Construct "I" contains TIM3 extracellular ("EC") and transmembrane ("TM") domains and a CD28 intracellular ("IC") signaling domain (TIM3tm-CD28). Construct "II" contains the extracellular domain of TIM3 and the transmembrane and intracellular domains of CD28 (TIM3-CD28tm). Constructs "III" and "IV" also incorporate a portion of the extracellular domain of CD28 adjacent to the transmembrane-proximal cysteine to promote multimerization and enhance CD28 signaling. To account for the extra extracellular amino acids (*e.g.*, extra nine (9) amino acids for murine constructs, or twelve (12) amino acids for human constructs), construct IV has a truncated portion of TIM3, wherein the TIM3 extracellular domain is truncated 9 amino acids. Constructs "I", "II", and "IV" maintain the short spatial distance between the cells (*e.g.*, between a T cell and an antigen presenting cell) and may co-localize with the TCR within the cSMAC and deliver a strong co-stimulatory signal. (B) Expression of TIM3-CD28 constructs by murine $CD8^+$ T cells, as determined by anti-TIM3 antibody staining and flow cytometry. T cells transduced to express TIM3-CD28 constructs (TIM3tm-CD28; TIM3-CD28tm; TIM3-CD28Cys; TIM3-9aas-CD28Cys) typically exhibited expression of the constructs, in contrast with control T cells that received empty vector.

DETAILED DESCRIPTION

The instant disclosure provides fusion proteins that modulate signaling in a host cell, such as an immune cell. For example, fusion proteins of this disclosure can provide an activation or co-stimulatory signal in a human T cell, wherein the T cell may

optionally be engineered to have a preferred antigen-specific TCR. These immunomodulatory fusion proteins (IFPs) can interact with ubiquitously expressed targets or with targets that are commonly upregulated or overexpressed in non-normal cells (*e.g.*, a cancer cell). Such IFPs have an extracellular binding domain and an intracellular signaling domain. By transducing T cells with engineered TCRs (*e.g.*, high affinity TCRs) and fusion proteins of this disclosure that generate activation signals, certain embodiments of T cells may no longer require exogenous co-stimulation upon interaction with, for example, a tumor cell.

In certain aspects, the present disclosure provides host cells (*e.g.*, immune cells such as T cells, dendritic cells, NK cells or the like) comprising an IFP, vectors encoding IFPs, and methods of activating T cells comprising an IFP for various therapeutic applications, including the treatment of a disease in subject (*e.g.*, cancer, infectious disease).

Prior to setting forth this disclosure in more detail, it may be helpful to an understanding thereof to provide definitions of certain terms to be used herein. Additional definitions are set forth throughout this disclosure.

In the present description, any concentration range, percentage range, ratio range, or integer range is to be understood to include the value of any integer within the recited range and, when appropriate, fractions thereof (such as one tenth and one hundredth of an integer), unless otherwise indicated. Also, any number range recited herein relating to any physical feature, such as polymer subunits, size or thickness, are to be understood to include any integer within the recited range, unless otherwise indicated. As used herein, the term "about" means ± 20% of the indicated range, value, or structure, unless otherwise indicated. It should be understood that the terms "a" and "an" as used herein refer to "one or more" of the enumerated components. The use of the alternative (*e.g.*, "or") should be understood to mean either one, both, or any combination thereof of the alternatives. As used herein, the terms "include," "have" and "comprise" are used synonymously, which terms and variants thereof are intended to be construed as non-limiting.

The term "consisting essentially of" limits the scope of a claim to the specified materials or steps, or to those that do not materially affect the basic characteristics of a claimed invention. For example, a protein domain, region, or module (*e.g.*, a binding domain, hinge region, linker module) or a protein (which may have one or more domains, regions, or modules) "consists essentially of" a particular amino acid sequence when the amino acid sequence of a domain, region, or module or protein includes extensions, deletions, mutations, or any combination thereof (*e.g.*, amino acids at the amino- or carboxy-terminus or between domains) that, in combination, contribute to at most 20% (*e.g.*, at most 15%, 10%, 8%, 6%, 5%, 4%, 3%, 2%, or 1%) of the length of a domain, region, or module or protein and do not substantially affect (*i.e.*, do not reduce the activity by more than 50%, such as no more than 40%, 30%, 25%, 20%, 15%, 10%, 5%, or 1%) the activity of the domain(s), region(s), module(s), or protein (*e.g.*, the target binding affinity of a binding protein).

As used herein, "heterologous" or "non-endogenous" or "exogenous" refers to any gene, protein, compound, molecule, or activity that is not native to a host cell or a subject, or is any gene, protein, compound, molecule, or activity native to a host or host cell that has been altered or mutated such that the structure, activity or both is different as between the native and mutated molecules. In certain embodiments, heterologous, non-endogenous or exogenous molecules (*e.g.*, receptors, ligands) may not be endogenous to a host cell or subject, but instead nucleic acids encoding such molecules may have been added to a host cell by conjugation, transformation, transfection, electroporation, or the like, wherein the added nucleic acid molecule may integrate into a host cell genome or can exist as extra-chromosomal genetic material (*e.g.*, as a plasmid or other self-replicating vector). The term "homologous" or "homolog" refers to a molecule or activity found in or derived from a host cell, species, or strain. For example, a heterologous or exogenous molecule or gene encoding the molecule may be homologous to a native host or host cell molecule or gene that encodes the molecule, respectively, but may have an altered structure, sequence, expression level or combinations thereof. A non-endogenous molecule may be from the same species, a different species, or a combination thereof.

As used herein, the term "endogenous" or "native" refers to a gene, protein, compound, molecule, or activity that is normally present in a host or host cell and has no engineered alterations.

A "binding domain" (also referred to as a "binding region" or "binding moiety"), as used herein, refers to a molecule, such as a peptide, oligopeptide, polypeptide or protein, that possesses the ability to specifically and non-covalently associate, unite, or combine with a target molecule (*e.g.*, CD200, CD47, CD19, CD20, CD22, ROR1, mesothelin, PD-L1, PD-L2, PSMA, WT-1, cyclin-A1). A binding domain includes any naturally occurring, synthetic, semi-synthetic, or recombinantly produced binding partner for a biological molecule or other target of interest or binding protein thereof. In some embodiments, the binding domain is an antigen-binding domain, such as an antibody or T cell receptor (TCR) or functional binding domain or antigen-binding fragment thereof. Exemplary binding domains include receptor ectodomains (*e.g.*, those of CD200R, PD-1, CTLA4, BTLA, CD2, Fas) or binding portions thereof, ligands (*e.g.*, cytokines such as IL35, chemokines) or binding portions thereof, single chain antibody variable regions (*e.g.*, domain antibodies, sFv, scFv, Fab) or binding portions thereof, antigen-binding regions of T cell receptors (TCRs), such as single chain TCRs (scTCRs), or synthetic polypeptides selected for the specific ability to bind to a biological molecule.

In some embodiments, "specifically binds" refers to an association or union of a binding domain, or a fusion protein thereof, to a target molecule with an affinity or K_a (*i.e.*, an equilibrium association constant of a particular binding interaction with units of 1/M) equal to or greater than 10^5 M^{-1}, or binds to such target molecule while not significantly associating or uniting with any other molecules or components in a sample. Binding domains (or fusion proteins thereof) may be classified as "high affinity" binding domains (or fusion proteins thereof) or "low affinity" binding domains (or fusion proteins thereof). "High affinity" binding domains refer to those binding domains with a K_a of at least 10^7 M^{-1}, at least 10^8 M^{-1}, at least 10^9 M^{-1}, at least 10^{10} M^{-1}, at least 10^{11} M^{-1}, at least 10^{12} M^{-1}, or at least 10^{13} M^{-1}. "Low affinity" binding domains refer to those binding domains with a K_a of up to 10^7 M^{-1}, up to 10^6 M^{-1}, up to 10^5 M^{-1}.

Alternatively, affinity may be defined as an equilibrium dissociation constant (K_d) of a particular binding interaction with units of M (*e.g.*, 10^{-5} M to 10^{-13} M). In certain embodiments, a binding domain may have "enhanced affinity," which refers to a selected or engineered binding domain with stronger binding to a target antigen than a wild type (or parent) binding domain. For example, enhanced affinity may be due to a Ka (equilibrium association constant) for the target antigen that is higher than the wild type binding domain, or due to a K_d (dissociation constant) for the target antigen that is less than that of the wild type binding domain, or due to an off-rate (K_{off}) for the target antigen that is less than that of the wild type binding domain. A variety of assays are known for identifying binding domains of the present disclosure that specifically bind a particular target, as well as determining binding domain or fusion protein affinities, such as Western blot, ELISA, and Biacore® analysis (*see also*, *e.g.*, Scatchard *et al.*, *Ann. N.Y. Acad. Sci. 51*:660, 1949; and U.S. Patent Nos. 5,283,173, 5,468,614, or the equivalent).

As used herein, a "fusion protein" refers to a polypeptide that, in a single chain, has at least two distinct domains, wherein the domains are not naturally found together in a protein. A nucleic acid molecule encoding a fusion protein may be constructed using PCR, recombinantly engineered, or the like, or such fusion proteins can be made using methods of protein synthesis. A fusion protein may further contain other components (*e.g.*, covalently bound), such as a tag or bioactive molecule. In certain embodiments, a fusion protein expressed or produced by a host cell (*e.g.*, T cell) locates to the cell surface, where the fusion protein is anchored to the cell membrane with a portion of the fusion protein located extracellularly (*e.g.*, containing a binding domain) and a portion of the fusion protein located intracellularly (*e.g.*, containing a signaling domain).

A "hydrophobic component," as used herein, means any amino acid sequence having a three-dimensional structure that is thermodynamically stable in a cell membrane, and generally ranges in length from about 15 amino acids to about 30 amino acids. The structure of a hydrophobic component may comprise an alpha helix, a beta barrel, a beta sheet, a beta helix, or any combination thereof. In certain embodiments, a

hydrophobic component is comprised of a "transmembrane domain" from a known transmembrane protein, which is a portion of a transmembrane protein that can insert into or span a cell membrane. In further embodiments, a hydrophobic component or transmembrane domain can be disposed between and connect the extracellular and intracellular portions of a fusion protein. Additionally, the hydrophobic component may be modified to contain charged regions or hydrophilic residues to facilitate intermolecular interactions.

As used herein, an "intracellular signaling domain" is an intracellular portion of molecule, such as one used in a fusion protein of this disclosure, that can directly or indirectly promote a response such as a co-stimulatory, positive, or activating biological or physiological response in a cell when receiving the appropriate signal. In certain embodiments, an intracellular signaling domain is part of a protein or protein complex that receives a signal when bound, or itself can bind directly to a target molecule to transmit a signal to other components in the cell. An intracellular signaling domain may directly promote a cellular response when it contains one or more signaling domains or motifs, such as an immunoreceptor tyrosine-based activation motif (ITAM), a kinase domain, a co-stimulatory domain, or the like. In other embodiments, an intracellular signaling domain will indirectly promote a cellular response by associating with one or more other proteins that in turn directly promote a cellular response. In some embodiments, an intracellular signaling domain or functional fragment thereof may be from a CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD40, CD47, CD79A, CD79B, CD134 (OX40), CD137 (4 1BB), CD150 (SLAMF1), CD278 (ICOS), CD357 (GITR), CARD11, DAP10, DAP12, FcRα, FcRβ, FcRγ, Fyn, Lck, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, ROR2, Ryk, Slp76, pTα, TCRα, TCRβ, TRIM, Zap70, PTCH2, or any combination thereof. In some embodiments, an intracellular signaling domain or functional fragment thereof does not comprise a CD3ζ.

A "multimerization domain," as used herein, refers to a polypeptide molecule or region that preferentially interacts or associates with another polypeptide molecule or region, directly or indirectly, wherein the interaction of multimerization domains

substantially contribute to or efficiently promote multimerization (*i.e.*, the formation of a dimer, trimer, tetramer, or higher order multimers, which may be a homodimer, heterodimer, homotrimer, heterotrimer, homomultimer, heteromultimer, or the like). For example, multimerization may be due to one or more types of molecular forces, including covalent bonds (*e.g.*, disulfide bonds or bridges), ionic bonds, metallic bonds, electrostatic interactions, salt bridges, dipole-dipole forces, hydrogen bonding, Van der Waals forces, hydrophobic interactions, or any combination thereof. A multimer is stable under appropriate conditions (*e.g.*, physiological conditions, in an aqueous solution suitable for expressing, purifying, or storing recombinant or engineered proteins, or under conditions for non-denaturing or non-reducing electrophoresis). Exemplary multimerization domains may comprise one or more disulfide bonds, zinc finger motif, a leucine zipper motif, helix-turn-helix, helix-loop-helix, or the like.

In certain embodiments, a fusion protein may contain a "linker," which can provide a spacer function to facilitate the interaction of two single chain fusion proteins, or positioning of one or more binding domains, so that the resulting polypeptide structure maintains a specific binding affinity to a target molecule or maintains signaling activity (*e.g.*, effector domain activity) or both. Exemplary linkers include from one to about ten repeats of Gly_xSer_y, wherein x and y are independently an integer from 1 to 5.

"Junction amino acids" or "junction amino acid residues" refer to one or more (*e.g.*, about 2-20) amino acid residues between two adjacent motifs, regions, or domains of a fusion protein, such as between a binding domain and an adjacent hydrophobic component, or on one or both ends of a hydrophobic component. Junction amino acids may result from the construct design of a fusion protein (*e.g.*, amino acid residues resulting from the use of a restriction enzyme site during the construction of a nucleic acid molecule encoding a fusion protein). In certain embodiments, junction amino acids form a linker, such as those having from one to about ten repeats of Gly_xSer_y, wherein x and y are independently an integer from 1 to 5.

As used herein, an "immune system cell" means any cell of the immune system that originates from a hematopoietic stem cell in the bone marrow, which gives rise to

two major lineages, a myeloid progenitor cell (which give rise to myeloid cells such as monocytes, macrophages, dendritic cells, megakaryocytes and granulocytes) and a lymphoid progenitor cell (which give rise to lymphoid cells such as T cells, B cells and natural killer (NK) cells). Exemplary immune system cells include a CD4+ T cell, a CD8+ T cell, a CD4- CD8- double negative T cell, a γδ T cell, a regulatory T cell, a natural killer cell, and a dendritic cell. Macrophages and dendritic cells may be referred to as "antigen presenting cells" or "APCs," which are specialized cells that can activate T cells when a major histocompatibility complex (MHC) receptor on the surface of the APC complexed with a peptide interacts with a TCR on the surface of a T cell.

A "T cell" is an immune system cell that matures in the thymus and produces T cell receptors (TCRs). T cells can be naïve (not exposed to antigen; increased expression of CD62L, CCR7, CD28, CD3, CD127, and CD45RA, and decreased expression of CD45RO as compared to T_{CM}), memory T cells (T_M) (antigen-experienced and long-lived), and effector cells (antigen-experienced, cytotoxic). T_M can be further divided into subsets of central memory T cells (T_{CM}, increased expression of CD62L, CCR7, CD28, CD127, CD45RO, and CD95, and decreased expression of CD54RA as compared to naïve T cells) and effector memory T cells (T_{EM}, decreased expression of CD62L, CCR7, CD28, CD45RA, and increased expression of CD127 as compared to naïve T cells or T_{CM}). Effector T cells (T_E) refers to antigen-experienced CD8+ cytotoxic T lymphocytes that have decreased expression of CD62L ,CCR7, CD28, and are positive for granzyme and perforin as compared to T_{CM}. Other exemplary T cells include regulatory T cells, such as CD4+ CD25+ (Foxp3+) regulatory T cells and Treg17 cells, as well as Tr1, Th3, CD8+CD28-, and Qa-1 restricted T cells.

"T cell receptor" (TCR) refers to a molecule found on the surface of T cells (or T lymphocytes) that, in association with CD3, is generally responsible for recognizing antigens bound to major histocompatibility complex (MHC) molecules. The TCR has a disulfide-linked heterodimer of the highly variable α and β chains (also known as TCRα and TCRβ, respectively) in most T cells. In a small subset of T cells, the TCR is made up of a heterodimer of variable γ and δ chains (also known as TCRγ and TCRδ,

respectively). Each chain of the TCR is a member of the immunoglobulin superfamily and possesses one N-terminal immunoglobulin variable domain, one immunoglobulin constant domain, a transmembrane region, and a short cytoplasmic tail at the C-terminal end (*see* Janeway *et al.*, *Immunobiology: The Immune System in Health and Disease*, 3rd Ed., Current Biology Publications, p. 4:33, 1997). TCR, as used in the present disclosure, may be from various animal species, including human, mouse, rat, cat, dog, goat, horse, or other mammals. TCRs may be cell-bound (*i.e.*, have a transmembrane region or domain) or in soluble form.

"Major histocompatibility complex molecules" (MHC molecules), which is used interchangeably and is understood to also refer to the human counterpart human leukocyte antigen (HLA molecules), refer to glycoproteins that deliver peptide antigens to a cell surface. MHC class I molecules are heterodimers consisting of a membrane spanning α chain (with three α domains) and a non-covalently associated β2 microglobulin. MHC class II molecules are composed of two transmembrane glycoproteins, α and β, both of which span the membrane. Each chain has two domains. MHC (HLA) class I molecules deliver peptides originating in the cytosol to the cell surface, where peptide:MHC (or peptide:HLA in humans) complex is recognized by $CD8^+$ T cells. MHC (HLA) class II molecules deliver peptides originating in the vesicular system to the cell surface, where they are recognized by $CD4^+$ T cells. An MHC molecule may be from various animal species, including human, mouse, rat, or other mammals.

"Nucleic acid molecule", or polynucleotide, may be in the form of RNA or DNA, which includes cDNA, genomic DNA, and synthetic DNA. A nucleic acid molecule may be double stranded or single stranded, and if single stranded, may be the coding strand or non-coding (anti-sense strand). A coding molecule may have a coding sequence identical to a coding sequence known in the art or may have a different coding sequence, which, as the result of the redundancy or degeneracy of the genetic code, or by splicing, can encode the same polypeptide.

Variants of the nucleic acid molecules or polynucleotides of this disclosure are also contemplated. Variant polynucleotides are at least 90%, and preferably 95%, 99%,

or 99.9% identical to one of the polynucleotides of defined sequence as described herein, or that hybridizes to one of those polynucleotides of defined sequence under stringent hybridization conditions of 0.015M sodium chloride, 0.0015M sodium citrate at about 65-68°C or 0.015M sodium chloride, 0.0015M sodium citrate, and 50% formamide at about 42°C. The polynucleotide variants retain the capacity to encode a binding domain or fusion protein thereof having the functionality described herein.

The term "stringent" is used to refer to conditions that are commonly understood in the art as stringent. Hybridization stringency is principally determined by temperature, ionic strength, and the concentration of denaturing agents such as formamide. Examples of stringent conditions for hybridization and washing are 0.015M sodium chloride, 0.0015M sodium citrate at about 65-68°C or 0.015M sodium chloride, 0.0015M sodium citrate, and 50% formamide at about 42°C (*see* Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989).

More stringent conditions (such as higher temperature, lower ionic strength, higher formamide, or other denaturing agent) may also be used; however, the rate of hybridization will be affected. In instances wherein hybridization of deoxyoligonucleotides is concerned, additional exemplary stringent hybridization conditions include washing in 6x SSC, 0.05% sodium pyrophosphate at 37°C (for 14-base oligonucleotides), 48°C (for 17-base oligonucleotides), 55°C (for 20-base oligonucleotides), and 60°C (for 23-base oligonucleotides).

A "vector" is a nucleic acid molecule that is capable of transporting another nucleic acid. Vectors may be, for example, plasmids, cosmids, viruses, or phage. An "expression vector" is a vector that is capable of directing the expression of a protein encoded by one or more genes carried by the vector when it is present in the appropriate environment.

"Retroviruses" are viruses having an RNA genome. "Gammaretrovirus" refers to a genus of the retroviridae family. Exemplary gammaretroviruses include mouse stem cell virus, murine leukemia virus, feline leukemia virus, feline sarcoma virus, and avian reticuloendotheliosis viruses.

"Lentivirus" refers to a genus of retroviruses that are capable of infecting dividing and non-dividing cells. Several examples of lentiviruses include HIV (human immunodeficiency virus: including HIV type 1, and HIV type 2); equine infectious anemia virus; feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV).

The terms "identical" or "percent identity," in the context of two or more polypeptide or nucleic acid molecule sequences, means two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same over a specified region (*e.g.*, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity), when compared and aligned for maximum correspondence over a comparison window, or designated region, as measured using methods known in the art, such as a sequence comparison algorithm, by manual alignment, or by visual inspection. For example, preferred algorithms 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) Nucleic Acids Res. 25:3389 and Altschul *et al.* (1990) J. Mol. Biol. 215:403, respectively.

"Treat" or "treatment" or "ameliorate" refers to medical management of a disease, disorder, or condition of a subject (*e.g.*, a human or non-human mammal, such as a primate, horse, dog, mouse, or rat). In general, an appropriate dose or treatment regimen comprising a host cell expressing a fusion protein of this disclosure, and optionally an adjuvant or adjunctive therapy, is administered in an amount sufficient to elicit a therapeutic or prophylactic benefit. Therapeutic or prophylactic/preventive benefit includes improved clinical outcome; lessening or alleviation of symptoms associated with a disease; decreased occurrence of symptoms; improved quality of life; longer disease-free status; diminishment of extent of disease, stabilization of disease state; delay of disease progression; remission; survival; prolonged survival; or any combination thereof.

A "therapeutically effective amount" or "effective amount" of a fusion protein or cell expressing a fusion protein of this disclosure (*e.g.*, CD200R-CD28, SIRPα-CD28,

CD200R-41BB, SIRPα-41BB, CD200R-CD28-41BB, SIRPα-CD28-4-1BB or other such fusion proteins), in the context of a disease or condition being treated, refers to that amount of fusion protein or number of cells sufficient to result in amelioration of one or more symptoms of the disease being treated in a statistically significant manner (*e.g.*, reducing infection, reducing tumor size, inhibiting cancer growth or the like).

Immunomodulatory Fusion Proteins (IFPs)

In certain aspects, the present disclosure provides a fusion protein, comprising an extracellular component, a hydrophobic component, and an intracellular component. In some embodiments, the extracellular component includes a binding domain such as one that specifically binds to a target. In some embodiments, the binding domain is from a molecule that ordinarily, *e.g.*, in its natural setting, is capable of delivering a negative or inhibitory signal when bound to its binding partner or ligand or receptor, such as an immunoinhibitory receptor or checkpoint molecule, or the target is an inhibitory receptor or ligand or checkpoint molecule or other inhibitory ligand. In some embodiments, the intracellular component includes a signaling domain, such as a costimulatory signaling domain or signaling region of a molecule generally capable of delivering a costimulatory or positive signal, *e.g.*, to an immune cell. Thus, in some aspects, the fusion proteins are capable of delivering a positive or costimulatory signal in response to a binding event that in a natural setting would result in an inhibitory signal.

In some embodiments, the fusion protein is such that a particular distance is achieved. For example, in some embodiments, a fusion protein::target complex (such as one comprised of an extracellular portion of a complex formed between the fusion protein and the target by specific binding thereto) is of a particular length or spans a particular distance, such as a distance of up to a distance between membranes in an immunological synapse, or that spanned by the extracellular portion of a cognate complex between a TCR and MHC molecule, *e.g.*, following specific recognition thereof by a TCR, or the distance spanned by the extracellular portion of a complex formed between the natural molecule and its natural binding partner. In some embodiments, the distance or length is sufficient to promote the colocalization of a

fusion protein with antigen receptor or other signaling molecule when expressed in an immune cell, such as a T cell, or entry into an immunologic synapse.

By way of background, an immunological synapse is an interface between cells, which can form between a variety of cells, such as between immune cells (Rossy *et al.*, *Frontiers in Immunol. 3*: 1-12, 2012; Hatherley *et al.*, *Structure 21*:820, 2013). For example, in the case of a T cell contacting an antigen-presenting cell (APC), an immunological synapse can be formed by the binding of a TCR (found on the surface of a T cell) with an HLA-peptide (MHC-peptide for non-human) complex (found on the surface of, for example, APCs; HLA class I molecules can be found on the surface of all nucleated cells, while HLA class II can conditionally be expressed on all cell types but are regularly found on APCs). In addition, an immunological synapse may be organized into supramolecular activation clusters (SMACs), which can affect lymphocyte activation, direct antigen-HLA (or antigen-MHC) complex presentation to lymphocytes, and direct secretion of cytokines or lytic granules between cells. A SMAC can be comprised of three structures arranged in concentric circles: a central region (cSMAC) containing a high number of TCRs as well as co-stimulatory and inhibitory molecules, a peripheral region (pSMAC) where LFA-1 and talins are clustered, and a distal region (dSMAC) that is enriched for CD43 and CD45 molecules. In certain embodiments, an immunological synapse will span from about 10nm to about 15nm. For example, protein interactions found within the immunological synapse, such as the TCR::HLA-peptide interaction or a fusion protein-target interaction, generally span about 14nm between membranes. In certain embodiments, the width of a SMAC in an immunological synapse does not exceed 15nm.

In some embodiments, the extracellular span of a fusion protein::target complex is such that it can localize to a particular compartment of an immunological synapse. Some complexes thought to localize to various compartments of the immunological synapse are well-characterized with regard to the length of their extracellular span. For example, the MHC-TCR complex is thought to have an extracellular span of approximately 10-15 nm and more integrin-based complexes are thought to have extracellular spans on the order of approximately 40 nm (Alakoskela *et al.*, *Biophys J*

100:2865, 2011). Additional exemplary complexes include the CD2-CD48 complex, which is thought to have an extracellular span of approximately 12.8 nm (Milstein *et al.*, *J Biol Chem 283*:34414, 2008). Additionally, exemplary ligand-binding molecules thought to localize to the cSMAC include the TCR and MHC complexes, CD2, CD4, CD8, CD28, and ligands thereof (Dustin *et al.*, *CSH Perspectives in Biology 2*:a002311, 2010); thus, it is contemplated that these molecules complexed with their natural ligands are of an appropriate size to localize to the cSMAC.

In some aspects, the length or distance or approximate length or distance of a particular construct or engineered extracellular portion thereof such as an extracellular portion of a fusion protein, or complex of any of the foregoing such as with a binding partner thereof, may be determined or modeled by known methods. In some exemplary models, a protein's tertiary structure, binding domains, and other characteristics may be approximated using an input amino acid or nucleic acid sequence. The tertiary structure of a protein may be used to approximate extracellular portion size, flexibility, and other characteristics useful for determining the approximate length of the extracellular portion of the protein or complex thereof. In general, methods for modeling or approximating the length of the extracellular portion of a protein are known. For example, molbiol-tools.ca and Swiss-Model contain multiple tools useful for predicting protein structure (*see also* Schwede, T., *Structure 21*:1531, 2013).

In certain embodiments, a fusion protein of this disclosure complexed, associated or interacting with a target is capable of residing within an immunological synapse. In some embodiments, the extracellular portion of a fusion protein::target complex spans an immunological synapse. In other embodiments, a fusion protein::target complex is localized in a supramolecular activation cluster (SMAC), such as a cSMAC. In further embodiments, the extracellular portion of a fusion protein::target complex spans an immunological synapse defined by the extracellular portion of a TCR::HLA-peptide interaction. In still further embodiments, the length of the extracellular portion of a fusion protein::target complex is about 12nm to about 15nm, or is about 14 nm.

The distance between the cell membranes of cells interacting in an immunological synapse may be measured by any method known in the art. For example, in particular embodiments, the distance may be measured by a subdiffraction-resolution method or electron microscopy (James and Vale, *Nature 487*:64-69, 2012).

In particular embodiments, a fusion protein as disclosed herein comprises an extracellular portion that extends less than 40 nm from the cell membrane. In some embodiments, a fusion protein as disclosed herein comprises an extracellular portion that extends less than 30 nm from the cell membrane. In some embodiments, a fusion protein as disclosed herein comprises an extracellular portion that extends less than 20 nm from the cell membrane. In some embodiments, a fusion protein as disclosed herein comprises an extracellular portion that extends less than 15 nm from the cell membrane.

In some embodiments, the provided fusion proteins provide the advantage of having an extracellular length or spatial distance as compared to the distance between cell membrane(s) that allows for entry into a synapse or co-localization with antigen receptor, or that mimic a distance or length present in the natural proteins. In some embodiments, where the extracellular portion of the fusion protein includes domain(s) from an additional molecule, which is from a different molecule from which a binding domain is obtained, the length of the extracellular component containing the binding domain is reduced, *e.g.*, truncated, as compared to the extracellular region of the natural molecule, to provide for such similar length or distance. In some embodiments, a fusion protein as described herein comprises an extracellular component comprising an extracellular domain of a cell-surface receptor and a second domain (*e.g.*, a linker or an extracellular domain of a second cell-surface receptor). In some such embodiments, to maintain an extracellular component capable of residing within an immunological synapse or spanning an immunological synapse when complexed with a target molecule, one or more domains of the extracellular component may be truncated.

In some diseases (*e.g.*, cancer), the amplitude and quality of a T cell response resulting from antigen recognition by a T cell receptor (TCR) can be dysregulated (*e.g.*, reduced) due to an imbalance between co-stimulatory and inhibitory signals, which can result in immune resistance. One advantage of certain fusion proteins of the instant

disclosure is that a first signal can be converted into a qualitatively different second signal. For example, in some embodiments, the fusion proteins are such that a negative or inhibitory signal can effectively be converted into a positive or co-stimulatory signal to thereby relieve or minimize immune resistance associated with a disease, such as cancer. For example, upon binding to a target that, if bound by its natural binding partner, would result in inhibition or delivery of a negative signal, a fusion protein as provided herein, in some embodiments, is capable of instead delivering a positive, *e.g.*, costimulatory signal, to a cell in which it is expressed, such as in a T cell. In certain embodiments, a fusion protein of this disclosure comprises an extracellular component associated with a negative signal and an intracellular component associated with a positive signal. An exemplary receptor found on the surface of T cells, cytotoxic T-lymphocyte-associated antigen 4 (CTLA4 or CD152), can receive an inhibitory signal when bound by one of its ligands, CD80 or CD86, found on APCs. CTLA4 regulates the amplitude of early stage T cell activation by counteracting the T cell co-stimulatory receptor CD28 (*see* Rudd *et al.*, *Immunol. Rev. 229*:12, 2009). Another exemplary receptor found on the surface of T cells, programmed cell death protein 1 (PD-1 or CD279), can receive an inhibitory signal when bound by one of its ligands, PD-L1 (B7-H1, CD274) or PD-L2 (B7-DC, CD73), found on APCs. PD-1 limits the activity of T cells in peripheral tissues during inflammation and to minimize autoimmunity (*see* Keir *et al.*, *Annu. Rev. Immunol. 26*:677, 2008). Representative fusion proteins of this disclosure comprising an extracellular component associated with a negative signal (*e.g.*, CTLA4 or PD-1) and an intracellular component associated with a positive signal (*e.g.*, CD28, CD137) include a CTLA4-CD28 fusion protein, a CTLA4-CD137 fusion protein, a CTLA4-CD28-CD137 fusion protein, a PD1-CD28 fusion protein, a PD1-CD137 fusion protein, or a PD1-CD28-CD137 fusion protein.

Fusion proteins of the instant disclosure may block or reduce the number of inhibitory signals received by an immune cell. For example, in some embodiments, a fusion protein as disclosed herein converts an inhibitory signal into a positive signal, thereby reducing the total number of inhibitory signals received by an immune cell or converting an ordinarily negative or inhibitory signal to a positive one. In other

embodiments, a fusion protein as disclosed herein blocks the signaling of a wild-type receptor. For example, dominant negative fusion proteins are included within the scope of the disclosure. In some embodiments, a fusion protein as disclosed herein binds to a wild-type receptor and blocks signaling of the wild-type receptor by forming an oligomer with the wild-type receptor.

Yet another advantage of certain fusion proteins of the instant disclosure is that more than one such fusion protein may be expressed by a cell, providing multiple stimulatory signals. It has been observed that recombinant TCRs possessing multiple co-stimulatory domains may not produce adequate co-stimulatory signaling. Co-expressing multiple immunomodulatory fusion proteins, especially those capable of residing within an immunological synapse, may provide the co-stimulatory signaling necessary for T cells to avoid anergy and proliferate.

In some embodiments, a fusion protein of the instant disclosure operates *in trans* relative to a TCR or chimeric antigen receptor (CAR) or other antigen receptor. In some embodiments, a fusion protein as disclosed herein operates outside of the immunological synapse.

In yet another aspect, a fusion protein of the instant disclosure allows for enrichment of transduced T cells by restimulation with tumor cells expressing a ligand that binds to the fusion protein, without the need for sorting.

In one exemplary embodiment, a fusion protein comprising (a) an extracellular portion of a CD200R, (b) a transmembrane domain of a CD28, and (c) an intracellular signaling domain of a CD28 is provided. In some embodiments, the extracellular portion further comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain. In further embodiments, the extracellular portion of the CD200R comprises at least about 231 amino acids from the N-terminus of CD200R. In still further embodiments, the fusion protein further comprises an intracellular signaling domain of a CD137 (4-1BB).

In another exemplary embodiment, the present disclosure provides a fusion protein comprising (a) an extracellular portion of a SIRPα, (b) a transmembrane domain of a CD28, and (c) an intracellular signaling domain of a CD28. In some embodiments,

the fusion protein further comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain. In further embodiments, the extracellular portion of the SIRPα comprises at least about 361 amino acids from the N-terminus of SIRPα. In still further embodiments, the fusion protein further comprises an intracellular signaling domain of a CD137 (4-1BB).

Component parts of the fusion proteins of the present disclosure are further described in detail herein.

Extracellular Component

As described herein, a fusion protein of the present disclosure generally comprises an extracellular component comprising a binding domain that specifically binds a target. Binding of a target by the fusion protein binding domain may (1) block the interaction of target with another molecule (*e.g.*, block or interfere with a receptor-ligand interaction), (2) interfere, reduce or eliminate certain functions of the target (*e.g.*, inhibitory signal transduction), (3) induce certain biological pathways not normally induced when the target is bound (*e.g.*, converting an inhibitory or negative signal into a stimulatory or positive signal), such as in a cell in which the fusion protein is expressed, or any combination thereof. In some embodiments, the fusion proteins as described herein comprise an extracellular portion, wherein the extracellular portion comprises an extracellular portion of protein associated with a negative signal.

Exemplary binding domains of this disclosure may be ectodomains of cell-surface receptors, or binding portions thereof, ectodomains of cell-surface ligands, cytokines (*e.g.*, IL35), chemokines, antibody-based binding domains, TCR-based binding domains, non-conventional binding domains, or any combination thereof. For example, binding domains comprising an ectodomain of CD200R, SIRPα, CD279 (PD-1), CD2, CD95 (Fas), CTLA4 (CD152), CD223 (LAG3), CD272 (BTLA), A2aR, KIR, TIM3, CD300, or LPA5 are within the scope of this disclosure. As used herein, an "ectodomain" from a cell-surface receptor or ligand includes a complete extracellular domain or a functional (binding) fragment thereof. In certain embodiments, an ectodomain comprises a mutated extracellular domain or a functional (binding) fragment thereof that has a higher avidity for target as compared to a wild-type or

reference protein. In certain embodiments, an ectodomain comprises a variable-like domain or a CDR of a variable-like domain.

In some embodiments, a fusion protein contains an extracellular component comprising a CD200-binding domain, such as a CD200R ectodomain or CD200-binding portion thereof. By way of background, CD200R is a receptor that binds to CD200, a type-1 membrane protein of the immunoglobulin superfamily (Tonks *et al.*, *Leukemia 21*:566-568, 2007). CD200 has been reported to be upregulated on various malignancies, including leukemias, multiple myeloma, and various solid tumors (*e.g.*, melanoma, breast, and squamous cell carcinoma). In fact, high levels of CD200 expression have been linked with poor prognosis for acute myeloid leukemia (AML), and CD200R signaling has been shown to have an inhibitory effect on T cells (Coles *et al.*, *Leukemia* 26: 2148-2151, 2012). In certain embodiments, a CD200R ectodomain includes a full length extracellular portion of a CD200R protein, a full length mature extracellular portion of a CD200R protein, a binding fragment of an extracellular portion of a CD200R protein, or a binding fragment of an extracellular portion of a CD200R protein along with a portion of the transmembrane domain of CD200R, or any combination thereof.

In further embodiments, a CD200R is encoded by a nucleic acid molecule as set forth in SEQ ID NO.:2. In certain other embodiments, a CD200R ectodomain comprises at least 200 amino acids from the N-terminus of CD200R. In some other embodiments, a CD200R is encoded by a nucleic acid molecule as set forth in SEQ ID NO.:11. In yet other embodiments, an extracellular portion of the CD200R comprises at least 180, 190, 200, 210, 220, 230, 231, 234, or 243 amino acids from the N-terminus of CD200R. For example, in certain embodiments, a CD200R is encoded by the nucleic acid molecule as set forth in SEQ ID NO.:8. In any of the aforementioned embodiments, a CD200R, a CD200R ectodomain, or any CD200R fragment thereof used in a fusion protein of this disclosure is a human CD200R. In further embodiments, there are provided CD200R ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least

100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:2.

In some embodiments, a CD200R comprises an amino acid sequence as set forth in SEQ ID NO.:25. In some embodiments, a CD200R comprises an amino acid sequence as set forth in SEQ ID NO.:34. In certain embodiments, a CD200R comprises an amino acid sequence as set forth in SEQ ID NO.:31. In any of the aforementioned embodiments, a CD200R, a CD200R ectodomain, or any CD200R fragment thereof used in a fusion protein of this disclosure is a human CD200R. In further embodiments, there are provided CD200R ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence as set forth in SEQ ID NO.:25.

In some embodiments, a fusion protein contains an extracellular component comprising a CD47-binding domain such as a SIRPα ectodomain or binding portion thereof. By way of background, CD47 is a widely expressed transmembrane protein that plays a role in protecting cells from phagocytosis (Willingham *et al.*, *PNAS 109*: 6662–6667, 2012). Binding of CD47 to SIRPα initiates SIRPα signaling, which inhibits phagocytosis by macrophages. Accordingly, downregulation of SIRPα will result in increased phagocytosis by macrophages. SIRPα is expressed on multiple human tumor types including AML, chronic myelogenous leukemia (CML), acute lymphoblastic leukemia (ALL), Non-Hodgkin lymphoma (NHL), multiple myeloma (MM), lung, bladder, and other solid tumors. In certain embodiments, a SIRPα ectodomain includes a full length extracellular portion of a SIRPα protein, a full length mature extracellular portion of a SIRPα protein, a binding fragment of an extracellular portion of a SIRPα protein, and a binding fragment of an extracellular portion of a SIRPα protein along with a portion of the transmembrane domain of SIRPα, or any combination thereof.

In further embodiments, a SIRPα ectodomain or binding portion thereof is encoded by a nucleic acid molecule as set forth in SEQ ID NO.:17. In certain

embodiments, a SIRPα ectodomain comprises at least 300, 310, 320, 330, 340, 350, 360, 361, 370, 373, or more amino acids from the N-terminus of SIRPα. In some other embodiments, a SIRPα is encoded by a nucleic acid molecule as set forth in SEQ ID NO.:21. In any of the aforementioned embodiments, a SIRPα, a SIRPα ectodomain, or any SIRPα fragment thereof used in a fusion protein of this disclosure is a human SIRPα. In further embodiments, there are provided SIRPα ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:17.

In further embodiments, a SIRPα ectodomain comprises an amino acid sequence as set forth in SEQ ID NO.:40. In some embodiments, a SIRPα comprises an amino acid sequence as set forth in SEQ ID NO.:44. In any of the aforementioned embodiments, a SIRPα, a SIRPα ectodomain, or any SIRPα fragment thereof used in a fusion protein of this disclosure is a human SIRPα. In further embodiments, there are provided SIRPα ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence as set forth in SEQ ID NO.:40.

In some embodiments, a fusion protein contains an extracellular component comprising a binding domain that binds to PD-L1, PD-L2, or both. In some embodiments, a fusion protein contains an extracellular component comprising a PD-1 ectodomain or ligand-binding portion thereof. In certain embodiments, a PD-1 ectodomain includes a full length extracellular portion of a PD-1 protein, a full length mature extracellular portion of a PD-1 protein, a binding fragment of an extracellular portion of a PD-1 protein, or a binding fragment of an extracellular portion of a PD-1 protein along with a portion of the transmembrane domain of PD-1, or any combination thereof. In certain embodiments, a PD-1 ectodomain comprises at least 80, 90, 100,

110, 120, 125, 130, 132, 135, 137, 140, 149, 150, 155, 158, 160, or 170 amino acids from the N-terminus of PD-1. For example, in certain embodiments, a PD-1 ectodomain is encoded by the nucleic acid molecule as set forth in SEQ ID NO.:91, 93, or 95. In further embodiments, a PD-1 ectodomain comprises at least from about 90 amino acids to at least about 130 amino acids from a PD-1 as set forth in SEQ ID NO.:60. In still further embodiments, a PD-1 ectodomain comprises 170 amino acids from the N-terminus of a PD-1 ectodomain, as set forth in SEQ ID NO.:90. In some embodiments, a PD-1 is encoded by a nucleic acid molecule as set forth in SEQ ID NO.:89. In any of the aforementioned embodiments, a PD-1, a PD-1 ectodomain, or any PD-1 fragment thereof used in a fusion protein of this disclosure is a human PD-1. In further embodiments, there are provided PD-1 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence as set forth in SEQ ID NO.:60. In further embodiments, there are provided PD-1 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence as set forth in SEQ ID NO.:90. In still further embodiments, there are provided PD-1 binding domains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:89.

In certain embodiments, a PD-1 ectodomain comprises the amino acid sequence as set forth in SEQ ID NO.:92, 94, or 96. In further embodiments, there are provided PD-1 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an

ectodomain of a molecule having an amino acid sequence as set forth in SEQ ID NO.: 92, 94, or 96. In any of the aforementioned embodiments, a PD-1, a PD-1 ectodomain, or any PD-1 fragment thereof used in a fusion protein of this disclosure is a human PD-1.

In some embodiments, a fusion protein contains an extracellular component comprising a CD2 ectodomain. In certain embodiments, a CD2 ectodomain is encoded by a nucleic acid molecule as set forth in SEQ ID NO.:61. In certain embodiments, a CD2 ectodomain includes a full length extracellular portion of a CD2 protein, a full length mature extracellular portion of a CD2 protein, a binding fragment of an extracellular portion of a CD2 protein, or a binding fragment of an extracellular portion of a CD2 protein along with a portion of the transmembrane domain of CD2, or any combination thereof. In any of the aforementioned embodiments, a CD2, a CD2 ectodomain, or any CD2 fragment thereof used in a fusion protein of this disclosure is a human CD2. In further embodiments, there are provided CD2 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in GenBank Accession No. NM_001767.3. In further embodiments, there are provided CD2 ectodomains s that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:61.

In some embodiments, a CD2 ectodomain comprises an amino acid sequence as set forth in SEQ ID NO.:62. In further embodiments, there are provided CD2 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence as set forth in SEQ ID

NO.:62. In any of the aforementioned embodiments, a CD2, a CD2 ectodomain, or any CD2 fragment thereof used in a fusion protein of this disclosure is a human CD2.

In some embodiments, a fusion protein contains an extracellular component comprising a binding domain that binds to FasL. In some embodiments, a fusion protein contains an extracellular component comprising a Fas (CD95) ectodomain. Fas is expressed on tumor-associated vasculature and prevents CD8 cell infiltration by inducing cell death. In certain embodiments, a Fas ectodomain includes a full length extracellular portion of a Fas protein, a full length mature extracellular portion of a Fas protein, a binding fragment of an extracellular portion of a Fas protein, and a binding fragment of an extracellular portion of a Fas protein along with a portion of the transmembrane domain of Fas, or any combination thereof. In some embodiments, a Fas ectodomain is encoded by a nucleic acid molecule as set forth in SEQ ID NO.:71. In yet other embodiments, a Fas ectodomain comprises at least 150, 160, 161, 166, 170, or 173 amino acids from the N-terminus of Fas. For example, in certain embodiments, a Fas is encoded by the nucleic acid molecule as set forth in SEQ ID NO.:73. In certain other embodiments, a Fas is encoded by the nucleic acid molecule as set forth in SEQ ID NO.:75. In any of the aforementioned embodiments, a Fas, a Fas ectodomain, or any Fas fragment thereof used in a fusion protein of this disclosure is a human Fas. In further embodiments, there are provided Fas ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in GenBank Accession No. NM_000043.4. In still further embodiments, there are provided Fas ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:71.

In some embodiments, a Fas ectodomain comprises an amino acid sequence as set forth in SEQ ID NO.:72. In certain embodiments, a Fas ectodomain comprises the amino acid sequence as set forth in SEQ ID NO.:74. In certain other embodiments, a Fas ectodomain comprises the amino acid sequence as set forth in SEQ ID NO.:76. In any of the aforementioned embodiments, a Fas, a Fas ectodomain, or any Fas fragment thereof used in a fusion protein of this disclosure is a human Fas. In further embodiments, there are provided Fas ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence as set forth in SEQ ID NO.:72.

In some embodiments, a fusion protein contains an extracellular component comprising a LAG3 (CD223) ectodomain. In certain embodiments, a LAG3 ectodomain includes a full length extracellular portion of a LAG3 protein, a full length mature extracellular portion of a LAG3 protein, a binding fragment of an extracellular portion of a LAG3 protein, and a binding fragment of an extracellular portion of a LAG3 protein along with a portion of the transmembrane domain of LAG3, or any combination thereof. For example, in some embodiments, a LAG3 ectodomain comprises about 420, 416, 415, 413, or 410 amino acids from the N terminus of LAG3. In any of the aforementioned embodiments, a LAG3, a LAG3 ectodomain, or any LAG3 fragment thereof used in a fusion protein of this disclosure is a human LAG3. In further embodiments, there are provided LAG3 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in GenBank Accession No. NM_ 002286.5.

In further embodiments, a LAG3 is encoded by a nucleic acid molecule as set forth in SEQ ID NO.:153. In certain other embodiments, a LAG3 ectodomain comprises at least 430, 435, 438, 440, 445, or 450 amino acids from the N-terminus of

LAG3. For example, in certain embodiments, a LAG3 is encoded by the nucleic acid molecule as set forth in SEQ ID NO.:161. In any of the aforementioned embodiments, a LAG3, LAG3 ectodomain, or any LAG3 fragment thereof used in a fusion protein of this disclosure is a human LAG3. In further embodiments, there are provided LAG3 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:153.

In some embodiments, a LAG3 comprises an amino acid sequence as set forth in SEQ ID NO.:154. In some embodiments, a LAG3 comprises an amino acid sequence as set forth in SEQ ID NO.:162. In any of the aforementioned embodiments, a LAG3, a LAG3 ectodomain, or any LAG3 fragment thereof used in a fusion protein of this disclosure is a human LAG3. In further embodiments, there are provided LAG3 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence as set forth in SEQ ID NO.:154.

In some embodiments, a fusion protein contains an extracellular component comprising a TIM3 ectodomain. In certain embodiments, a TIM3 ectodomain includes a full length extracellular portion of a TIM3 protein, a full length mature extracellular portion of a TIM3 protein, a binding fragment of an extracellular portion of a TIM3 protein, and a binding fragment of an extracellular portion of a TIM3 protein along with a portion of the transmembrane domain of TIM3, or any combination thereof. In any of the aforementioned embodiments, a TIM3, a TIM3 ectodomain, or any TIM3 fragment thereof used in a fusion protein of this disclosure is a human TIM3. In further embodiments, there are provided TIM3 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at

least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in GenBank Accession No. NM_032782.4.

In further embodiments, a TIM3 is encoded by a nucleic acid molecule as set forth in SEQ ID NO.:167. In certain other embodiments, a TIM3 ectodomain comprises at least 180, 185, 190, 195, or 200 amino acids from the N-terminus of TIM3. For example, in certain embodiments, a TIM3 is encoded by the nucleic acid molecule as set forth in SEQ ID NO.:177. In any of the aforementioned embodiments, a TIM3, TIM3 ectodomain, or any TIM3 fragment thereof used in a fusion protein of this disclosure is a human TIM3. In further embodiments, there are provided TIM3 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:167.

In some embodiments, a TIM3 comprises an amino acid sequence as set forth in SEQ ID NO.:168. In some embodiments, a TIM3 comprises an amino acid sequence as set forth in SEQ ID NO.:178. In any of the aforementioned embodiments, a TIM3, a TIM3 ectodomain, or any TIM3 fragment thereof used in a fusion protein of this disclosure is a human TIM3. In further embodiments, there are provided TIM3 ectodomains that have a sequence that is at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or at least 100% identical to an ectodomain of a molecule having an amino acid sequence as set forth in SEQ ID NO.:168.

A binding domain may be any peptide that specifically binds a target of interest. Sources of binding domains include antibody variable regions from various species (which can be in the form of antibodies, sFvs, scFvs, Fabs, scFv-based grababody, or soluble VH domain or domain antibodies), including human, rodent, avian, or ovine. Additional sources of binding domains include variable regions of antibodies from

other species, such as camelid (from camels, dromedaries, or llamas; Ghahroudi *et al.*, *FEBS Lett. 414*:521, 1997; Vincke *et al.*, *J. Biol. Chem. 284*:3273, 2009; Hamers-Casterman *et al.*, *Nature 363*:446, 1993 and Nguyen *et al.*, *J. Mol. Biol. 275*:413, 1998), nurse sharks (Roux *et al.*, *Proc. Nat'l. Acad. Sci. (USA) 95*:11804, 1998), spotted ratfish (Nguyen *et al.*, *Immunogen. 54*:39, 2002), or lamprey (Herrin *et al.*, *Proc. Nat'l. Acad. Sci. (USA) 105*:2040, 2008 and Alder *et al. Nat. Immunol. 9*:319, 2008). These antibodies can form antigen-binding regions using only a heavy chain variable region, *i.e.*, these functional antibodies are homodimers of heavy chains only (referred to as "heavy chain antibodies") (Jespers *et al.*, *Nat. Biotechnol. 22*:1161, 2004; Cortez-Retamozo *et al.*, *Cancer Res. 64*:2853, 2004; Baral *et al.*, *Nature Med. 12*:580, 2006; and Barthelemy *et al.*, *J. Biol. Chem. 283*:3639, 2008).

An alternative source of non-conventional binding domains of this disclosure includes sequences that encode random peptide libraries or sequences that encode an engineered diversity of amino acids in loop regions of alternative non-antibody scaffolds, such as scTCR (*see, e.g.*, Lake *et al.*, *Int. Immunol.*11:745, 1999; Maynard *et al.*, *J. Immunol. Methods 306*:51, 2005; U.S. Patent No. 8,361,794), fibrinogen domains (*see, e.g.*, Weisel *et al.*, *Science 230*:1388, 1985), Kunitz domains (*see, e.g.*, US Patent No. 6,423,498), designed ankyrin repeat proteins (DARPins) (Binz *et al.*, *J. Mol. Biol. 332*:489, 2003 and Binz *et al.*, *Nat. Biotechnol. 22*:575, 2004), fibronectin binding domains (adnectins or monobodies) (Richards *et al.*, *J. Mol. Biol. 326*:1475, 2003; Parker *et al.*, *Protein Eng. Des. Selec. 18*:435, 2005 and Hackel *et al.* (2008) *J. Mol. Biol. 381*:1238-1252), cysteine-knot miniproteins (Vita *et al.* (1995) *Proc. Nat'l. Acad. Sci. (USA) 92*:6404-6408; Martin *et al.* (2002) *Nat. Biotechnol. 21*:71, 2002 and Huang *et al.* (2005) *Structure 13*:755, 2005), tetratricopeptide repeat domains (Main *et al.*, *Structure 11*:497, 2003 and Cortajarena *et al.*, *ACS Chem. Biol. 3*:161, 2008), leucine-rich repeat domains (Stumpp *et al.*, *J. Mol. Biol. 332*:471, 2003), lipocalin domains (*see, e.g.*, WO 2006/095164, Beste *et al.*, *Proc. Nat'l. Acad. Sci. (USA) 96*:1898, 1999 and Schönfeld *et al.*, *Proc. Nat'l. Acad. Sci. (USA) 106*:8198, 2009), V-like domains (*see, e.g.*, US Patent Application Publication No. 2007/0065431), C-type lectin domains (Zelensky and Gready, *FEBS J. 272*:6179, 2005; Beavil *et al.*, *Proc. Nat'l. Acad. Sci.*

(USA) 89:753, 1992 and Sato *et al.*, *Proc. Nat'l. Acad. Sci. (USA) 100*:7779, 2003), mAb^2 or Fcab™ (*see*, *e.g.*, PCT Patent Application Publication Nos. WO 2007/098934; WO 2006/072620), armadillo repeat proteins (*see*, *e.g.*, Madhurantakam *et al.*, *Protein Sci. 21*: 1015, 2012; PCT Patent Application Publication No. WO 2009/040338), affilin (Ebersbach *et al.*, *J. Mol. Biol. 372*: 172, 2007), affibody, avimers, knottins, fynomers, atrimers, cytotoxic T-lymphocyte associated protein-4 (Weidle *et al.*, *Cancer Gen. Proteo. 10*:155, 2013) or the like (Nord *et al.*, *Protein Eng. 8*:601, 1995; Nord *et al.*, *Nat. Biotechnol. 15*:772, 1997; Nord *et al.*, *Euro. J. Biochem. 268*:4269, 2001; Binz *et al.*, *Nat. Biotechnol. 23*:1257, 2005; Boersma and Plückthun, *Curr. Opin. Biotechnol. 22*:849, 2011).

In some embodiments, a binding domain is a single chain T cell receptor (scTCR) comprising $V_{\alpha/\beta}$ and $C_{\alpha/\beta}$ chains (*e.g.*, V_α-C_α, V_β-C_β, V_α-V_β) or comprising V_α-C_α, V_β-C_β, V_α-V_β pair specific for a target of interest (*e.g.*, peptide-MHC complex or peptide-HLA complex).

In certain embodiments, a binding domain comprises or is a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, at least 99.5%, or 100% identical to an ectodomain of a molecule having an amino acid sequence of a TCR V_α, V_β, C_α, or C_β, wherein each CDR comprises zero changes or at most one, two, or three changes, from a TCR or fragment or derivative thereof that specifically binds to a target of interest.

In certain embodiments, a binding domain V_α, V_β, C_α, or C_β region of the present disclosure can be derived from or based on a V_α, V_β, C_α, or C_β of a known TCR (*e.g.*, a high-affinity TCR) and contains one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10) insertions, one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10) deletions, one or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10) amino acid substitutions (*e.g.*, conservative amino acid substitutions or non-conservative amino acid substitutions), or a combination of the above-noted changes, when compared with the V_α, V_β, C_α, or C_β of a known TCR. An insertion, deletion or substitution may be anywhere in a V_α, V_β, C_α, or C_β region, including at the amino- or carboxy-terminus or both ends of these regions, provided that each CDR comprises zero changes or at most one, two, or three changes and provided a binding

domain containing a modified V_α, V_β, C_α, or C_β region can still specifically bind its target with an affinity similar to wild type. In certain embodiments, a TCR has an affinity for a peptide-HLA complex ranging from about 10μM to about 500μM. In further embodiments, a TCR has a high affinity for a peptide-HLA complex ranging from about 10nM to about 200pM.

In certain aspects, a fusion protein according to the present disclosure has an extracellular component comprised of a binding domain that specifically binds a target (*e.g.*, a ligand or receptor), wherein the extracellular component optionally includes one or more other functional subcomponents or domains, such as a multimerization domain, a linker, junction amino acids, or any combination thereof.

In certain embodiments, a fusion protein disclosed herein further comprises an additional extracellular region in addition to the binding domain or in addition to the portion derived from the molecule from which the binding domain is derived, such as a spacer or a multimerization domain. For example, in some aspects a multimerization domain is contained in or is a part of the extracellular component of the fusion protein. For example, a multimerization domain may be created by altering (*e.g.*, mutating) the extracellular component, or a multimerization domain may be created by adding 1 to about 50 amino acid residues to the extracellular component. A multimerization domain may be located between the binding domain of the extracellular component and hydrophobic component of a fusion protein of this disclosure. In certain embodiments, a fusion protein expressed on a cell surface comprises a multimerization domain within the extracellular component and is proximal to the cell membrane, within one to 50 amino acids from the hydrophobic component. For example, a fusion protein multimerization domain may comprise one or more cysteine residues located within 30, 25, 20, 15, 14, 13, 12, 11, 10, 9 8, 7, 6, 5, 4, 3, 2, 1 or 0 amino acids from the fusion protein hydrophobic component, wherein such one or more cysteine residues from one fusion protein can form one or more disulfide bridges with one or more other fusion proteins. In some embodiments, the additional extracellular portion is derived from the same molecule from which a transmembrane or stimulatory region of the fusion protein is derived.

In further embodiments, interaction(s) between multimerization domains of two or more fusion proteins substantially contribute to or efficiently promote signal transduction (*e.g.*, immune cell stimulation or activation) as compared to a fusion protein monomer. In certain embodiments, multimerization of fusion proteins promote signal transduction in a host cell in a statistically significant manner over fusion protein monomers. In further embodiments, multimerization of fusion proteins that promotes or enhances signal transduction in a host cell is via a disulfide bridge.

An exemplary multimer is a "dimer," which refers to a biological entity containing two molecules, such as two fusion proteins, associated with each other. Such a dimer is considered a "homodimer" when the two associated fusion proteins have substantially similar or identical amino acid sequences. Similarly, multimerization of three substantially or fully identical fusion proteins is referred to as a "homotrimer." In some embodiments, a multimerization domain comprises at least one cysteine residue, wherein a multimerization domain cysteine residue from a first fusion protein can form a disulfide bridge with a multimerization domain cysteine residue from a second fusion protein. In certain embodiments, a fusion protein dimer forms via a disulfide bridge. In other embodiments, a fusion protein trimer forms via two or more disulfide bridges. Alternatively, a dimer, homodimer, trimer or homotrimer may multimerize via a zinc finger motif or a leucine zipper motif. In still further embodiments, a fusion protein comprises a plurality of multimerization domains, which can be located extracellularly, intracellularly or both.

In some embodiments, a multimerization domain contained in the extracellular component of a fusion protein comprises an extracellular portion extending from the hydrophobic component. For example, in some embodiments, a multimerization domain contained in the extracellular component of a fusion protein comprises an extracellular portion of a CD28 extending from a CD28 transmembrane domain. In some embodiments, an extracellular portion of the CD28 comprises about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or up to about 25 amino acids adjacent to the transmembrane domain. In some embodiments, the extracellular portion of the CD28 comprises 9 amino acids or 12 amino acids adjacent to the transmembrane domain. In some

embodiments, the extracellular portion of a CD28 comprises the amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9. In some embodiments, the extracellular portion of a CD28 comprises the amino acid sequence as set forth in SEQ ID NO.:32. In yet another exemplary embodiment, a multimerization domain contained in the extracellular component of a fusion protein comprises an extracellular portion of a CD137 (4-1BB) (*e.g.*, ranging from one to about 50 amino acids) extending from a CD137 (4-1BB) transmembrane domain. In certain embodiments, the multimerization domain and the hydrophobic component are from different proteins. For example, a multimerization domain contained in the extracellular component of a fusion protein comprises an extracellular portion of a CD28 extending from a CD137 transmembrane domain, or comprises an extracellular portion of a CD137 extending from a CD28 transmembrane domain. In any of the aforementioned embodiments, a multimerization domain may further comprise a glycosylation site.

In some embodiments, a fusion protein may contain a linker or junction amino acids connecting, for example, an extracellular component with a multimerization domain or connecting an extracellular component with a hydrophobic component or connecting a hydrophobic component with an intracellular component. In some embodiments, the linker is a Gly_xSer_y, wherein x and y are independently an integer from 1 to 5.

A target molecule, which is specifically bound by a binding domain contained in a fusion protein of the present disclosure, may be found on or in association with a cell of interest ("target cell"). Exemplary target cells include an immune cell, a cancer cell, a cell associated with an autoimmune disease or disorder or with an inflammatory disease or disorder, and an infectious organism or cell (*e.g.*, bacteria, virus, virus-infected cell), or any cell presenting antigen complexed with a MHC or human leukocyte antigen (HLA). A cell of an infectious organism, such as a mammalian parasite, is also contemplated as a target cell. In some embodiments, the target is an immunosuppressive ligand. In some embodiments, the target is selected from a CD47,

CD58, CD80, CD86, CD95L (FasL), CD200, CD270 (HVEM), CD274 (PD-L1), or GAL9.

Intracellular Component

An intracellular component contained in a fusion protein of the present disclosure will have an intracellular signaling domain, such as an activating domain or a co-stimulatory domain, capable of transmitting functional signals to a cell. In certain embodiments, an intracellular signaling domain will indirectly promote a cellular response by associating with one or more other proteins that directly promote a cellular response. An intracellular signaling domain may include one, two, three or more receptor signaling domains, costimulatory domains, or combinations thereof. Any intracellular component comprising an activating domain, co-stimulatory domain, or both from any of a variety of signaling molecules (*e.g.*, signal transduction receptors) may be used in the fusion proteins of this disclosure.

As used herein, an "intracellular signaling domain" from a cell-surface receptor or ligand includes a complete intracellular domain, a portion comprising an intracellular signaling domain, or a functional (signaling) fragment thereof. In certain embodiments, an intracellular signaling domain comprises a mutated intracellular domain or a functional (signaling) fragment thereof that has increased signaling activity as compared to a wild-type or reference intracellular signaling domain.

A "co-stimulatory molecule" as used herein refers to a receptor or cell-surface molecule that can transduce signals into T cells to positively modulate T cell activation (Chen and Flies, *Nat. Rev. Immunol. 13*: 227-242, 2013). By way of background, T cell activation and proliferation requires two signals mediated through engagement of the T cell antigen-specific receptor (TCR) and a co-stimulatory signal, most typically binding of CD28 by CD80 and CD86 (Ledbetter *et al.*, *Blood 75*:1531, 1990).

An intracellular signaling domain or functional fragment thereof useful in the fusion proteins of this disclosure may be from a CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD40, CD47, CD79A, CD79B, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD278 (ICOS), CD357 (GITR), CARD11, DAP10, DAP12, FcRα, FcRβ, FcRγ, Fyn, Lck, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4,

ROR2, Ryk, Slp76, pTα, TCRα, TCRβ, TRIM, Zap70, PTCH2, or any combination thereof. In some embodiments, an intracellular signaling domain or functional fragment thereof does not comprise a primary signal. In some embodiments, an intracellular signaling domain does not comprise a CD3ζ.

In some embodiments, an intracellular signaling domain of a fusion protein of this disclosure comprises a CD28. CD28 signaling promotes proliferation of T cells stimulated via the TCR (Chen and Flies, *Nat. Rev. Immunol. 13*: 227-242, 2013). CD28 forms disulfide-linked homodimers, as a result of the cysteine residue proximal to the transmembrane domain (Lazar-Molnar *et al.*, *Cell Immunol. 244*: 125–129, 2006). In certain embodiments, a CD28 signaling domain includes a full length intracellular portion of a CD28 protein, a full length mature intracellular portion of a CD28 protein, a signaling fragment of an intracellular portion of a CD28 protein, and a signaling fragment of an intracellular portion of a CD28 protein along with a transmembrane domain or fragment thereof of CD28, or any combination thereof.

In some embodiments, an intracellular signaling domain of a fusion protein contains an intracellular signaling domain of a CD137 (4-1BB). CD137 is a co-stimulatory molecule, wherein binding of CD137 to its ligand (4-1BBL or CD137L) is associated with T cell activation and proliferation (Cheuk *et al.*, *Cancer Gene Therapy* 11: 215-226, 2004). In certain embodiments, a CD137 signaling domain includes a full length intracellular portion of a CD137 protein, a full length mature intracellular portion of a CD137 protein, a signaling fragment of an intracellular portion of a CD137 protein, and a signaling fragment of an intracellular portion of a CD137 protein along with a transmembrane domain or fragment thereof of CD137, or any combination thereof.

In certain embodiments, an intracellular signaling domain comprises a lymphocyte receptor signaling domain or comprises an amino acid sequences having one or a plurality of immunoreceptor tyrosine-based activation motifs (ITAMs). In still further embodiments, an intracellular signaling domain comprises a cytoplasmic portion that associates with a cytoplasmic signaling protein, wherein the cytoplasmic signaling protein is a lymphocyte receptor or signaling domain thereof, a protein comprising a plurality of ITAMs, a costimulatory factor, or any combination thereof.

In some exemplary embodiments, the present disclosure provides a fusion protein having an extracellular component comprising an extracellular portion of a CD200R that specifically binds CD200, an intracellular component comprising an intracellular portion of CD28, and a hydrophobic component connecting the extracellular and intracellular components, provided that a fusion protein::target complex spans a distance similar to a distance between membranes in an immunological synapse.

In particular embodiments, an intracellular component of a fusion protein of the instant disclosure comprises a CD28, a CD137 (4-1BB) or both. For example, in some embodiments, an intracellular component comprises the amino acid sequence encoded by a nucleic acid molecule as set forth in in SEQ ID NO.:5. In some other embodiments, an intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:13. In some embodiments, an intracellular component comprises two intracellular signaling domains, for example, a CD28 and a CD137 (4-1BB). In some embodiments, an intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5 and the amino acid sequence encoded by the nucleotide sequence as set SEQ ID NO.:13.

Hydrophobic Component

A hydrophobic portion contained in a single chain fusion protein of the present disclosure will allow a fusion protein of this disclosure to associate with a cellular membrane such that a portion of the fusion protein will be located extracellularly and a portion will be located intracellularly (*e.g.*, intracellular signaling domain). A hydrophobic component will generally be disposed within the cellular membrane phospholipid bilayer. In certain embodiments, one or more junction amino acids may be disposed between and connecting a hydrophobic portion with an intracellular signaling domain.

In certain embodiments, a hydrophobic domain is a transmembrane domain, such as one derived from an integral membrane protein (*e.g.*, receptor, cluster of differentiation (CD) molecule, enzyme, transporter, cell adhesion molecule, or the like).

In some embodiments, the hydrophobic domain comprises a transmembrane domain found in or derived from an integral membrane protein, wherein the transmembrane domain has been modified by the addition, removal, or replacement of one or more amino acids with at least one different amino acid, or any combination thereof, such as charged or hydrophilic residues that facilitate intermolecular interactions. Thus, the term "hydrophobic domain" includes transmembrane domains having, for example, modifications that may reduce hydrophobicity.

In some embodiments, the hydrophobic component comprises a transmembrane domain of a CD2, CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD40, CD47, CD79A, CD79B, CD80, CD86, CD95 (Fas), CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD200R, CD223 (LAG3), CD270 (HVEM), CD272 (BTLA), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), CD279 (PD-1), TIM3, CD300, CD357 (GITR), A2aR, DAP10, FcRα, FcRβ, FcRγ, Fyn, GAL9, KIR, Lck, LAT, LPA5, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPα, pTα, TCRα, TCRβ, TIM3, TRIM, or Zap70. In particular embodiments, a hydrophobic portion is a transmembrane domain from CD28, CD4, CD8, CD27, or CD137 (4-1BB). In certain embodiments, a transmembrane domain is a CD28 transmembrane domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4. In certain other embodiments, a transmembrane domain is a CD200R transmembrane domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:3. In still other embodiments, a transmembrane domain is a SIRPα transmembrane domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:18. In further embodiments, a transmembrane domain is a CD2 transmembrane domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:63. In still further embodiments, a transmembrane domain is a Fas transmembrane domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:77. In still further embodiments, a transmembrane domain is a TIM3 transmembrane domain. In still further embodiments, a transmembrane domain is a TIM3 transmembrane domain having an amino acid

sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:169. In still further embodiments, a transmembrane domain is a LAG3 transmembrane domain. In some embodiments, a transmembrane domain is a LAG3 transmembrane domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:155.

Nucleic Acids and Host Cells

In certain aspects, the present disclosure provides nucleic acid molecules that encode any one or more of the fusion proteins described herein, which may be immunomodulatory fusion proteins (IFPs). Such nucleic acid molecules can be inserted into an appropriate vector (*e.g.*, viral vector or non-viral plasmid vector) for introduction in a host cell of interest (*e.g.*, hematopoietic progenitor cell, T cell).

As used herein, the term "recombinant" or "non-natural" refers to an organism, microorganism, cell, nucleic acid molecule, or vector that includes at least one genetic alteration or has been modified by introduction of an exogenous nucleic acid molecule, wherein such alterations or modifications are introduced by genetic engineering. Genetic alterations include, for example, modifications introducing expressible nucleic acid molecules encoding proteins, fusion proteins or enzymes, or other nucleic acid molecule additions, deletions, substitutions or other functional disruption of a cell's genetic material. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression of a gene or operon. In certain embodiments, a cell, such as a T cell, obtained from a subject may be converted into a non-natural or recombinant cell (*e.g.*, a non-natural or recombinant T cell) by introducing a nucleic acid that encodes a fusion protein as described herein and whereby the cell expresses a fusion protein.

In certain embodiments, nucleic acid molecules encoding fusion proteins may be codon optimized to enhance or maximize expression in certain types of cells, such as T cells (Scholten *et al.*, *Clin. Immunol. 119*: 135-145, 2006).

In one exemplary embodiment, the present disclosure provides a nucleic acid molecule that encodes a CD200R-CD28 construct (huCD200Rtm-CD28), wherein the extracellular component comprises a CD200R ectodomain, the hydrophobic component

comprises the transmembrane domain of a CD200R, and the intracellular component comprises the intracellular signaling domain of a CD28. For example, in one embodiment, a nucleic acid molecule as set forth in SEQ ID NO.:1 is provided.

In another exemplary embodiment, the present disclosure provides a nucleic acid molecule that encodes a CD200R-CD28 construct (huCD200R-CD28tm), wherein the hydrophobic component comprises the transmembrane domain of a CD28. For example, in one embodiment, the disclosure provides a nucleic acid molecule as set forth in SEQ ID NO.:6.

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a CD200R-CD28 construct, wherein the extracellular comprises a truncated extracellular domain of CD200R and an extracellular portion of CD28. For example, the CD200R extracellular domain may be truncated by 9 amino acids (*e.g.*, huCD200R-9aas-CD28Cys, SEQ ID NO.:7) or by 12 amino acids (*e.g.*, huCD200R-12aas-CD28Cys, SEQ ID NO.:10).

In one exemplary embodiment, the present disclosure provides a nucleic acid molecule that encodes a CD200R-CD28-4-1BB construct (huCD200R-9aas-CD28Cystm-41BBic or huCD200R-12aas-CD28Cystm-41BBic), wherein the intracellular component comprises the intracellular signaling domain of CD137 (4-1BB). For example, in one embodiment, the nucleic acid molecule has the nucleotide sequence as set forth in SEQ ID NO.:12 or SEQ ID NO.:14.

In another exemplary embodiment, the present disclosure provides a nucleic acid molecule that encodes a CD200R-CD28-4-1BB construct (huCD200R-9aas-CD28Cys tm ic 41BBic or huCD200R-12aas-CD28Cys tm ic-41BBic), wherein the intracellular component comprises the intracellular signaling domain of CD28 and of CD137 (4-1BB). In one embodiment, for example, the nucleic acid of the present disclosure has the nucleotide sequence as set forth in SEQ ID NO.:9 or SEQ ID NO.:15.

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a SIRPα-CD28 construct. For example, the present disclosure includes a nucleic acid molecule as set forth in SEQ ID NO.:16 (huSIRPαtm-CD28) or SEQ ID NO.:19 (huSIRPα-CD28tm).

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a SIRPα-CD28 construct, wherein the extracellular component comprises a truncated extracellular domain of SIRPα and an extracellular portion of CD28. For example, the SIRPα extracellular domain may be truncated by 12 amino acids (*e.g.*, huSIRPα-12aas-CD28Cys, SEQ ID NO.:20).

In one exemplary embodiment, the present disclosure provides a nucleic acid molecule that encodes a SIRPα-CD28-4-1BB construct (huSIRPα-12aas-CD28Cystm-41BBic), wherein the intracellular component comprises the intracellular signaling domain of CD137 (4-1BB). For example, in one embodiment, the nucleic acid of the present disclosure has the nucleotide sequence as set forth in SEQ ID NO.:22.

In another exemplary embodiment, the present disclosure provides a nucleic acid molecule that encodes a SIRPα-CD28-4-1BB construct (huSIRPα-12aas-CD28Cys tm ic-41BBic), wherein the intracellular component comprises the intracellular signaling domain of CD28 and of CD137 (4-1BB). In one embodiment, for example, the nucleic acid of the present disclosure has the nucleotide sequence as set forth in SEQ ID NO.:23.

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a PD-1-CD28 construct. For example, the present disclosure includes a nucleic acid molecule as set forth in SEQ ID NO.:97 (huPD1-CD28Cys).

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a PD-1-CD28 construct, wherein the extracellular component comprises a truncated extracellular domain of PD-1 and an extracellular portion of CD28. For example, the PD-1 extracellular domain may be truncated by 12 amino acids (*e.g.*, huPD1-12aas-CD28Cys, SEQ ID NO.:99), 15 amino acids (*e.g.*, huPD1-15aas-CD28Cys, SEQ ID NO.:101), or 21 amino acids (*e.g.*, huPD1-21aas-CD28Cys, SEQ ID NO.:103).

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a CD2-CD28 construct. For example, the present disclosure includes a nucleic acid molecule as set forth in SEQ ID NO.:69 (huCD2-CD28Cys).

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a Fas-CD28 construct. For example, the present disclosure includes a nucleic acid molecule as set forth in SEQ ID NO.:83 (huFas-CD28Cys).

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a Fas-CD28 construct, wherein the extracellular component comprises a truncated extracellular domain of Fas and an extracellular portion of CD28. For example, the Fas extracellular domain may be truncated by 7 amino acids (*e.g.*, huFas-7aas-CD28Cys, SEQ ID NO.:85) or 12 amino acids (*e.g.*, huFas-12aas-CD28Cys, SEQ ID NO.:87).

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a TIM3-CD28 construct. For example, the present disclosure includes a nucleic acid molecule as set forth in SEQ ID NO.:173 (huTIM3-CD28Cys). Also included within the scope of the disclosure is a TIM3-CD28 fusion protein, wherein the extracellular component comprises a truncated extracellular domain of TIM3 and an extracellular portion of CD28. For example, the TIM3 extracellular domain may be truncated by 12 amino acids (*e.g.*, huTIM3-12aas-CD28Cys, SEQ ID NO.:175).

In other exemplary embodiments, the present disclosure provides a nucleic acid molecule that encodes a LAG3-CD28 construct. For example, the present disclosure includes a nucleic acid molecule as set forth in SEQ ID NO.:163 (huLAG3-CD28Cys). Also included within the scope of the disclosure is a LAG3-CD28 fusion protein, wherein the extracellular component comprises a truncated extracellular domain of LAG3 and an extracellular portion of CD28. For example, the LAG3 extracellular domain may be truncated by 12 amino acids (*e.g.*, huLAG3-12aas-CD28Cys, SEQ ID NO.:159).

A vector that encodes a core virus is referred to herein as a "viral vector." There are a large number of available viral vectors suitable for use with the compositions of the instant disclosure, including those identified for human gene therapy applications (*see* Pfeifer and Verma, *Ann. Rev. Genomics Hum. Genet. 2*:177, 2001). Suitable viral vectors include vectors based on RNA viruses, such as retrovirus-derived vectors, *e.g.*,

Moloney murine leukemia virus (MLV)-derived vectors, and include more complex retrovirus-derived vectors, *e.g.*, lentivirus-derived vectors. HIV-1-derived vectors belong to this category. Other examples include lentivirus vectors derived from HIV-2, FIV, equine infectious anemia virus, SIV, and Maedi-Visna virus (ovine lentivirus). Methods of using retroviral and lentiviral viral vectors and packaging cells for transducing mammalian host cells with viral particles containing chimeric antigen receptor transgenes are known in the art and have been previous described, for example, in U.S. Patent 8,119,772; Walchli *et al.*, *PLoS One 6*:327930, 2011; Zhao *et al.*, *J. Immunol. 174*:4415, 2005; Engels *et al.*, *Hum. Gene Ther. 14*:1155, 2003; Frecha *et al.*, *Mol. Ther. 18*:1748, 2010; Verhoeyen *et al.*, *Methods Mol. Biol. 506*:97, 2009. Retroviral and lentiviral vector constructs and expression systems are also commercially available.

In certain embodiments, a viral vector is used to introduce a non-endogenous nucleic acid sequence encoding a fusion protein or a non-endogenous nucleic acid sequence encoding a fusion protein specific for a target. A viral vector may be a retroviral vector or a lentiviral vector. A viral vector may also include nucleic acid sequences encoding a marker for transduction. Transduction markers for viral vectors are known in the art and include selection markers, which may confer drug resistance, or detectable markers, such as fluorescent markers or cell surface proteins that can be detected by methods such as flow cytometry. In particular embodiments, a viral vector further comprises a gene marker for transduction comprising green fluorescent protein (GFP), an extracellular domain of human CD2, or a truncated human EGFR (huEGFRt; *see* Wang *et al.*, *Blood 118*:1255, 2011). When a viral vector genome comprises a plurality of nucleic acid sequences to be expressed in a host cell as separate transcripts, the viral vector may also comprise additional sequences between the two (or more) transcripts allowing bicistronic or multicistronic expression. Examples of such sequences used in viral vectors include internal ribosome entry sites (IRES), furin cleavage sites, viral 2A peptide, or any combination thereof.

Other vectors also can be used for polynucleotide delivery including DNA viral vectors, including, for example adenovirus-based vectors and adeno-associated virus

(AAV)-based vectors; vectors derived from herpes simplex viruses (HSVs), including amplicon vectors, replication-defective HSV and attenuated HSV (Krisky *et al.*, *Gene Ther. 5*: 1517, 1998).

Other vectors recently developed for gene therapy uses can also be used with the compositions and methods of this disclosure. Such vectors include those derived from baculoviruses and α-viruses (Jolly, D J. 1999. Emerging Viral Vectors. pp 209-40 in Friedmann T. ed. The Development of Human Gene Therapy. New York: Cold Spring Harbor Lab), or plasmid vectors (such as sleeping beauty or other transposon vectors). In some embodiments, a viral or plasmid vector further comprises a gene marker for transduction (*e.g.* green fluorescent protein, huEGFRt).

In some embodiments, a vector encoding a fusion protein as disclosed herein may encode more than one fusion protein. For example, a vector may encode two different fusion proteins (*e.g.*, a first fusion protein comprising a PD-1 ectodomain and a second fusion protein comprising a TIM3 ectodomain).

In some embodiments, a vector encoding a fusion protein as disclosed herein may further comprise an antigen-specific TCR. In some embodiments, the antigen-specific TCR is exogenous. In some embodiments, the antigen-specific TCR is specific to a HLA (MHC) class I restricted antigen. In some embodiments, the antigen is a cancer-specific antigen. Embodiments wherein the cancer-specific antigen comprises WT-1, mesothelin, or cyclin-A1 are also within the scope of the disclosure. In still other embodiments, a vector that encodes a fusion protein as disclosed herein further encodes a ligand, which may be CD200, CD47, PD-L1, or CD58. In yet further embodiments, a vector that encodes a fusion protein as disclosed herein further encodes an siRNA for reducing the expression of an endogenous receptor. In some particular embodiments, the endogenous receptor is CD200R, SIRPα, CD279 (PD-1), CD95 (Fas) or CD2.

In some embodiments, host cells capable of expressing a fusion protein of this disclosure on the cell surface are immune cells. In some embodiments, host cells capable of expressing a fusion protein of this disclosure on the cell surface are T cells, including primary cells or cell lines derived from human, mouse, rat, or other mammals.

If obtained from a mammal, a T cell can be obtained from numerous sources, including blood, bone marrow, lymph node, thymus, or other tissues or fluids. A T cell may be enriched or purified. T cell lines are well known in the art, some of which are described in Sandberg *et al.*, *Leukemia 21*:230, 2000. In certain embodiments, T cells that lack endogenous expression of TCRα and β chains are used. Such T cells may naturally lack endogenous expression of TCRα and β chains or may have been modified to block expression (*e.g.*, T cells from a transgenic mouse that does not express TCR α and β chains or cells that have been manipulated to inhibit expression of TCR α and β chains) or to knockout TCRα chain, TCRβ chain, or both genes. In some embodiments, T cells may be engineered to express a TCR specific to a particular antigen.

In certain embodiments, a host cell transfected to express a fusion protein of this disclosure is a functional T cell, such as a virus-specific T cell, a tumor antigen specific cytotoxic T cell, a naïve T cell, a memory stem T cell, a central or effector memory T cell, γδ T cells, or a CD4+ CD25+ regulatory T cell. In further embodiments, a nucleic acid molecule encoding a fusion protein of this disclosure is introduced into bulk CD8+ T cells, naïve CD8+ T cells, CD8+ T_{CM} cells, CD8+ T_{EM} cells, or any combination thereof. In still further embodiments, a nucleic acid molecule encoding a fusion protein of this disclosure is introduced into bulk CD4+ T cells, naïve CD4+ T cells, CD4+ T_{CM} cells, CD4+ T_{EM} cells, or any combination thereof. In other embodiments, a nucleic acid molecule encoding a fusion protein of this disclosure is introduced into a population of T cells enriched for naïve CD8+ T cells and CD8+ T_{CM} cells. In still other embodiments, a nucleic acid molecule encoding a fusion protein of this disclosure is introduced into a population of T cells enriched for naïve CD4+ T cells and CD4+ T_{CM} cells. In any of the aforementioned embodiments, the T cells further contain a nucleic acid molecule encoding an engineered antigen-specific T cell receptor (TCR), an engineered antigen-specific high affinity TCR, an exogenous co-stimulatory molecule, a chimeric antigen receptor (CAR), or any combination thereof.

In certain embodiments, a host cell transfected to express a fusion protein of this disclosure is a functional natural killer cell.

One or more growth factor cytokines that promote proliferation of T cells expressing a fusion protein of this disclosure may be added to the culture used to expand T cells. The cytokines may be human or non-human. Exemplary growth factor cytokines that may be used promote T cell proliferation include IL2, IL15, or the like.

In certain embodiments, a host T cell transfected to express a fusion protein of this disclosure is a $CD4^+$ T cell that also expresses an antigen-specific high-affinity TCR specific to a HLA (MHC) class I restricted antigen (see Soto *et al.*, *Cancer Immunol Immunother.* 62: 359–369, 2013).

In certain embodiments, a host T cell transfected to express a fusion protein of this disclosure also expresses a recombinant TCR specific to a cancer antigen. In some embodiments, the cancer antigen is a WT1. "WT1" refers to Wilm's tumor 1, a transcription factor that contains four zinc-finger motifs at the C-terminus and a proline/glutamine-rich DNA binding domain at the N-terminus. WT1 has an essential role in the normal development of the urogenital system and is mutated in a small subset of patients with Wilm's tumors. High expression of WT1 has been observed in various cancers, including, breast cancer, ovarian cancer, acute leukemias, vascular neoplasms, melanomas, colon cancer, lung cancer, thyroid cancer, bone and soft tissue sarcoma, and esophageal cancer. Alternative splicing has been noted for WT1.

In certain embodiments, a host T cell transfected to express a fusion protein of this disclosure also expresses a recombinant TCR specific to mesothelin. "Mesothelin" (MSLN) refers to a gene that encodes a precursor protein that is cleaved into two products, megakaryocyte potentiating factor and mesothelin. Megakaryocyte potentiation factor functions as a cytokine that can stimulate colony formation in bone marrow megakaryocytes. Mesothelin is a glycosylphosphatidylinositol-anchored cell-surface protein that may function as a cell adhesion protein. This protein is overexpressed in epithelial mesotheliomas, ovarian cancers and in specific squamous cell carcinomas. Alternative splicing results in multiple transcript variants.

In certain embodiments, a host T cell transfected to express a fusion protein of this disclosure also expresses a recombinant TCR specific to cyclin-A1.

In certain embodiments, a host T cell transfected to express a fusion protein of this disclosure also expresses a CAR.

In still other embodiments, a host cell that expresses a fusion protein as disclosed herein further comprises a ligand, which may be CD200, CD47, PD-L1, or CD58. In yet further embodiments, a host cell that expresses a fusion protein as disclosed herein further expresses an siRNA for reducing the expression of an endogenous receptor. In some particular embodiments, the endogenous receptor is CD200R, SIRPα, CD279 (PD-1), CD95 (Fas), or CD2.

In some embodiments, a host cell that expresses a fusion protein as disclosed herein may express more than one fusion protein. For example, the host cell may express two different fusion proteins (*e.g.*, a first fusion protein comprising a PD-1 ectodomain and a second fusion protein comprising a TIM3 ectodomain).

Uses

Diseases that may be treated with cells expressing fusion proteins as described in the present disclosure include cancer, infectious diseases (viral, bacterial, protozoan infections), immune diseases (*e.g.*, autoimmune), or aging-related diseases (*e.g.*, senescence). Adoptive immune and gene therapy are promising treatments for various types of cancer (Morgan *et al.*, *Science 314*:126, 2006; Schmitt *et al.*, *Hum. Gene Ther. 20*:1240, 2009; June, *J. Clin. Invest. 117*:1466, 2007) and infectious disease (Kitchen *et al.*, *PLoS One 4*:38208, 2009; Rossi *et al.*, *Nat. Biotechnol. 25*:1444, 2007; Zhang *et al.*, *PLoS Pathog. 6*:e1001018, 2010; Luo *et al.*, *J. Mol. Med. 89*:903, 2011).

A wide variety of cancers, including solid tumors and leukemias are amenable to the compositions and methods disclosed herein. Exemplary types of cancer that may be treated include adenocarcinoma of the breast, prostate, and colon; all forms of bronchogenic carcinoma of the lung; myeloid leukemia; melanoma; hepatoma; neuroblastoma; papilloma; apudoma; choristoma; branchioma; malignant carcinoid syndrome; carcinoid heart disease; and carcinoma (*e.g.*, Walker, basal cell, basosquamous, Brown-Pearce, ductal, Ehrlich tumor, Krebs 2, Merkel cell, mucinous, non-small cell lung, oat cell, papillary, scirrhous, bronchiolar, bronchogenic, squamous cell, and transitional cell). Additional types of cancers that may be treated include

histiocytic disorders; malignant histiocytosis; leukemia; Hodgkin's disease; immunoproliferative small; non-Hodgkin's lymphoma; plasmacytoma; reticuloendotheliosis; melanoma; chondroblastoma; chondroma; chondrosarcoma; fibroma; fibrosarcoma; giant cell tumors; histiocytoma; lipoma; liposarcoma; mesothelioma; myxoma; myxosarcoma; osteoma; osteosarcoma; chordoma; craniopharyngioma; dysgerminoma; hamartoma; mesenchymoma; mesonephroma; myosarcoma; ameloblastoma; cementoma; odontoma; teratoma; thymoma; trophoblastic tumor. Further, the following types of cancers are also contemplated as amenable to treatment: adenoma; cholangioma; cholesteatoma; cyclindroma; cystadenocarcinoma; cystadenoma; granulosa cell tumor; gynandroblastoma; hepatoma; hidradenoma; islet cell tumor; Leydig cell tumor; papilloma; sertoli cell tumor; theca cell tumor; leimyoma; leiomyosarcoma; myoblastoma; myomma; myosarcoma; rhabdomyoma; rhabdomyosarcoma; ependymoma; ganglioneuroma; glioma; medulloblastoma; meningioma; neurilemmoma; neuroblastoma; neuroepithelioma; neurofibroma; neuroma; paraganglioma; paraganglioma nonchromaffin. The types of cancers that may be treated also include angiokeratoma; angiolymphoid hyperplasia with eosinophilia; angioma sclerosing; angiomatosis; glomangioma; hemangioendothelioma; hemangioma; hemangiopericytoma; hemangiosarcoma; lymphangioma; lymphangiomyoma; lymphangiosarcoma; pinealoma; carcinosarcoma; chondrosarcoma; cystosarcoma phyllodes; fibrosarcoma; hemangiosarcoma; leiomyosarcoma; leukosarcoma; liposarcoma; lymphangiosarcoma; myosarcoma; myxosarcoma; ovarian carcinoma; rhabdomyosarcoma; sarcoma; neoplasms; nerofibromatosis; and cervical dysplasia.

Exemplifying the variety of hyperproliferative disorders amenable to a fusion protein T cell therapy are B-cell cancers, including B-cell lymphomas (such as various forms of Hodgkin's disease, non-Hodgkins lymphoma (NHL) or central nervous system lymphomas), leukemias (such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), Hairy cell leukemia, B cell blast transformation of chronic myeloid leukemia) and myelomas (such as multiple myeloma). Additional B cell cancers include small lymphocytic lymphoma, B-cell prolymphocytic leukemia,

lymphoplasmacytic lymphoma, splenic marginal zone lymphoma, plasma cell myeloma, solitary plasmacytoma of bone, extraosseous plasmacytoma, extra-nodal marginal zone B-cell lymphoma of mucosa-associated (MALT) lymphoid tissue, nodal marginal zone B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, diffuse large B-cell lymphoma, mediastinal (thymic) large B-cell lymphoma, intravascular large B-cell lymphoma, primary effusion lymphoma, Burkitt's lymphoma/leukemia, B-cell proliferations of uncertain malignant potential, lymphomatoid granulomatosis, and post-transplant lymphoproliferative disorder.

Inflammatory and autoimmune diseases include arthritis, rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, polychondritis, psoriatic arthritis, psoriasis, dermatitis, polymyositis/dermatomyositis, inclusion body myositis, inflammatory myositis, toxic epidermal necrolysis, systemic scleroderma and sclerosis, CREST syndrome, inflammatory bowel disease, Crohn's disease, ulcerative colitis, respiratory distress syndrome, adult respiratory distress syndrome (ARDS), meningitis, encephalitis, uveitis, colitis, glomerulonephritis, allergic conditions, eczema, asthma, conditions involving infiltration of T cells and chronic inflammatory responses, atherosclerosis, autoimmune myocarditis, leukocyte adhesion deficiency, systemic lupus erythematosus (SLE), subacute cutaneous lupus erythematosus, discoid lupus, lupus myelitis, lupus cerebritis, juvenile onset diabetes, multiple sclerosis, allergic encephalomyelitis, neuromyelitis optica, rheumatic fever, Sydenham's chorea, immune responses associated with acute and delayed hypersensitivity mediated by cytokines and T-lymphocytes, tuberculosis, sarcoidosis, granulomatosis including Wegener's granulomatosis and Churg-Strauss disease, agranulocytosis, vasculitis (including hypersensitivity vasculitis/angiitis, ANCA and rheumatoid vasculitis), aplastic anemia, Diamond Blackfan anemia, immune hemolytic anemia including autoimmune hemolytic anemia (AIHA), pernicious anemia, pure red cell aplasia (PRCA), Factor VIII deficiency, hemophilia A, autoimmune neutropenia, pancytopenia, leukopenia, diseases involving leukocyte diapedesis, central nervous system (CNS) inflammatory disorders, multiple organ injury syndrome, myasthenia gravis, antigen-antibody complex mediated diseases, anti-glomerular basement membrane disease, anti-

phospholipid antibody syndrome, allergic neuritis, Behcet disease, Castleman's syndrome, Goodpasture's syndrome, Lambert-Eaton Myasthenic Syndrome, Reynaud's syndrome, Sjorgen's syndrome, Stevens-Johnson syndrome, solid organ transplant rejection, graft versus host disease (GVHD), bullous pemphigoid, pemphigus, autoimmune polyendocrinopathies, seronegative spondyloarthropathies, Reiter's disease, stiff-man syndrome, giant cell arteritis, immune complex nephritis, IgA nephropathy, IgM polyneuropathies or IgM mediated neuropathy, idiopathic thrombocytopenic purpura (ITP), thrombotic throbocytopenic purpura (TTP), Henoch-Schonlein purpura, autoimmune thrombocytopenia, autoimmune disease of the testis and ovary including autoimmune orchitis and oophoritis, primary hypothyroidism; autoimmune endocrine diseases including autoimmune thyroiditis, chronic thyroiditis (Hashimoto's Thyroiditis), subacute thyroiditis, idiopathic hypothyroidism, Addison's disease, Grave's disease, autoimmune polyglandular syndromes (or polyglandular endocrinopathy syndromes), Type I diabetes also referred to as insulin-dependent diabetes mellitus (IDDM) and Sheehan's syndrome; autoimmune hepatitis, lymphoid interstitial pneumonitis (HIV), bronchiolitis obliterans (non-transplant), non-specific interstitial pneumonia (NSIP), Guillain-Barré Syndrome, large vessel vasculitis (including polymyalgia rheumatica and giant cell (Takayasu's) arteritis), medium vessel vasculitis (including Kawasaki's disease and polyarteritis nodosa), polyarteritis nodosa (PAN) ankylosing spondylitis, Berger's disease (IgA nephropathy), rapidly progressive glomerulonephritis, primary biliary cirrhosis, Celiac sprue (gluten enteropathy), cryoglobulinemia, cryoglobulinemia associated with hepatitis, amyotrophic lateral sclerosis (ALS), coronary artery disease, familial Mediterranean fever, microscopic polyangiitis, Cogan's syndrome, Whiskott-Aldrich syndrome and thromboangiitis obliterans.

In particular embodiments, a method of treating a subject with the fusion protein as disclosed herein include acute myelocytic leukemia, acute lymphocytic leukemia, and chronic myelocytic leukemia.

Infectious diseases include those associated with infectious agents and include any of a variety of bacteria (*e.g.*, pathogenic *E. coli, S. typhimurium, P. aeruginosa, B.*

anthracis, C. botulinum, C. difficile, C. perfringens, H. pylori, V. cholerae, Listeria spp., Rickettsia spp., Chlamydia spp., and the like), mycobacteria, and parasites (including any known parasitic member of the Protozoa). Infectious viruses include eukaryotic viruses, such as adenovirus, bunyavirus, herpesvirus, papovavirus, papillomavirus (*e.g.*, HPV), paramyxovirus, picornavirus, rhabdovirus (*e.g.*, Rabies), orthomyxovirus (*e.g.*, influenza), poxvirus (*e.g.*, Vaccinia), reovirus, retrovirus, lentivirus (*e.g.*, HIV), flavivirus (*e.g.*, HCV, HBV) or the like. In certain embodiments, infection with cytosolic pathogens whose antigens are processed and displayed with HLA (MHC) Class I molecules, are treated with fusion proteins of this disclosure.

A fusion protein of this disclosure may be administered to a subject in cell-bound form (*e.g.*, gene therapy of target cell population (mature T cells (*e.g.*, $CD8^+$ or $CD4^+$ T cells) or other cells of T cell lineage)). In a particular embodiment, cells of T cell lineage expressing fusion proteins administered to a subject are syngeneic, allogeneic, or autologous cells.

Pharmaceutical compositions including fusion proteins of this disclosure may be administered in a manner appropriate to the disease or condition to be treated (or prevented) as determined by persons skilled in the medical art. An appropriate dose, suitable duration, and frequency of administration of the compositions will be determined by such factors as the condition of the patient, size, type and severity of the disease, particular form of the active ingredient, and the method of administration. The present disclosure provides pharmaceutical compositions comprising cells expressing a fusion protein as disclosed herein and a pharmaceutically acceptable carrier, diluents, or excipient. Suitable excipients include water, saline, dextrose, glycerol, or the like and combinations thereof.

In some embodiments, the disclosure is directed to a method of increasing the activity of an immune cell, enhancing or prolonging an immune response, stimulating an antigen-specific T cell response, inhibiting an immunosuppressive signaling pathway, treating cancer or a tumor, inhibiting immune resistance of cancer cells, or treating an infection, comprising administering to a subject in need thereof an effective amount of a host cell expressing a fusion protein as described herein. In further

embodiments, a host cell for use in any of the aforementioned methods further expresses an engineered antigen-specific TCR, an engineered antigen-specific high affinity TCR, a CAR, a co-stimulatory molecule, or any combination thereof. In particular embodiments, methods of treating leukemia are provided, comprising co-expressing a fusion protein as disclosed herein and a recombinant, antigen-specific TCR.

In some embodiments, there are provided methods of inducing or enhancing a Class I HLA response by a CD4+ T cell, comprising administering to a subject in need thereof an effective amount of a CD4+ T cell expressing a fusion protein as described herein. In further embodiments, a host cell for use in inducing or enhancing a Class I HLA response by a CD4+ T cell further expresses an engineered antigen-specific TCR, an engineered antigen-specific high affinity TCR, a CAR, a co-stimulatory molecule, or any combination thereof.

In any of the aforementioned embodiments, the methods are effective in the absence of administering exogenous IL-2.

In still other embodiments, a subject of any of the aforementioned methods is further treated with an adjunctive therapy, such as a chemotherapy. Exemplary chemotherapeutic agents include, for example, alkylating agents such as thiotepa and cyclosphosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramide, triethylenethiophosphaoramide and trimethylolomelamine; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, ranimustine; antibiotics such as aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, calicheamicin, carabicin, caminomycin, carzinophilin, chromomycins, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin, epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins,

mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine, 5-FU; androgens such as calusterone, dromostanolone propionate, epitiostanol, mepitiostane, testolactone; anti-adrenals such as aminoglutethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidamine; mitoguazone; mitoxantrone; mopidamol; nitracrine; pentostatin; phenamet; pirarubicin; podophyllinic acid; 2-ethylhydrazide; procarbazine; PSKTM; razoxane; sizofiran; spirogermanium; tenuazonic acid; triaziquone; 2, 2′,2″-trichlorotriethylamine; urethan; vindesine; dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); cyclophosphamide; thiotepa; taxanes, e.g. paclitaxel (Taxol™, Bristol-Myers Squibb Oncology, Princeton, N.J.) and docetaxel (Taxotere™, Rhone-Poulenc Rorer, Antony, France); chlorambucil; gemcitabine; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine; platinum; etoposide (VP-16); ifosfamide; mitomycin C; mitoxantrone; vincristine; vinorelbine; navelbine; novantrone; teniposide; daunomycin; aminopterin; xeloda; ibandronate; CPT-11; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoic acid; esperamicins, capecitabine; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

In some embodiments, the adjunctive therapy is a vaccine, an inhibitor of an immunosuppression signal, a B-Raf inhibitor, a MEK inhibitor, a tyrosine kinase inhibitor, a cytotoxic agent, a chemotherapeutic, or any combination thereof. In some embodiments, the inhibitor of an immunosuppression signal is an antibody or siRNA. In some embodiments, the antibody or siRNA is specific for PD-1, PD-L1, PD-L2,

CTLA4, LAG3, KIR, CD244, B7-H3, B7-H4, BTLA, HVEM, GAL9, TIM3, A2aR, or any combination thereof.

EXAMPLES

EXAMPLE 1

CD200R-CD28 FUSION PROTEIN CONSTRUCTIONS

Exemplary fusion proteins as described herein are illustrated using schematic representations in Figure 1A. Exemplary fusion proteins include immunomodulatory fusion proteins (IFPs) comprised of the extracellular domain of CD200R or a portion thereof, and an intracellular signaling domain of CD28 or a portion thereof (Figure 1A, constructs I-V). The hydrophobic component may be comprised of the transmembrane domain of either CD200R (Figure 1A, construct I) or CD28 (Figure 1A, constructs II-V), or portions thereof. In some exemplary CD200R-CD28 fusion proteins, the hydrophobic component comprises the transmembrane domain of CD28 and the extracellular component further comprises an extracellular portion of CD28, particularly an extracellular cysteine residue adjacent to the hydrophobic component (*e.g.*, Figure 1A construct III, CD200R-CD28Cys; construct IV, CD200R-3aas-CD28Cys; and construct V, CD200R-9aas-CD28Cys). The extracellular component may comprise all or a portion of the extracellular domain of CD200R. In some embodiments, the extracellular component comprises the entire extracellular domain of CD200R (Figure 1A, constructs I-III). In other examples, the extracellular component comprises the first 235 amino acids (preserving an N-linked glycosylation site) (*e.g.*, Figure 1A, construct IV, CD200R-3aas-CD28Cys) or the first 229 amino acids (*e.g.*, Figure 1A, construct V, CD200R-9aas-CD28Cys) from the N-terminus of CD200R. The size of the extracellular component, which may be manipulated by adjusting the fusion protein construct, may affect the ability of the fusion protein to enter the immunological synapse and co-localize with the TCR within the cSMAC to deliver a strong co-stimulatory signal. Additionally, the CD200R-CD28 construct has the

capacity to convert what would typically be an inhibitory signal from the binding of CD200R to its target into a positive signal generated by the CD28 intracellular signaling domain.

An exemplary nucleic acid molecule encoding a CD200R-CD28 fusion protein comprises the following elements (5' to 3'): Extracellular Component (CD200R)-Multimerization Domain (CD28 Cysteine)-Hydrophobic Component (CD28 transmembrane)-Intracellular Component (CD28 intracellular). In some embodiments, a nucleic acid molecule encoding a CD200R-CD28 fusion protein comprises a nucleic acid molecule as set forth in any one of SEQ ID NOS.:47-51 or 1, 6, 7, 10, 12, 14, or 15.

Nucleic acids encoding the constructs were ordered from Invitrogen or generated in-house by PCR then directionally TOPO-cloned into the pENTR™/D-TOPO® vector (Invitrogen), and transferred into the retroviral vector pMP71-attR using Gateway® technology (Invitrogen). In certain embodiments, the nucleic acid molecules encoding IFPs of the instant disclosure were codon optimized before cloning into the pMP71-attR retroviral vector.

EXAMPLE 2

TRANSGENIC EXPRESSION OF CD200R-CD28 CONSTRUCTS

A preclinical mouse model for disseminated leukemia, based on the murine C57BL/6 Friend virus-induced erythroleukemia (FBL) and TCR_{gag} transgenic mice, was used to determine if CD200R-CD28 chimeric receptors can improve T cell function.

TCR transgenic mice were generated to produce $CD8^+$ T cells specific for the gag epitope (TCR_{gag}). C57BL/6 (B6) mice were purchased from the Jackson Laboratory. TCR_{gag} transgenic mice express a TCR transgene specific for the Friend virus gag epitope in $CD8^+$ T cell (Öhlén *et al.*, *J. Immunol. 166*: 2863-2870, 2001). All animal studies performed were approved under the University of Washington Institutional Animal Care and Use Committee protocol (Protocol # 2013-01). The

murine B6 Friend virus induced erythroleukemia (FBL) expresses the F-MuLV encoded gag epitope (peptide CCLCLTVFL).

CD200R-CD28 chimeric constructs based on murine genes were inserted into the pMP71 retroviral vector and used to transduce primary mouse splenocytes stimulated with anti-CD3 and anti-CD28 antibodies. Constructs were designed as described in Example 1, and ordered from Invitrogen or generated in-house by PCR. The constructs were then directionally TOPO-cloned into the pENTR™/D-TOPO® vector (Invitrogen), and transferred into the retroviral vector pMP71-attR using Gateway® technology (Invitrogen). The retroviral packaging cell line Plat-E (Morita *et al.*, 2000, *Gene Therapy* 7:1063-1066, 2000; Cell Biolabs, Inc.) was transduced with the retroviral vector using effectene transduction reagent (Qiagen). Viral supernatant was collected on days 2 and 3 and then used to transduce TCR_{gag} T cells.

One day prior to the transfection, TCR_{gag} T cells were stimulated with anti-CD3/CD28 and 100 U/mL rhIL-2. Transduction of TCR_{gag} T cells was performed in 12 well plates in the presence of IL-2 and polybrene by spinfection for 90 minutes at 1000*g*. FBL cells were transduced with CD200 with polybrene spinfection, similar to T cell transduction, and subsequently sorted to generate a homogenous population.

Five days after transduction, $CD8^+$ T cells were analyzed for construct expression by anti-CD200R antibody staining and flow cytometry (Figure 1B). A vector encoding green fluorescent protein (GFP) was used as a control. Transduction efficiency ranged from 4-36% and the mean fluorescent intensity (MFI) of the transduced cells was similar between constructs.

EXAMPLE 3

CD200R-CD28 CONSTRUCTS PROMOTE *IN VITRO* PROLIFERATION, ACCUMULATION, AND EFFECTOR FUNCTION OF TRANSDUCED T CELLS

The CD200R-CD28 constructs described in Examples 1 and 2 were assessed for their abilities to promote proliferation, accumulation, and effector function of TCR_{gag} T cells.

Expansion of effector cells in vitro

TCR_{gag} effector cells were generated *in vitro* as previously described (Stromnes *et al.*, *J. Clin. Invest. 120*: 3722-34, 2010). Irradiated antigen presenting splenocytes ($5x10^6$), irradiated FBL ($3x10^6$), and TCR_{gag} *tg* cells (10^6) were cultured together with IL-2 (50 U/mL) in 10 mL of culture media (IMDM supplemented with non-essential amino acids, 2 μM glutamine, 100 U/mL penicillin/streptomycin, 10% FBS, and 50 μM 2-mercapatoethanol). T cells were restimulated weekly and assessed by flow cytometry 5-7 days after the last stimulation.

In vitro *T cell proliferation assay*

TCR_{gag} T cells were transduced as in Example 2. To assess T cell proliferation *in vitro*, TCR_{gag} T cells were stained with CellTrace Violet (CTV, Life Technologies) according to the manufacturer's protocol. CTV-labeled Tg T cells (10^5) and GFP control T cells were stimulated with titrating numbers of $CD200^-$ FBL or $CD200^+$ FBL cells. After 3 days, CTV dilution of TCR_{gag} T cells was assessed by flow cytometry.

Flow cytometry results indicating the number of TCR_{gag} T cells after stimulation with titrating numbers of CD200- FBL cells (upper) or $CD200^+$ FBL (lower) are shown in Figure 2A. Four of the five CD200R-CD28 constructs tested dramatically improved proliferation of TCR_{gag} T cells in response to $CD200^+$ FBL (blue lines) compared to GFP control-transduced T cells (red lines).

In vitro *T cell accumulation assay*

To determine if the enhanced proliferation also resulted in increased accumulation of transduced cells, the proportion of transduced cells in the total TCR_{gag} population over multiple cycles of stimulation with irradiated $CD200^+$ FBL was measured.

Several of the constructs promoted accumulation of transduced T cells, including CD200R-CD28tm, CD200R-CD28Cys, CD200R-3aas-CD28Cys, and CD200R-9aas-CD28Cys (Figure 2B). Of these constructs, CD200R-9aas-CD28Cys exhibited the greatest increase in transduced T cells over multiple stimulations, resulting in more than a 3-fold expansion over 3 stimulations.

In vitro *T cell enrichment assay*

A mixed population of transduced and nontransduced $CD8^+$ T cells were restimulated with $CD200^+$ or $CD200^-$ irradiated FBL cells to determine if restimulation would enrich the population for the transduced CD200R-9aas-CD28Cys IFP^+ T cells. Repeated restimulation with irradiated $CD200^+$ tumor cells enriched the cells transduced with the IFP compared to wild type T cells, demonstrating that recognition of a target expressing the ligand for the CD200R-9aas-CD28Cys IRP enhances the response (Figure 2C).

In vitro *colocalization assay*

Transduced T cells were imaged by microscopy to determine if the CD200R-9aas-CD28Cys IFP colocalized with the cognate ligand in the immunological synapse (IS) during T cell activation. CTxB was used to stain lipids within the cell membrane, which are enriched at the synapse (Figure 2D, panel I). Labeled antibodies that target CD200 expressed by the FBL cell (Figure 2D, panel II) or CD200R expressed by the T cell (Figure 2D, panel III) were used to visualize the location of the molecules in relation to the IS. CD200 ligand and CD200R colocalized within the IS (Figure 2D, panel IV), demonstrating that the construct is sized appropriately to be accommodated by the immunologic synapse.

CFSE-based cytotoxicity assay

Increased CD28 signaling also promotes effector function (Chen and Flies, *Nat. Rev. Immunol. 13*: 227-242, 2013). CD200R-CD28 fusion protein-transduced T cells were tested for increased killing of target tumor cells. FBL and control EL4 tumors were incubated for 10 minutes at room temperature with 2.5 μM ($CFSE_{hi}$) or 0.25 μM ($CFSE^{lo}$) CFSE in PBS, respectively. Excess dye was removed by washing tumor cells in serum-containing media. A 1:1 mixture of EL4 and FBL tumor cells was incubated with titrated numbers of CD200R-CD28 or GFP vector transduced TCR_{gag} *in vitro* expanded effector T cells for 4 hours in 96-well, round-bottom plates at 37°C and 5% CO_2. Specific FBL lysis was determined by flow cytometric analyses of the % $CFSE_{hi}$ (FBL) of total CFSE positive cells (FBL+ EL4) remaining in the well.

TCR_{gag} T cells transduced with CD200R-CD28 constructs displayed an enhanced ability to lyse FBL tumor *in vitro* compared to TCR_{gag} T cells transduced with

an empty vector (Figures 2E, 2G). Target tumor cells were labeled with different dilutions of the fluorescent dyes CellTrace Violet (CTV) or CFSE to generate a 1:1:1 mix of EL4 cells (CTV+), $CD200^+$ FBL ($CFSE^{hi}$) and non-specific EL4 ($CFSE^{lo}$) control targets (Figure 2F). Additionally, control GFP-transduced TCR_{gag} T cells lysed $CD200^-$ FBL and $CD200^+$ FBL at equal efficiencies (Figure 2G). By contrast, TCR_{gag} T cells transduced with CD200R-9aas-CD28Cys exhibited increased killing of $CD200^+$ FBL cells compared to control T cells, lysing over 40% of $CD200^+$ FBL at the lowest E:T ratio tested (Figure 2G).

Taken together, these data show that CD200R-CD28 constructs function to increase accumulation and the lytic activity of transduced T cells in response to tumor cell stimulation.

EXAMPLE 4

T CELLS TRANSDUCED WITH CD200R-9AAS-CD28CYS EXHIBIT ENHANCED ACCUMULATION *IN VIVO* IN RESPONSE TO RECOGNITION OF FBL

B6 mice were injected with 4×10^6 live FBL leukemia intraperitoneal (i.p.) as previously described (Stromnes *et al.*, *J. Clin. Invest. 120*: 3722-34, 2010). After allowing 5 days for the FBL to disseminate, mice received 180 mg/kg cyclophosphamide (Cy, "Cytoxan") i.p. at least 6 hours before transfer of the effector T cells. For survival studies, 10^5 TCR_{gag} T cells which previously underwent 1-3 stimulations *in vitro* were transferred into tumor-bearing mice. To assess short-term proliferation and accumulation, $2x10^6$ of each of fusion protein-transduced and a GFP-control-transduced T cells were co-injected into tumor-bearing mice and the mice euthanized for analysis 8 days later. Mice were regularly monitored for tumor burden and euthanized if evidence of tumor progression predicted mortality would occur within 24-48 hours.

To assess whether CD200R-9aas-CD28Cys fusion protein-transduced T cells exhibited greater proliferation and accumulation *in vivo* in response to recognition of FBL, a mixed population of fusion protein-transduced and control cells were transferred into tumor-bearing mice and the ratio of cells by *ex vivo* analysis were compared 8 days

after transfer (Figure 3A). By use of congenic markers, transduced T cells were detected at a 1.2-1.4-fold greater ratio over control cells in both the spleen and lymph nodes relative to the ratio that was injected (Figure 3B). Transduced CD200R-9aas-CD28Cys$^+$ TCR_{gag} T cells exhibited reduced CD62L expression 3 days post-transfer to tumor-bearing mice, suggesting an effector T cell phenotype (Figure 3C). By day 15, transduced and control T cells exhibited similar phenotypes, including a lack of exhaustion markers (Figure 3D). Similar to the *in vitro* findings, T cells that expressed CD200R-9aas-CD28Cys displayed increased accumulation in response to tumor stimulation *in vivo*. Furthermore, they exhibited protein expression patterns consistent with an effector T cell phenotype for at least 3 days following transfer to tumor-bearing mice.

EXAMPLE 5

ADOPTIVE IMMUNOTHERAPY WITH CD200R-CD28$^+$ T CELLS EXHIBITS GREATER ACTIVITY IN THERAPY OF DISSEMINATED LEUKEMIA

Adoptive immunotherapy with T cells transduced with CD200R-CD28 mediated increased therapeutic activity in the preclinical mouse model of disseminated leukemia.

Mice were injected with a lethal dose of CD200$^+$ FBL leukemia and five days later, cohorts of Cy-treated mice received additional therapy with 10^5 T cells (Figure 4A). The contribution of the CD28 cysteine bond to efficacy mediated by the CD200R-CD28 construct was assessed by comparing T cells transduced with CD200R-CD28tm, CD200R-9aas-CD28Cys, and GFP control constructs as shown in Figure 1A. IL-2 was administered for 10 days as an additional therapeutic reagent to a cohort of mice to promote the activity of the T cells (Stromnes *et al.*, *J. Clin. Invest. 120*: 3722-34, 2010). Before injection, T cells were assessed for various surface proteins by flow cytometry. Transduced and control TCR_{gag} T cells displayed similar phenotypes, indicating that transduction did not alter the phenotype of the cells prior to injection (Figure 4B).

In the small cohort of mice that received IL-2 injections, T cells improved survival but a significant difference in the survival of mice that received the different groups of T cells could not be detected (Figure 4C). However, in the cohort of mice

that did not receive IL-2 injections, there was a significant improvement in the survival of mice that received T cells transduced with CD200R-CD28 constructs appropriately sized to fit within the immunological synapse (Figure 4D). The majority of the mice not receiving T cells, receiving T cells transduced with the GFP control vector or T cells transduced with the largest ectodomain (CD200R-CD28Cys IFP) did not survive beyond day 30 (Figures 4C and 4D, black solid, dashed, and orange lines, respectively). In contrast, 71% of mice that received CD200R-CD28tm$^+$ T cells and 83% of mice that received CD200R-9aas-CD28Cys$^+$ T cells survived more than 100 days post-therapy (Figures 4C and 4D, green and red lines, respectively). These data suggest that transduction of T cells with CD200R-CD28 constructs that span a distance similar to a distance between membranes in an immunological synapse provides sufficient costimulation to overcome the dependence of T cell immunotherapy on injection of exogenous IL-2. Furthermore, although there were differences in proliferation and accumulation between the CD200Rtm-CD28 and CD200R-9aas-CD28Cys constructs tested in mice that did not receive injections of exogenous IL-2, both IFPs effectively enhanced T cell immunotherapy to significantly improve the clinical outcome from otherwise progressive leukemia.

EXAMPLE 6

CD200R-9AAS-CD28CYS$^+$ T CELLS DO NOT CAUSE AUTOREACTIVITY WITH ENDOGENOUS TISSUES AND DO NOT EXHIBIT INFILTRATION OF NORMAL TISSUES *IN VIVO*

To determine if transduction of TCR_{gag} T cells lowered the threshold of activation sufficiently to result in autoreactivity with endogenous tissues, autoimmune toxicity was assessed in transgenic mice engineered to express the FBL gag tumor Ag as a self-antigen in hepatocytes, under control of the albumin promoter (Figure 5A). TCR_{gag} effectors were generated *in vitro* and 10^6 were transferred into Cytoxan-treated Alb:Gag mice with disseminated leukemia. At 3 and 7 days post-transfer, liver damage was assessed by quantification of serum levels of the liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Adoptive therapy with

control or CD200R-9aas-CD28Cys^{+} TCR$_{gag}$ cells in mice did not affect serum levels of AST or ALT at days 3 or 7 post-transfer, indicating that CD200R-9aas-CD28Cys does not induce detectable autoimmune liver damage in Alb:Gag mice (Figure 5B).

T cells transduced with IFP do not exhibit increased infiltration of normal tissues compared to control T cells. Mice were euthanized 7 days post-transfer and liver sections were stained with an antibody to the T cell marker CD3 to quantify T cell infiltration. Limited presence of T cells in liver tissue was observed, with no significant difference between recipients of CD200R-9aas-CD28Cys^{+} or control TCR$_{gag}$, indicating no increased lymphocytic cellular infiltration as a result of IFP expression (Figure 5C).

EXAMPLE 7

4-1BB CO-STIMULATORY SIGNALING DOMAIN PROMOTES ACCUMULATION OF TRANSDUCED T CELLS *IN VITRO*

Co-stimulatory receptor 4-1BB is upregulated on activated T cells, which promotes T cell survival and cytokine production (Chen and Flies, *Nat. Rev. Immunol.* *13*: 227-242, 2013). To assess if the intracellular signaling domain of 4-1BB, with or without the intracellular signaling domain of CD28, could induce increased T cell proliferation and accumulation, IFPs using 4-1BB (CD200R-9aas-4-1BB) or combining 4-1BB with CD28 (CD200R-9aas-CD28-4-1BB) were generated (Figure 6A) using the methods described in Example 2. TCR$_{gag}$ T cells were transduced as in Example 2, and TCR$_{gag}$ effector cells were generated *in vitro* as in Example 3.

As was observed with CD200R-9aas-CD28Cys, T cells transduced with the 4-1BB constructs accumulated over multiple rounds of stimulation *in vitro* (Figure 6B). These data indicate that 4-1BB IFPs also promote proliferation and survival of T cells.

TCR$_{gag}$ T cells transduced with a CD200R-4-1BB displayed an enhanced ability to lyse FBL tumor *in vitro* using the CFSE-based cytotoxicity assay described in Example 3 (Figure 6C). CD200R-41BB-transduced T cells also promote survival (Figure 6D).

EXAMPLE 8

CO-EXPRESSION OF CD200RTM-CD28 ENHANCES FUNCTION IN WT1-SPECIFIC TCR PRIMARY T CELLS

A human CD200Rtm-CD28 construct (SEQ ID NO.:1) was generated to determine if IFP expression enhanced T cell function of human primary T cells. The construct was combined with the beta and alpha chains of the HLA-A2-restricted $WT1_{126}$-specific TCR "C4" by linking the genes with P2A elements (Figure 7A). The first P2A sequence was codon optimized to prevent genetic recombination with the second P2A sequence. To generate lentiviruses, 293 T/17 cells ($3x10^6$ cells/plate) were transduced with human constructs in the pRRLSIN and the packaging vectors pMDLg/pRRE, pMD2-G, and pRSV-REV using Effectene (Qiagen). Culture media was changed on day 1 post-transfection and virus-containing supernatant collected on days 2 and 3, and aliquots frozen for future use.

The Jurkat human T cell subline, which lacks an endogenous TCR, was used to test expression of the IFP and TCRs. These Jurkat T cells were transduced by spinfection of $2x10^6$ cells with 2 ml of retroviral supernatant at 1000 g for 90 min at 32° C. Transduction of the Jurkat human T cell line with the three-gene construct resulted in high expression of the IFP and expression of the TCR at a similar MFI as T cells transduced with the TCR only (Figure 7A).

To transduce primary human T cells, peripheral blood mononuclear cells (PBMC) were harvested from HLA-A2+ donors. $CD8^+$ T cells were purified using Miltenyi magnetic beads and stimulated with Human T cell Expander CD3/CD28 Dynabeads (Life Technologies) and 50 IU/ml IL-2. Four hours following stimulation, T cells were transduced as described above for Jurkat T cells. T cells were restimulated every 10-14 days with a rapid expansion protocol (REP), as has been previously described (Ho et al., *J Immunol Methods 310*:40-52, 2006).

The human cell line T2 was used as an APC, because it is deficient in TAP and thus cannot present endogenous peptides, while low level MHCI expression allows presentation of exogenously loaded peptides. Expression of CD200 by the T2 cells was

assessed by flow cytometry (Figure 7B). T2 cells exhibited a low level of endogenous CD200 expression (Figure 7B).

Transduced T cells were stimulated with $WT1_{126}$-pulsed T2 cells. Despite a low level of CD200 expression on the target cells, CD200Rtm-CD28-transduced T cells exhibited enhanced proliferation as compared to T cells transduced with the C4 TCR alone (Figure 7C). In addition, stimulated CD200Rtm-CD28-transduced T cells (*i.e.*, IFP^+ T cells) produced increased levels of IFNγ and IL-2 compared to control T cells when exposed to CD200dim tumor cells (Figure 7D).

Overall, these results showed that primary T cells transduced to express a human CD200Rtm-CD28 construct and the beta and alpha chains of a $WT1_{126}$-specific TCR exhibited enhanced proliferation and increased cytokine production relative to T cells transduced with the TCR construct alone.

EXAMPLE 9

SIRPα-CD28 FUSION PROTEIN CONSTRUCTS PROMOTE ACCUMULATION OF TRANSDUCED T CELLS *IN VITRO*

Exemplary fusion proteins as described herein also include IFPs comprised of the extracellular domain of SIRPα, or portions thereof, and an intracellular signaling domain of CD28 (Figure 8A). The hydrophobic component may be comprised of the transmembrane domain of either SIRPα or CD28, or portions thereof. In some exemplary SIRPα-CD28 fusion proteins, the hydrophobic component comprises the transmembrane domain of CD28 and the extracellular component further comprises an extracellular portion of CD28, particularly an extracellular cysteine residue adjacent to the hydrophobic component (*e.g.*, SIRPα-CD28Cys, SIRPα-6aas-CD28Cys, SIRPα-9aas-CD28Cys, and SIRPα-9aas-CD28Cys). The extracellular component may comprise all or a portion of the extracellular domain of SIRPα. In some embodiments, the extracellular component comprises the entire extracellular domain of SIRPα. In other examples, the extracellular component comprises the first 367 amino acids (*e.g.*, SIRPα-6aas-CD28Cys), the first 364 amino acids (*e.g.*, SIRPα-9aas-CD28Cys), or the first 350 (SIRPα-23aas-CD28Cys) amino acids from the N-terminus of SIRPα. The

size of the extracellular component may affect the ability of the fusion protein to enter the immunological synapse and co-localize with the TCR within the cSMAC to deliver a strong co-stimulatory signal. In some examples, the extracellular component comprises a truncated SIRPα, which may alter the size of the extracellular component. For example, to account for the additional extracellular amino acids of the extracellular domain of the fusion protein (*e.g.*, an additional 9 or 12 amino acids), SIRPα-6aas-CD28 has a truncated portion of SIRPα that preserves a natural N-linked glycosylation site. In another example, SIRPα-23aas-CD28 has a truncated portion of SIRPα that lacks the entire stem region of the SIRPα extracellular domain. Additionally, a SIRPα-CD28 construct has the capacity to convert a signal initiated by the binding of SIRPα to its target into a positive (*e.g.*, costimulatory) signal generated by the CD28 intracellular signaling domain.

IFPs using SIRPα extracellular components were generated (Figure 8A) using the methods described in Example 2. TCR_{gag} T cells were transduced as in Example 2, and TCR_{gag} effector cells were generated *in vitro* as in Example 3. FBL cells were transduced with CD47 or mCherry with polybrene spinfection, similar to T cell transduction, and subsequently sorted to generate a homogenous population.

As was observed with CD200R-9aas-CD28Cys, T cells transduced with the SIRPα constructs accumulated over multiple rounds of stimulation *in vitro* (Figure 8B). These data suggest that SIRPα-CD28 IFPs also promote proliferation and survival of T cells.

To assess T cell proliferation *in vitro*, a CTV Dilution Proliferation assay was performed as described in Example 2. As was observed with CD200R-9aas-CD28Cys, T cells transduced with the SIRPα constructs engineered to maintain the T cell-tumor cell synapse distance exhibited enhanced proliferation as compared to control T cells (Figure 8C). In addition, $CD47^+$ tumor cells were efficiently killed after 3 days of co-culture with SIRPα-$CD28^+$ T cells but not control T cells or T cells transduced with a SIRPα construct that lacked an intracellular signaling domain (Figure 8D). To further assess the lytic capacity of SIRPα-$CD28^+$ T cells, an IncuCyte assay was used to quantify killing of $CD47^+$ FBL. A total of 10^5 $mCherry^+$ $CD47^+$ FBL were co-cultured

in 24-well plates with a titration of human T cells transduced with SIRPα-CD28 constructs. The plate was incubated in an IncuCyte (Essen BioScience) inside a cell culture incubator for 70 hours. Images were captured every hour to monitor killing of tumor cells, as determined by loss of red signal. SIRPα-CD28$^+$ T cells killed CD47$^+$ tumor cells, even at the lowest effector-to-target ratio tested (0.4:1; Figure 8E).

EXAMPLE 10

PD-1-CD28 FUSION PROTEIN CONSTRUCTS PROMOTE CYTOKINE PRODUCTION IN TRANSDUCED T CELLS *IN VITRO*

Exemplary fusion proteins as described herein also include IFPs comprised of the extracellular domain of PD-1, or portions thereof, and an intracellular signaling domain of CD28 (Figure 9A). The transmembrane component may be comprised of the transmembrane domain of either PD-1 or CD28, or portions thereof. In some exemplary PD1-CD28 fusion proteins, the transmembrane component comprises the transmembrane domain of CD28 and the extracellular component further comprises an extracellular portion of CD28, particularly an extracellular cysteine residue adjacent to the transmembrane component (*e.g.*, PD1-CD28Cys, PD1-9aas-CD28Cys, and PD1-21aas-CD28Cys) to promote inter-chain dimerization. The extracellular component may comprise all or a portion of the extracellular domain of PD-1, or may be truncated (*e.g.*, -9aas in murine constructs, -12aas or -15aas in human constructs; lacking the stem region of PD-1, -21aas) to maintain the short spatial distance between the cells to facilitate access of the liganded receptor to the immunologic synapse. Additionally, a PD1-CD28 construct has the capacity to convert what would typically be an inhibitory signal from the binding of PD1 to its target into a positive (*e.g.*, costimulatory) signal generated by the CD28 intracellular signaling domain.

IFPs comprising PD-1 extracellular components were generated (Figure 9A) using the methods described in Example 2. TCR_{gag} T cells were transduced as in Example 2, and TCR_{gag} effector cells were generated *in vitro* as in Example 3.

Murine PD1-CD28 IFPs were generated using constructs I-IV and VII (Fig. 9A). PD1-CD28$^+$ T cells were restimulated in the presence of Brefeldin A (to retain

produced cytokines) with FBL cells endogenously expressing the PD-1 ligands, PD-L1 and PD-L2. After 5 hours, cells were fixed and treated with the BD Cytofix/Cytoperm kit, to allow intracellular staining of the effector cytokines, IFNγ and TNFα. Transduction with each of the five PD1-CD28 constructs enhanced production of intracellular cytokines compared to control T cells (Figure 9B).

Human PD1-CD28 IFPs were generated using constructs I-III and V-VII (Figure 9A). Vectors containing the PD1-CD28 IFP and C4 TCR were generated as described above. Jurkat T cells were transduced as described above. T cells transduced with the TCR and PD1-12aas-CD28Cys or PD1-15aas-CD28Cys exhibited high transduction efficiencies and expression of both proteins (Figure 10).

EXAMPLE 11

FAS-CD28 FUSION PROTEIN CONSTRUCTS PROMOTE ACCUMULATION AND ENHANCED FUNCTION IN TRANSDUCED T CELLS *IN VITRO*

Exemplary fusion proteins as described herein also include IFPs comprised of the extracellular domain of Fas, or portions thereof, and an intracellular signaling domain of CD28 (Figure 11A). The transmembrane component may be comprised of the domain of either Fas or CD28, or portions thereof. In some exemplary Fas-CD28 fusion proteins, the transmembrane component comprises the transmembrane domain of CD28 and the extracellular component further comprises an extracellular portion of CD28, particularly an extracellular cysteine residue adjacent to the transmembrane component (*e.g.*, Fas-CD28Cys and Fas-9aas-CD28Cys). The extracellular component may comprise all or a portion of the extracellular domain of Fas or may be truncated to preserve maintain a short spatial distance between the cells (-9aas) upon receptor-ligand interaction. Additionally, a Fas-CD28 construct has the capacity to convert a signal initiated by the binding of Fas to its target into a positive (*e.g.*, costimulatory) signal generated by the CD28 intracellular signaling domain.

IFPs comprising Fas extracellular components were generated (Figure 11A) using the methods described in Example 2. TCR_{gag} T cells were transduced as in Example 2, and TCR_{gag} effector cells were generated *in vitro* as in Example 3.

To determine if expression of the Fas-CD28 IFP results in increased accumulation of transduced cells, the proportion of transduced cells from the mixed population in the total TCR_{gag} population was measured over multiple cycles of stimulation with irradiated FBL, as described in Example 3. All of the constructs promoted accumulation of transduced T cells compared to control T cells (Figure 11B). In addition, expression of Fas-CD28 constructs but not full-length (FL) Fas promoted survival or expansion of T cells upon multiple stimulations *in vitro* Figure 11C).

EXAMPLE 12

LAG3-CD28 FUSION PROTEIN CONSTRUCTS

Exemplary fusion proteins as described herein also include IFPs comprised of the extracellular domain of LAG3, or portions thereof, and an intracellular signaling domain of CD28 (Figure 12A). The transmembrane component may be comprised of the domain of either LAG3 or CD28, or portions thereof. In some exemplary LAG3-CD28 fusion proteins, the transmembrane component comprises the transmembrane domain of CD28 and the extracellular component further comprises an extracellular portion of CD28, particularly an extracellular cysteine residue adjacent to the transmembrane component (*e.g.*, LAG3-CD28Cys and LAG3-9aas-CD28Cys). The extracellular component may comprise all or a portion of the extracellular domain of LAG3 or may be truncated to maintain a short spatial distance between the cells (*e.g.*, -9aas) upon receptor-ligand interaction. Additionally, a LAG3-CD28 construct has the capacity to convert what would typically be an inhibitory signal from the binding of LAG3 to its target into a positive (*e.g.*, costimulatory) signal generated by the CD28 intracellular signaling domain.

IFPs using LAG3 extracellular components were generated (Figure 12A) using the methods described in Example 2. T cells were transduced with LAG3-eGFP constructs as described. Five days after transduction, $CD8^+$ T cells were analyzed for construct expression by anti-LAG3 antibody staining and flow cytometry (Figure 12B). A vector encoding only green fluorescent protein (GFP) was used as a control. All constructs exhibited expression of LAG3 (Figure 12B).

EXAMPLE 13

TIM3-CD28 FUSION PROTEIN CONSTRUCTS

Exemplary fusion proteins as described herein also include IFPs comprised of the extracellular domain of TIM3, or portions thereof, and an intracellular signaling domain of CD28 (Figure 13A). The transmembrane component may be comprised of the domain of either TIM3 or CD28, or portions thereof. In some exemplary TIM3-CD28 fusion proteins, the transmembrane component comprises the transmembrane domain of CD28 and the extracellular component further comprises an extracellular portion of CD28, particularly an extracellular cysteine residue adjacent to the transmembrane component (*e.g.*, TIM3-CD28Cys and TIM3-9aas-CD28Cys). The extracellular component may comprise all or a portion of the extracellular domain of TIM3 or may be truncated to maintain the short spatial distance between the cells (*e.g.*, -9aas). Additionally, a TIM3-CD28 construct has the capacity to convert what would typically be an inhibitory signal from the binding of TIM3 to its target into a positive signal generated by the CD28 intracellular signaling domain.

New IFPs using TIM3 extracellular components were generated (Figure 13A) using the methods described in Example 2. T cells were transduced with GFP-TIM3 constructs as described. Five days after transduction, $CD8^+$ T cells were analyzed for construct expression by anti-TIM3 antibody staining and flow cytometry (Figure 13B). A vector encoding only green fluorescent protein (GFP) was used as a control. Most constructs exhibited similar expression of TIM3 (Figure 13B).

While specific embodiments of the invention have been illustrated and described, it will be readily appreciated that the various embodiments described above can be combined to provide further embodiments, and that various changes can be made therein without departing from the spirit and scope of the invention.

All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications, and non-patent publications referred to in this specification or listed in the Application Data Sheet, including but not limited to U.S. Provisional Patent Application No. 62/128,979, are incorporated herein

by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

What is claimed is:

1. A fusion protein, comprising (a) an extracellular component comprised of a binding domain that specifically binds a target, (b) an intracellular component comprised of an intracellular signaling domain, and (c) a hydrophobic component connecting the extracellular and intracellular components,

wherein the extracellular portion of a complex formed by specific binding of the fusion protein to the target (fusion protein::target complex) is of a size, or spans a distance, of (i) up to about a distance between two cell membranes of an immunological synapse, (ii) up to about or substantially the same as a distance spanned by the extracellular portion of a complex between a T cell receptor (TCR) and an MHC-peptide complex specifically bound by the TCR, (iii) up to about or substantially the same as a distance spanned by the extracellular portion of a complex between a natural molecule comprising the binding domain and its cognate binding partner; (iii) less than or up to about 40 nm, 25 nm, 20 nm, 15 nm, or 14 nm; or (iv) any combination thereof.

2. The fusion protein according to claim 1, wherein the fusion protein::target complex localizes to a supramolecular activation cluster (SMAC).

3. The fusion protein according to claim 1 or 2, wherein the fusion protein::target complex localizes to a central region supramolecular activation cluster (cSMAC).

4. The fusion protein according to claim 2 or claim 3, wherein the SMAC has a width of a T cell receptor (TCR) associated with an antigen::human leukocyte antigen (HLA) complex.

5. The fusion protein according to any one of claims 1-4, wherein the extracellular component is associated with a negative signal and the intracellular component is associated with a positive signal.

6. A fusion protein, comprising (a) an extracellular component comprised of a binding domain that specifically binds a target, (b) an intracellular component comprised of an intracellular signaling domain, and (c) a hydrophobic component connecting the extracellular and intracellular components,

wherein the binding domain is, or has at least 95 % identity to, an inhibitory molecule binding domain and the intracellular signaling domain is, or contains at least 95 % identity to, a costimulatory or stimulatory molecule binding domain, and

wherein the inhibitory molecule is not a B7-CD28 superfamily member, is not CTLA4, is not PD1, does not bind to B7, is a glycoprotein, or any combination thereof.

7. The fusion protein according to any of claims 1-6, wherein the expression of the fusion protein in a T cell comprising a TCR or chimeric antigen receptor specific for an antigen results in at least about a 1.5-fold, 2-fold, or 3-fold increase in survival, expansion, cytotoxicity, cytokine secretion, and/or response to multiple rounds of stimulation, by the T cell, in response to binding of the antigen and/or following administration to a subject, and/or results in at least about a 1.5-fold, 2-fold, or 3-fold increase in time of survival, disease-free survival, or amelioration of one or more disease symptom, of a subject to which the cell is administered, as compared to a cell substantially the same as the T cell but not containing the fusion protein.

8. The fusion protein according to any of claims 1-7, wherein the fusion protein is capable, when expressed in a T cell, of co-localizing with a TCR or CAR expressed by the T cell.

9. The fusion protein according to any of claims 6-8, wherein the binding domain and/or the inhibitory molecule specifically binds to a CD200 or a CD47.

10. The fusion protein according to any of claims 6-9, wherein the binding domain is derived from, and/or the inhibitory molecule is, a CD200R, a SIRPα, a TIM3, a CD2, a CD95 (Fas), a CD223 (LAG3), an A2aR, a KIR, TIM3, a CD300, or a LPA5.

11. The fusion protein according to any of claims 6-9, wherein the inhibitory molecule is or comprises a CD200R.

12. The fusion protein according to any of claims 6-9, wherein the inhibitory molecule is or comprises a SIRPα.

13. The fusion protein according to any one of claims 1-12, wherein the binding domain is an antibody binding fragment, a receptor ectodomain, a cytokine, or a ligand.

14. The fusion protein according to any one of claims 1-13, wherein the extracellular component comprises an extracellular portion of a CD200R, SIRPα, CD279 (PD-1), CD2, CD95 (Fas), CD152 (CTLA4), CD223 (LAG3), CD272 (BTLA), A2aR, KIR, TIM3, CD300, or LPA5.

15. The fusion protein according to any of claims 1-14, wherein the extracellular component further comprises an additional extracellular portion, wherein the additional extracellular portion optionally is from or shares identity with an extracellular portion of a molecule that is distinct from the binding domain source molecule or does not contain the binding domain.

16. The fusion protein according to any one of claims 1-15, wherein the extracellular component further comprises an extracellular portion from a the

hydrophobic component, or that contains the hydrophobic component or a portion thereof.

17. The fusion protein according to claim 15 or 16, wherein the additional extracellular portion comprises a multimerization domain and/or a spacer.

18. The fusion protein according to any one of claims 1-17, wherein the extracellular component comprises an extracellular portion of a CD200R or wherein the binding domain comprises a CD200-binding portion of a CD200R or binding variant thereof.

19. The fusion protein according to any one of claims 1-17, wherein the extracellular component comprises an extracellular portion of a SIRPα or wherein the binding domain comprises a CD47 binding portion of a SIRPα or binding variant thereof.

20. The fusion protein according to any one of claims 1-19, wherein the extracellular component or the additional extracellular portion comprises a multimerization domain.

21. The fusion protein according to claim 20, wherein the multimerization domain comprises a cysteine residue.

22. The fusion protein according to claim 20, wherein the multimerization domain comprises an extracellular component modified to contain a cysteine residue within about 2 to about 15 amino acids from the hydrophobic component.

23. The fusion protein according to any one of claims 1-22, wherein the hydrophobic component comprises a transmembrane domain of a CD2, CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD40, CD79A, CD79B, CD80, CD86, CD95 (Fas),

CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD152 (CTLA4), CD200R, CD223 (LAG3), CD270 (HVEM), CD272 (BTLA), CD273 (PD-L2), CD274 (PD-L1), CD278 (ICOS), CD279 (PD-1), CD300, CD357 (GITR), A2aR, DAP10, FcRα, FcRβ, FcRγ, Fyn, GAL9, KIR, Lck, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, PTCH2, ROR2, Ryk, Slp76, SIRPα, pTα, TCRα, TCRβ, TIM3, TRIM, LPA5, or Zap70.

24. The fusion protein according to any one of claims 1-23, wherein the hydrophobic component comprises a transmembrane domain of a CD28.

25. The fusion protein according to any one of claims 1-23, wherein the hydrophobic component comprises a transmembrane domain of a 4-1BB.

26. The fusion protein according to any one of claims 1-25, wherein the intracellular signaling domain comprises an intracellular signaling domain of a costimulatory molecule.

27. The fusion protein of claim 26, wherein the costimulatory molecule comprises CD28, CD137 (4-1BB), or ICOS.

28. The fusion protein of any of claims 1-27, wherein the intracellular signaling domain comprises an intracellular signaling domain of a CD3ε, CD3δ, CD3ζ, CD25, CD27, CD28, CD40, CD47, CD79A, CD79B, CD134 (OX40), CD137 (4-1BB), CD150 (SLAMF1), CD278 (ICOS), CD357 (GITR), CARD11, DAP10, DAP12, FcRα, FcRβ, FcRγ, Fyn, Lck, LAT, LRP, NKG2D, NOTCH1, NOTCH2, NOTCH3, NOTCH4, ROR2, Ryk, Slp76, pTα, TCRα, TCRβ, TRIM, Zap70, PTCH2, or any combination thereof.

29. The fusion protein according to any one of claims 1-28, wherein the intracellular signaling domain comprises a costimulatory domain of a CD137 (4-1BB), CD27, CD28, ICOS, OX40 (CD134), or any combination thereof.

30. The fusion protein according to any one of claims 1-29, wherein the intracellular signaling domain comprises a costimulatory domain of a CD137 (4-1BB) or CD28, or any combination thereof.

31. The fusion protein according to any one of claims 1-30, wherein the intracellular signaling domain comprises a costimulatory domain of a CD28.

32. The fusion protein according to any one of claims 1-31, wherein the intracellular signaling domain comprises a costimulatory domain of a CD137 (4-1BB).

33. The fusion protein according to any one of claims 1-32, wherein the target is an immunosupressive ligand.

34. The fusion protein according to any one of claims 1-32, wherein the target is selected from a CD47, a CD58, a CD95L (FasL), a CD200, a CD270 (HVEM), a CD274 (PD-L1), and a GAL9.

35. The fusion protein according to any of claims 1-34, wherein (a) the extracellular component comprises an extracellular portion of a CD200R, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28 or a 41BB.

36. The fusion protein according to claim 35, wherein the extracellular portion of the CD200R comprises the entire extracellular domain of CD200R.

37. The fusion protein according to claim 35, wherein the extracellular portion of the CD200R comprises at least 200 amino acids from the N-terminus of CD200R.

38. The fusion protein according to claim 35, wherein the extracellular portion of the CD200R comprises at least about 225 amino acids to at least about 235 amino acids from the N-terminus of CD200R.

39. The fusion protein according to claim 35, wherein the extracellular portion of the CD200R comprises at least about 234 amino acids from the N-terminus of CD200R.

40. The fusion protein according to any of claims 35-39, wherein the CD200R is a human CD200R.

41. The fusion protein according to any one of claims 35-40, wherein the intracellular component comprises a second intracellular signaling domain.

42. The fusion protein according to claim 41, wherein the second intracellular signaling domain comprises an intracellular signaling domain of a CD137 (4-1BB).

43. The fusion protein according to any one of claims 35-42, wherein the extracellular component comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain.

44. The fusion protein according to claim 43, wherein the extracellular portion of the CD28 comprises a cysteine residue.

45. The fusion protein according to any of claims 1-44, wherein the intracellular signaling component does not contain a CD3ζ signaling domain and/or does not contain a signaling domain capable of delivering a primary signal to a T cell and/or does not contain a signaling domain from a molecule naturally capable of delivering a primary signal to a T cell.

46. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:2, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:3, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

47. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:2, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

48. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:8, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises the amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

49. The fusion protein according to claim 41, wherein the extracellular component further comprises a multimerization domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9.

50. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:11, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

51. The fusion protein according to claim 50, wherein the extracellular component further comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9.

52. The fusion protein according to claim 51, wherein the intracellular component further comprises a second intracellular signaling domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:13.

53. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an extracellular portion of a CD200R, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD137 (4-1BB).

54. The fusion protein according to claim 53, wherein the extracellular portion of the CD200R comprises the entire extracellular domain of CD200R.

55. The fusion protein according to claim 53, wherein the extracellular portion of the CD200R comprises at least 200 amino acids from the N-terminus of CD200R.

56. The fusion protein according to claim 53, wherein the extracellular portion of the CD200R comprises at least about 225 amino acids to at least about 235 amino acids from the N-terminus of CD200R.

57. The fusion protein according to any one of claims 53-56, wherein the extracellular component comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain.

58. The fusion protein according to claim 57, wherein the extracellular portion of the CD28 comprises a cysteine residue.

59. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an extracellular portion of a CD200R, (b) the hydrophobic component comprises a transmembrane domain of a CD137 (4-1BB), and (c) the intracellular component comprises an intracellular signaling domain of a CD137 (4-1BB).

60. The fusion protein according to claim 59, wherein the extracellular portion of the CD200R comprises the entire extracellular domain of CD200R.

61. The fusion protein according to claim 59, wherein the extracellular portion of the CD200R comprises at least a portion of the extracellular domain at least 230 amino acids from the N-terminus of CD200R.

62. The fusion protein according to any one of claims 59-61, wherein the extracellular component comprises an extracellular portion of a CD137 (4-1BB) extending from the CD137 (4-1BB) transmembrane domain.

63. The fusion protein according to claim 62, wherein the extracellular portion of the CD137 (4-1BB) comprises a cysteine residue.

64. The fusion protein according to claim 1, wherein (a) the extracellular component comprises a binding domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:8 and a multimerization domain with

an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:13.

65. The fusion protein according to claim 64, wherein the intracellular component further comprises a second intracellular signaling domain having an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

66. The fusion protein according to claim 1, wherein (a) the extracellular component comprises a binding domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:11 and a multimerization domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:13.

67. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an extracellular portion of a SIRPα, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

68. The fusion protein according to claim 67, wherein the extracellular portion of the SIRPα comprises the entire extracellular domain of SIRPα.

69. The fusion protein according to claim 67, wherein the extracellular portion of the SIRPα comprises at least 361 amino acids from the N-terminus of SIRPα.

70. The fusion protein according to any one of claims 67-69, wherein the intracellular component comprises a second intracellular signaling domain.

71. The fusion protein according to claim 70, wherein the second intracellular signaling domain comprises an intracellular signaling domain of a CD137 (4-1BB).

72. The fusion protein according to any one of claims 67-71, wherein the extracellular component comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain.

73. The fusion protein according to claim 72, wherein the extracellular portion of the CD28 comprises a cysteine residue.

74. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:17, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:18, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

75. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:17, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

76. The fusion protein according to claim 1, wherein (a) the extracellular component comprises a binding domain with an amino acid sequence encoded by a

nucleic acid molecule as set forth in SEQ ID NO.:21 and a multimerization domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises the amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises the amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

77. The fusion protein according to claim 1, wherein (a) the extracellular component comprises a binding domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:21 and a multimerization domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises the amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises the amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:13.

78. The fusion protein according to claim 70, wherein the intracellular component further comprises the amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ. ID. NO:5.

79. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an extracellular portion of a CD279 (PD-1), (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

80. The fusion protein according to claim 79, wherein the extracellular portion of the CD279 (PD-1) comprises the entire extracellular domain of CD279 (PD-1).

81. The fusion protein according to claim 79, wherein the extracellular portion of the CD279 (PD-1) comprises a portion of the extracellular domain at least 100 amino acids from the N-terminus of CD279 (PD-1).

82. The fusion protein according to claim 79, wherein the extracellular portion of the CD279 (PD-1) comprises a portion of the extracellular domain at least 149 amino acids from the N-terminus of CD279 (PD-1).

83. The fusion protein according to any one of claims 79-82, wherein the extracellular component comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain.

84. The fusion protein according to claim 83, wherein the extracellular portion of the CD28 comprises a cysteine residue.

85. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:89 and a multimerization domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

86. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:91 and a multimerization domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

87. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:95 and a multimerization domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

88. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an extracellular portion of a CD95 (Fas), (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

89. The fusion protein according to claim 88, wherein the extracellular portion of the CD95 (Fas) comprises the entire extracellular domain of CD95 (Fas).

90. The fusion protein according to claim 88, wherein the extracellular portion of the CD95 (Fas) comprises at least a portion of the extracellular domain of at least 175 amino acids from the N-terminus of CD95 (Fas).

91. The fusion protein according to claim 88, wherein the extracellular portion of the CD95 (Fas) comprises at least a portion of the extracellular domain of at least 173 amino acids from the N-terminus of CD95 (Fas).

92. The fusion protein according to claim 88, wherein the extracellular portion of the CD95 (Fas) comprises at least a portion of the extracellular domain of at least 166 amino acids from the N-terminus of CD95 (Fas).

93. The fusion protein according to claim 88, wherein the extracellular portion of the CD95 (Fas) comprises at least a portion of the extracellular domain of at least 161 amino acids from the N-terminus of CD95 (Fas).

94. The fusion protein according to any one of claims 88-93, wherein the extracellular component comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain.

95. The fusion protein according to claim 94, wherein the extracellular portion of the CD28 comprises a cysteine residue.

96. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:71 and a multimerization domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

97. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:73 and a multimerization domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

98. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:75 and a multimerization domain with an amino acid sequence

encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

99. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an extracellular portion of a CD2, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

100. The fusion protein according to claim 99, wherein the extracellular portion of the CD2 comprises the entire extracellular domain of CD2.

101. The fusion protein according to claim 99, wherein the extracellular portion of the CD2 comprises at least a portion of the extracellular domain of at least 175 amino acids from the N-terminus of CD2.

102. The fusion protein according to any one of claims 99-101, wherein the extracellular component comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain.

103. The fusion protein according to claim 102, wherein the extracellular portion of the CD28 comprises a cysteine residue.

104. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:61 and a multimerization domain with an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:9, (b) the hydrophobic component comprises an amino acid sequence encoded by a nucleic acid molecule as

set forth in SEQ ID NO.:4, and (c) the intracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:5.

105. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an extracellular portion of a TIM3, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

106. The fusion protein according to claim 105, wherein the extracellular portion of the TIM3 comprises the entire extracellular domain of TIM3.

107. The fusion protein according to claim 105, wherein the extracellular portion of the TIM3 comprises at least a portion of the extracellular domain of at least 180 amino acids from the N-terminus of TIM3.

108. The fusion protein according to any one of claims 105-107, wherein the extracellular component comprises an extracellular portion of a CD28 extending from the CD28 transmembrane domain.

109. The fusion protein according to claim 108, wherein the extracellular portion of the CD28 comprises a cysteine residue.

110. The fusion protein according to claim 1, wherein (a) the extracellular component comprises an extracellular portion of a LAG3, (b) the hydrophobic component comprises a transmembrane domain of a CD28, and (c) the intracellular component comprises an intracellular signaling domain of a CD28.

111. The fusion protein according to claim 110, wherein the extracellular portion of the LAG3 comprises the entire extracellular domain of LAG3.

112. The fusion protein according to claim 110, wherein the extracellular portion of the LAG3 comprises at least a portion of the extracellular domain of at least 410 amino acids from the N-terminus of LAG3.

113. The fusion protein according to any one of claims 110-112, wherein the extracellular component comprises an extracellular portion of a LAG3 extending from the CD28 transmembrane domain.

114. The fusion protein according to claim 113, wherein the extracellular portion of the CD28 comprises a cysteine residue.

115. A fusion protein according to claim 5, wherein the extracellular component comprises a binding domain with an amino acid sequence as set forth in SEQ ID NO.:31 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid as set forth in SEQ ID NO.:36.

116. The fusion protein according to claim 115, wherein the intracellular component further comprises a second intracellular signaling domain having an amino acid sequence as set forth in SEQ ID NO.:28.

117. The fusion protein according to claim 5, wherein (a) the extracellular component comprises a binding domain with an amino acid sequence as set forth in SEQ ID NO.:34 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:36.

118. The fusion protein according to claim 5, wherein (a) the extracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:40, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:41, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:28.

119. The fusion protein according to claim 5, wherein (a) the extracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:40, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:28.

120. The fusion protein according to claim 5, wherein (a) the extracellular component comprises a binding domain with an amino acid sequence as set forth in SEQ ID NO.:44 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises the amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises the amino acid sequence as set forth in SEQ ID NO.:28.

121. The fusion protein according to claim 5, wherein (a) the extracellular component comprises a binding domain with an amino acid sequence as set forth in SEQ ID NO.:44 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises the amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises the amino acid sequence as set forth in SEQ ID NO.:36.

122. The fusion protein according to claim 121, wherein the intracellular component further comprises the amino acid sequence as set forth in SEQ. ID. NO:28.

123. The fusion protein according to claim 5, wherein (a) the extracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:90 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:28.

124. The fusion protein according to claim 5, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:92 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:28.

125. The fusion protein according to claim 5, wherein (a) the extracellular component comprises an amino acid sequence encoded by a nucleic acid molecule as set forth in SEQ ID NO.:96 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:28.

126. The fusion protein according to claim 5, wherein (a) the extracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:72 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:28.

127. The fusion protein according to claim 5, wherein (a) the extracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:74 and a

multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:28.

128. The fusion protein according to claim 5, wherein (a) the extracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:76 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:28.

129. The fusion protein according to claim 5, wherein (a) the extracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:62 and a multimerization domain with an amino acid sequence as set forth in SEQ ID NO.:32, (b) the hydrophobic component comprises an amino acid sequence as set forth in SEQ ID NO.:27, and (c) the intracellular component comprises an amino acid sequence as set forth in SEQ ID NO.:28.

130. A nucleic acid molecule encoding a fusion protein according to any one of claims 1-129.

131. A vector comprising a nucleic acid molecule according to claim 130.

132. The vector according to claim 131, wherein the vector is a viral vector.

133. The vector according to claim 132, wherein the viral vector is a lentiviral or retroviral vector.

134. The vector according to claim 133, wherein the viral vector is a lentiviral vector.

135. The vector according to any one of claims 131-134, further encoding an antigen-specific TCR.

136. The vector according to claim 135, wherein the TCR is exogenous to a host cell.

137. The vector according to claim 135 or claim 136, wherein the TCR is specific to a HLA class I restricted antigen.

138. The vector according to any one of claims 135-137, wherein the antigen is a cancer-specific antigen.

139. The vector according to claim 138, wherein the cancer-specific antigen comprises WT-1, mesothelin, or cyclin-A1.

140. The vector according to any one of claims 131-139, further encoding a ligand.

141. The vector according to claim 140, wherein the ligand is CD200, CD47, PD-L1, or CD58.

142. The vector according to any one of claims 131-141, further encoding an siRNA for reducing the expression of an endogenous receptor.

143. The vector according to claim 142, wherein the endogenous receptor comprises CD200R, SIRPα, CD279 (PD-1), CD95 (Fas), or CD2 or is a TCR or portion thereof.

144. A host cell, comprising a fusion protein according to any one of claims 1-129.

145. A host cell, comprising at least two different fusion proteins according to any one of claims 1-129.

146. A host cell according to claim 145, comprising:
a fusion protein according to claim 79; and
a fusion protein according to claim 105.

147. A host cell, comprising a nucleic acid molecule according to claim 130.

148. A host cell, comprising a vector according to any one of claims 131-143.

149. The host cell according to any one of claims 144-148, wherein the host cell is an immune system cell.

150. The host cell according to claim 149, wherein the immune system cell is a T cell.

151. The host cell according to claim 150, wherein the T cell is a CD4+ T cell.

152. The host cell according to claim 150, wherein the T cell is a CD8+ T cell.

153. The host cell according to any one of claims 144-152, further comprising an antigen receptor, which optionally is an antigen-specific TCR.

154. The host cell according to any one of claims 153, wherein the antigen-specific TCR is exogenous to the cell or the host.

155. The host cell according to claim 153 or claim 154, wherein the TCR binds to an antigen::HLA complex with high affinity.

156. The host cell according to claim 155, wherein the high affinity binding has a K_a equal to or greater than 10^7 M^{-1}.

157. The host cell according to any one of claims 153-156, wherein the TCR is specific to a HLA class I restricted antigen.

158. The host cell according to any one of claims 153-156, wherein the antigen is a cancer-specific antigen.

159. The host cell according to claim 158, wherein the cancer-specific antigen comprises WT-1, mesothelin, or cyclin-A1.

160. The host cell according to any one of claims 153-156, wherein the antigen is a viral antigen.

161. The host cell according to claim 153, wherein the antigen receptor is a chimeric antigen receptor.

162. The host cell according to claim 161, wherein the chimeric antigen receptor comprises an extracellular antigen binding domain and an intracellular signaling domain capable of delivering a primary signal to a T cell and optionally a costimulatory domain.

163. The host cell according to any one of claims 144-162, further encoding a ligand.

164. The host cell according to claim 163, wherein the ligand is CD200, CD47, PD-L1, or CD58.

165. The host cell according to any one of claims 144-164, further encoding an siRNA for reducing the expression of an endogenous receptor.

166. The host cell according to claim 165, wherein the endogenous receptor comprises CD200R, SIRPα, CD279 (PD-1), CD95 (Fas) or CD2.

167. A method of treating a disease in a subject comprising administering a fusion protein according to any one of claims 1-129 to the subject.

168. A method of treating a disease in a subject comprising administering a vector according to any one of claims 131-143 to the subject.

169. A method of treating a disease in a subject comprising administering a host cell according to any one of claims 144-166 to the subject.

170. The method according to any one of claims 167-169, wherein the disease is selected from the group consisting of viral infection, bacterial infection, cancer, and autoimmune disease.

171. The method according to any one of claims 167-170, wherein the subject is human.

172. A fusion protein according to any one of claims 1-129, for use in treating cancer.

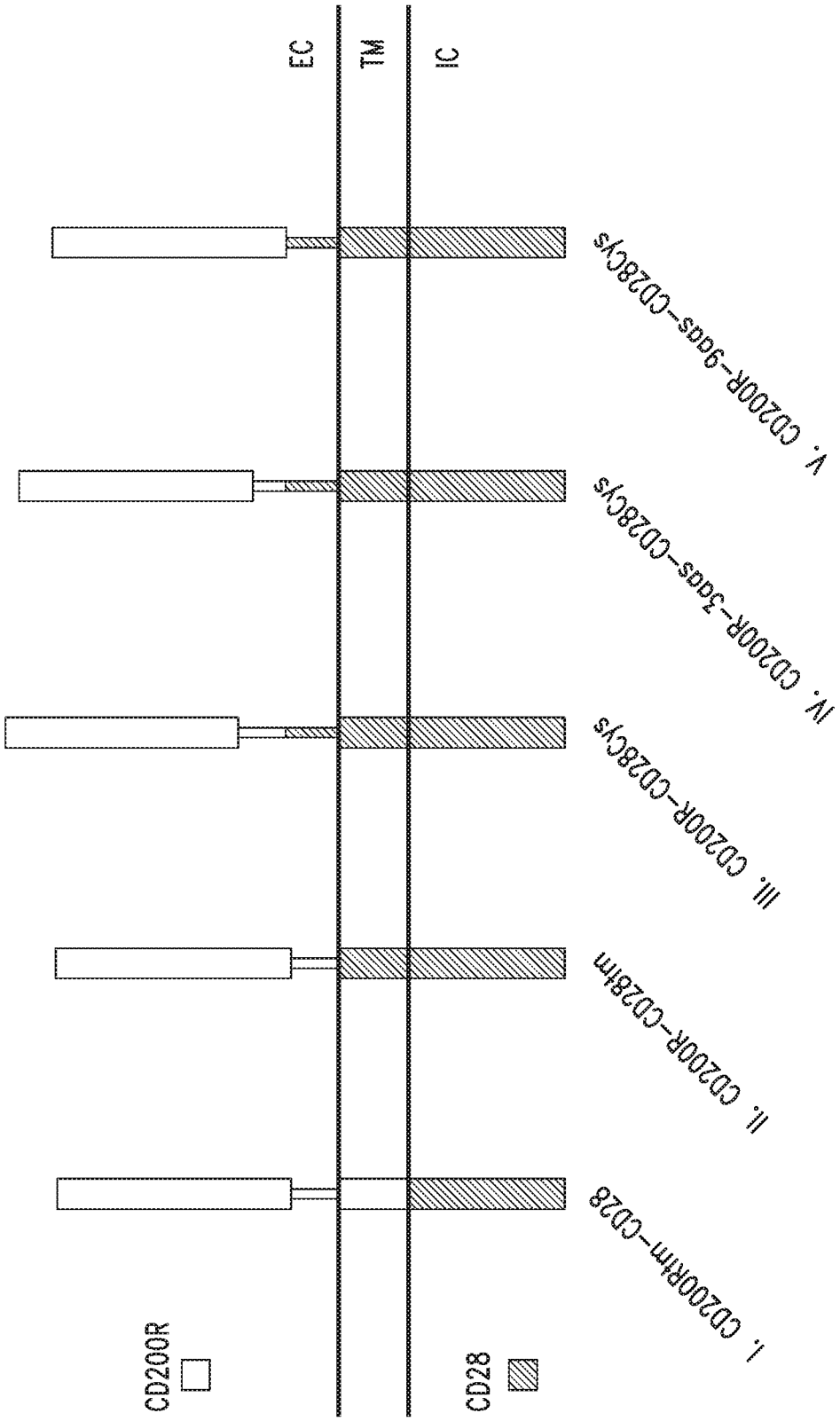

EC
TM
IC
I. CD200Rtm-CD28
II. CD200R-CD28tm
III. CD200R-CD28Cys
IV. CD200R-3Gas-CD28Cys
V. CD200R-9Gas-CD28Cys
CD200R
CD28

FIG. 1A

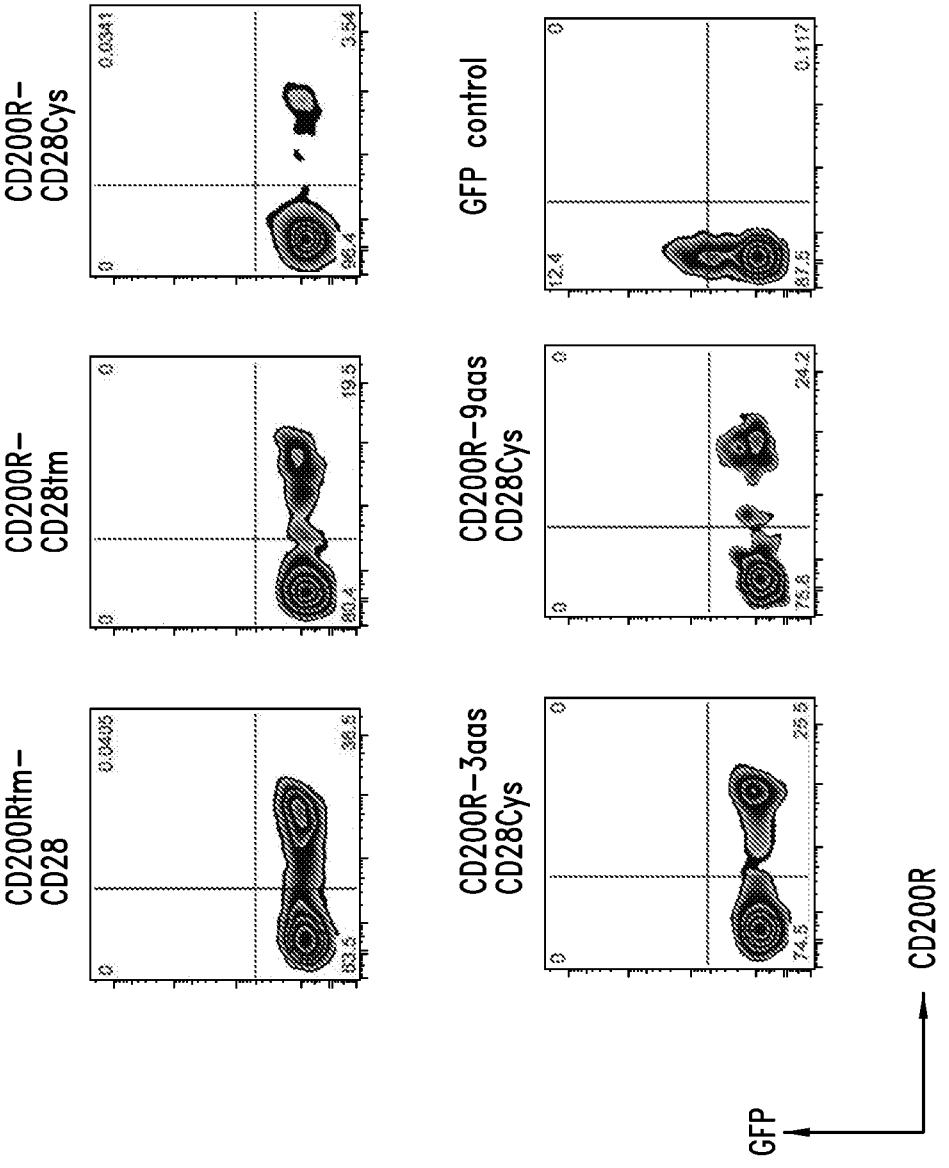

CD200Rtm-CD28
CD200R-CD28tm
CD200R-CD28Cys
CD200R-3aas CD28Cys
CD200R-9aas CD28Cys
GFP control
GFP
CD200R

FIG. 1B

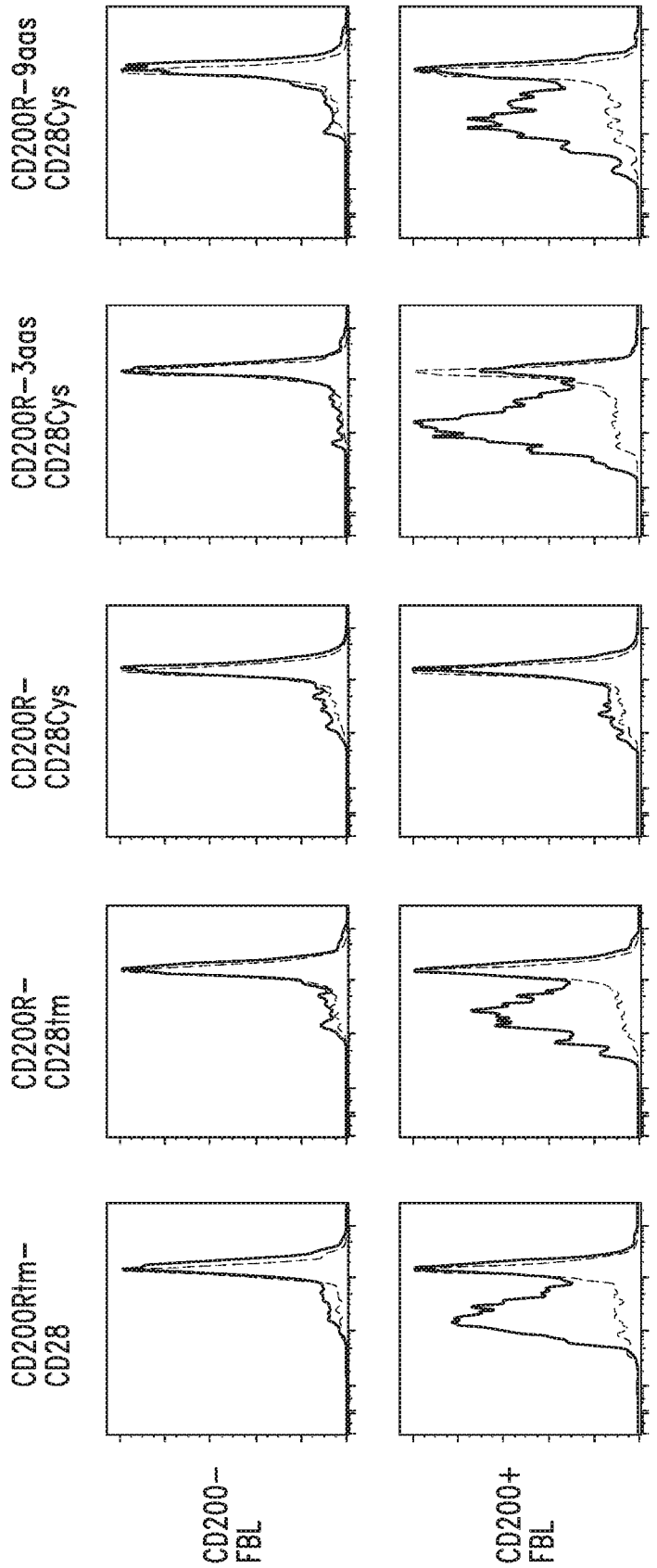

CD200Rtm-CD28
CD200R-CD28tm
CD200R-CD28Cys
CD200R-3aas CD28Cys
CD200R-9aas CD28Cys
CD200- FBL
CD200+ FBL
CTV
CD200R-CD28 transduced
GFP control

FIG. 2A

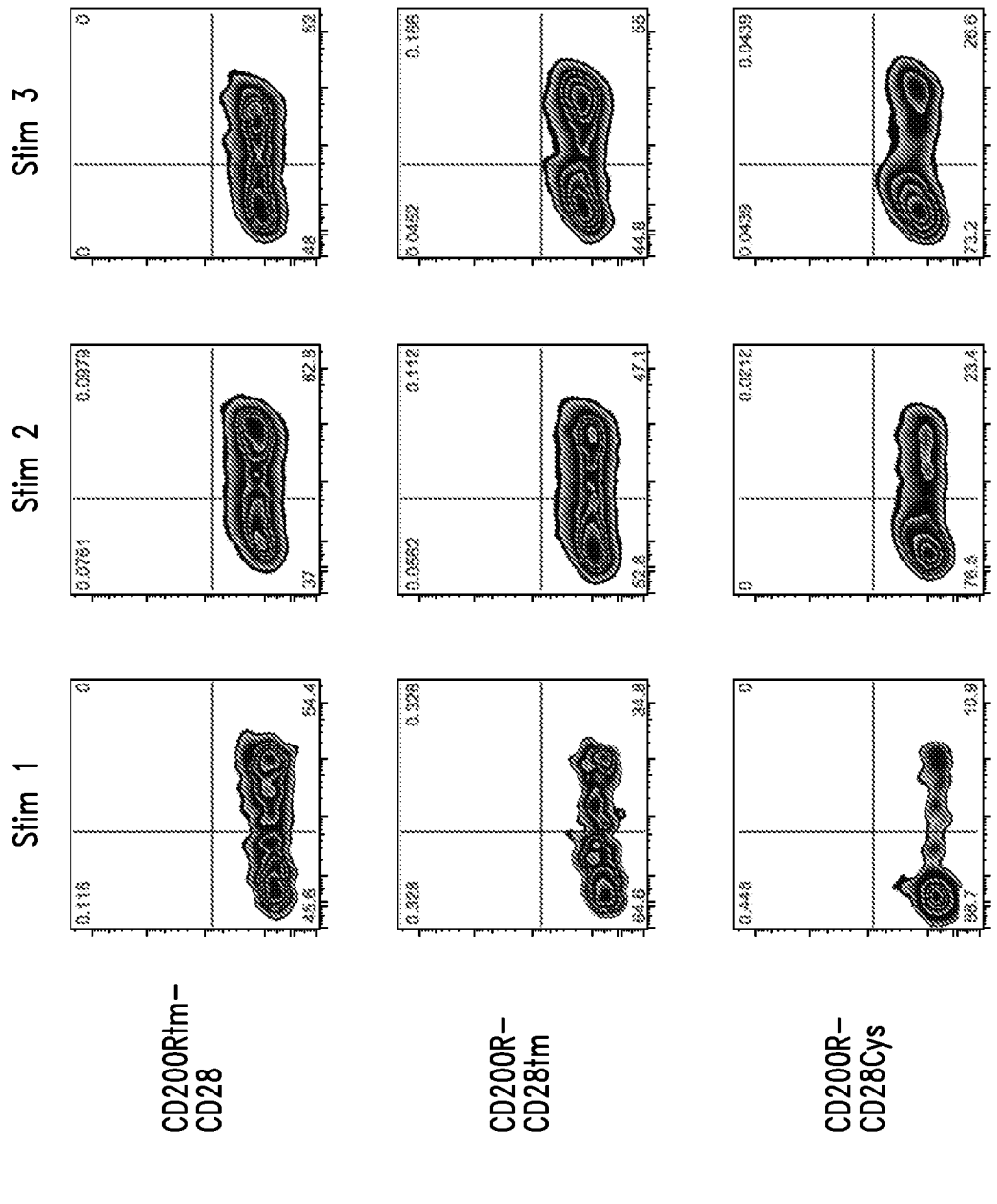

Stim 1
Stim 2
Stim 3
CD200Rtm-CD28
CD200R-CD28tm
CD200R-CD28Cys
CD200R

FIG. 2B

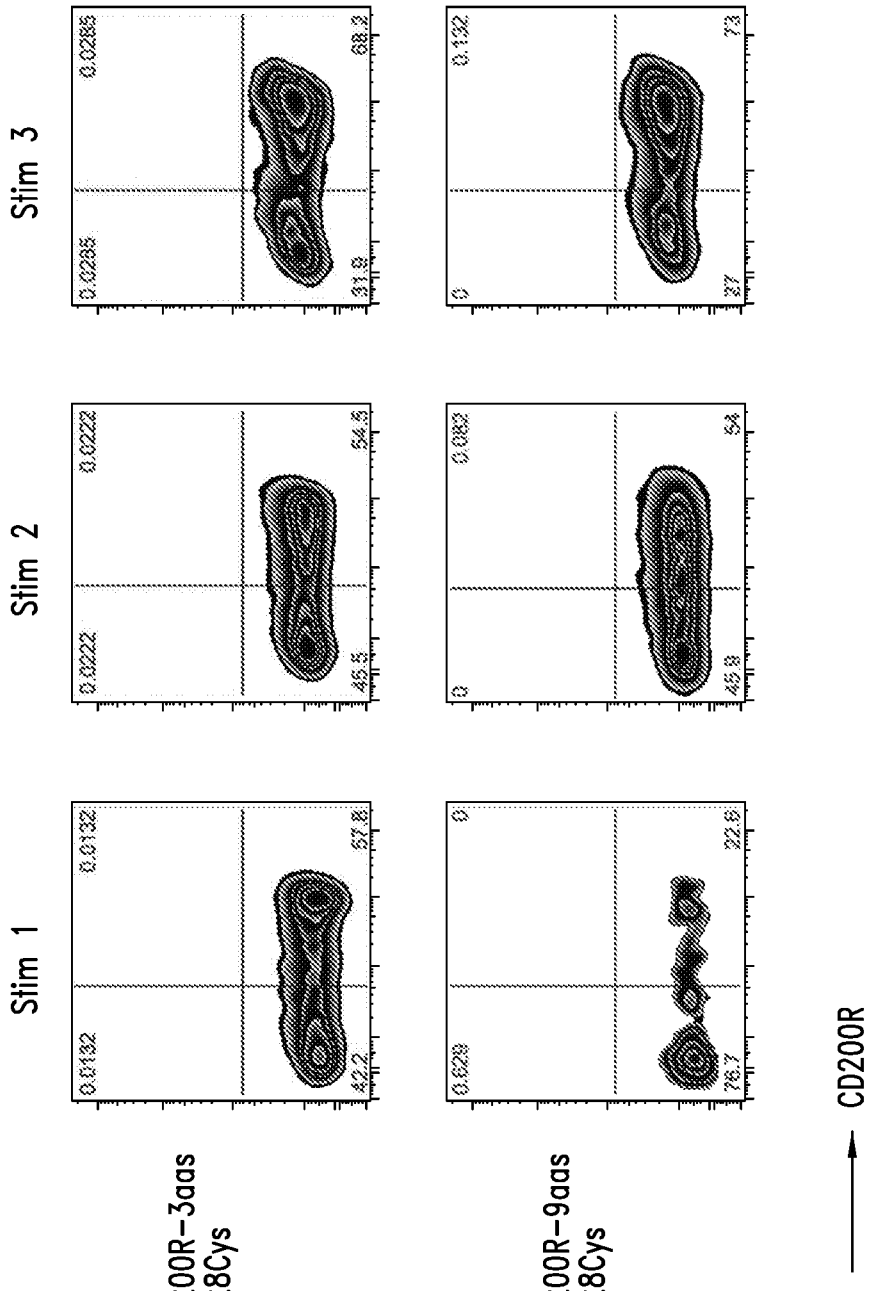

Stim 1
Stim 2
Stim 3
CD200R-3aas CD28Cys
CD200R-9aas CD28Cys
CD200R
0.0132
0.0132
57.8
42.2
0.0222
0.0222
54.5
45.5
0.0285
0.0285
68.2
31.9
0.629
0
22.8
76.7
0.082
0.082
54
45.8
0.132
0.132
73
27
0
0

FIG. 2B (Cont.)

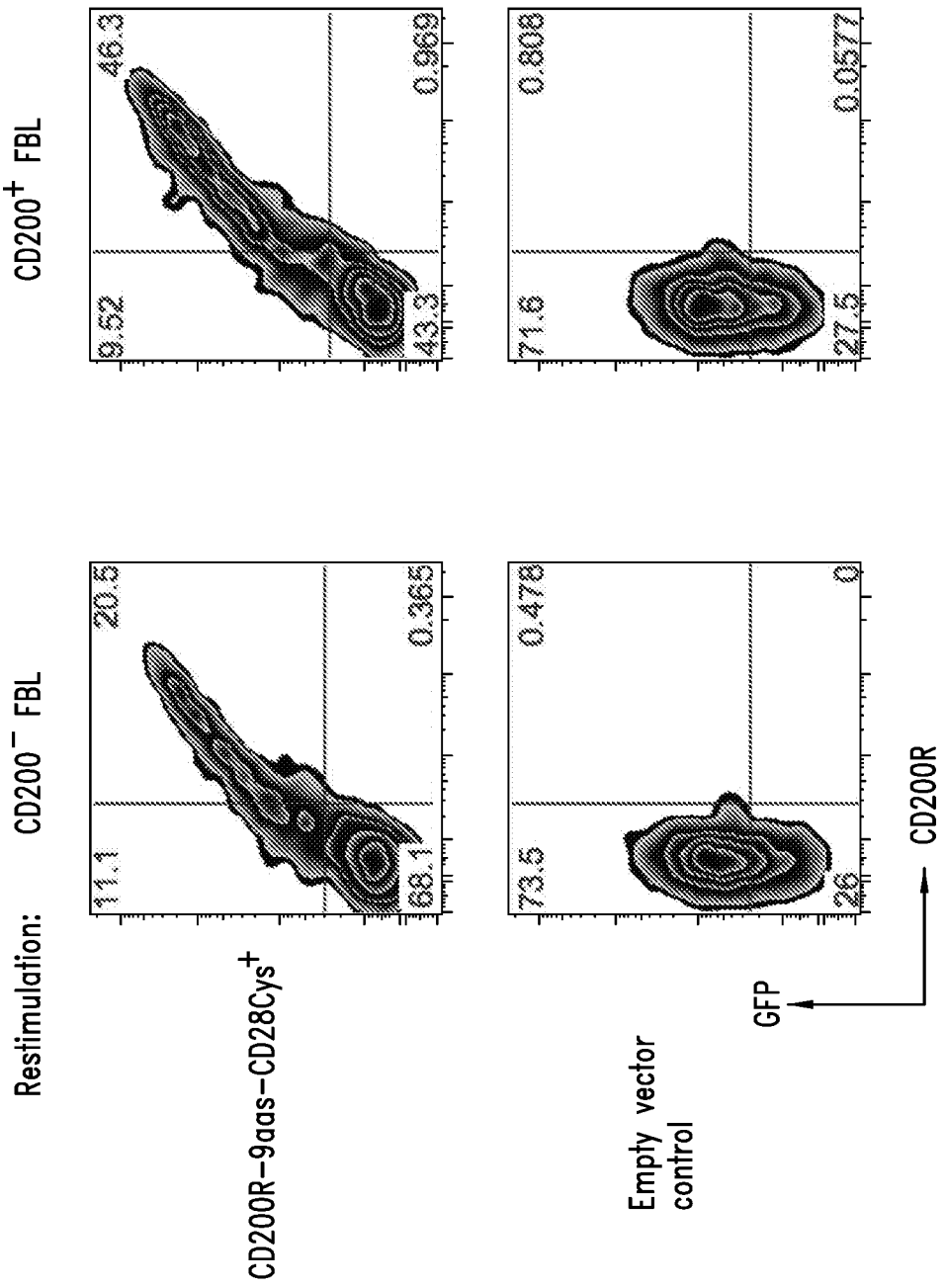

Restimulation:
CD200⁻ FBL
CD200⁺ FBL
CD200R-9aas-CD28Cys⁺
Empty vector control
GFP
CD200R
11.1
20.5
68.1
0.365
9.52
46.3
43.3
0.969
73.5
0.478
26
0
71.6
0.808
27.5
0.0577

FIG. 2C

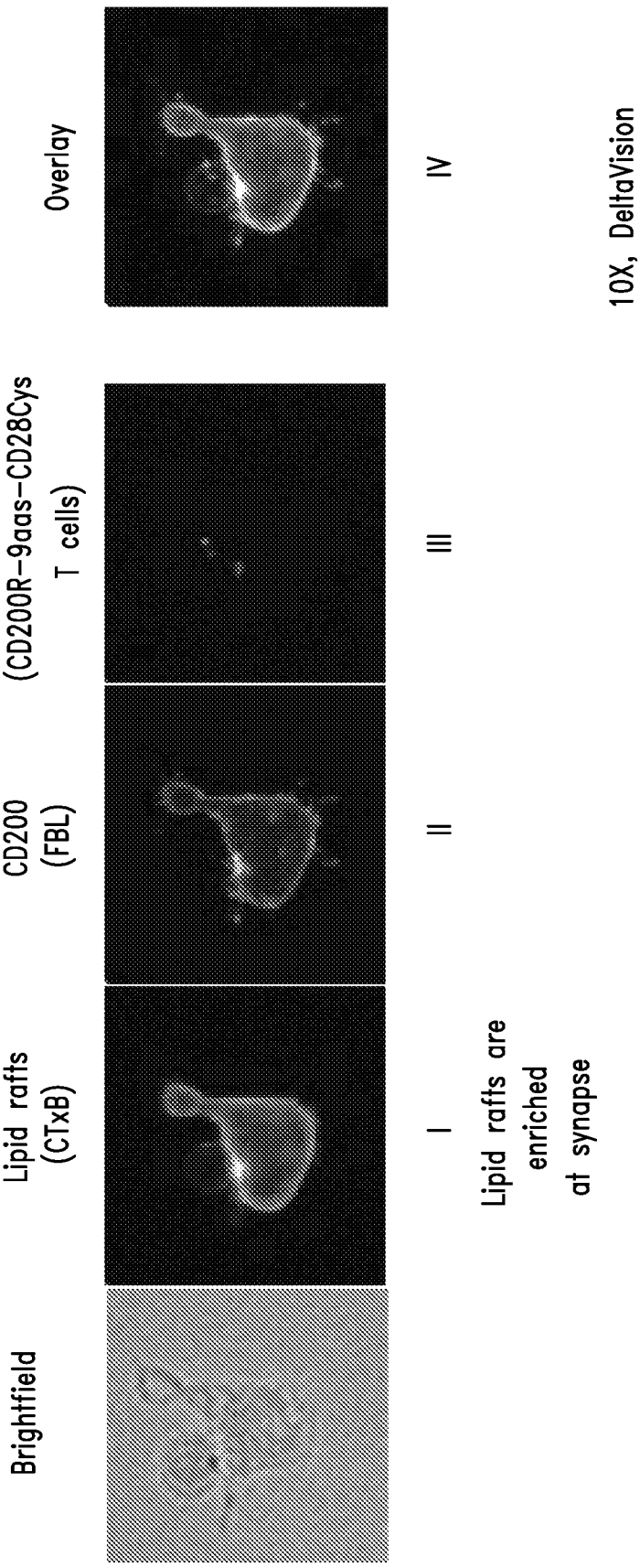

Brightfield
Lipid rafts (CTxB)
CD200 (FBL)
CD200R (CD200R-9aas-CD28Cys T cells)
Overlay
I
II
III
IV
Lipid rafts are enriched at synapse
10X, DeltaVision

FIG. 2D

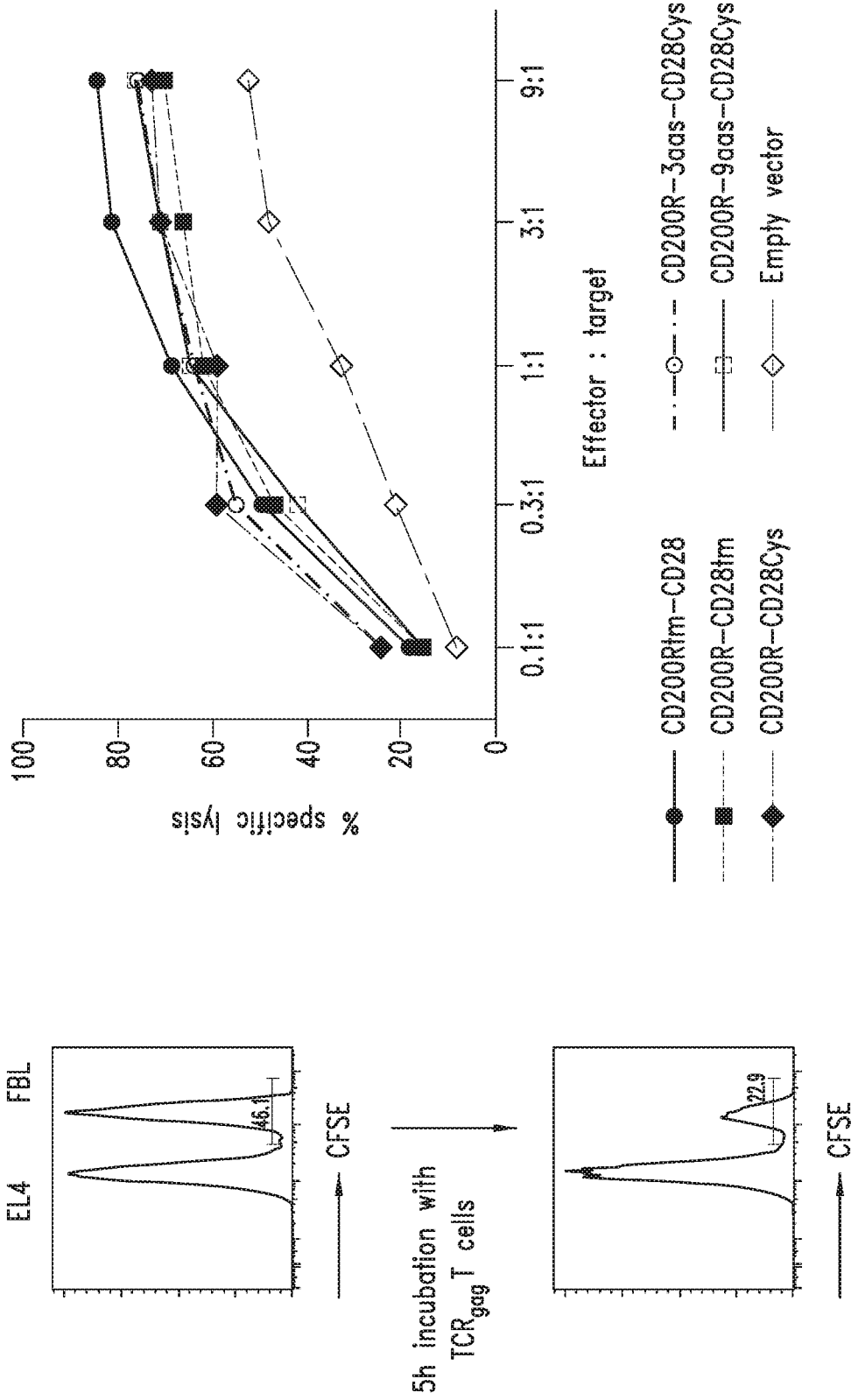

% specific lysis
100
80
60
40
20
0
0.1:1
0.3:1
1:1
3:1
9:1
Effector : target
CD200Rtm-CD28
CD200R-CD28tm
CD200R-CD28Cys
CD200R-3aas-CD28Cys
CD200R-9aas-CD28Cys
Empty vector
EL4
FBL
46.1
CFSE
5h incubation with
TCR$_{gag}$ T cells
22.9
CFSE

FIG. 2E

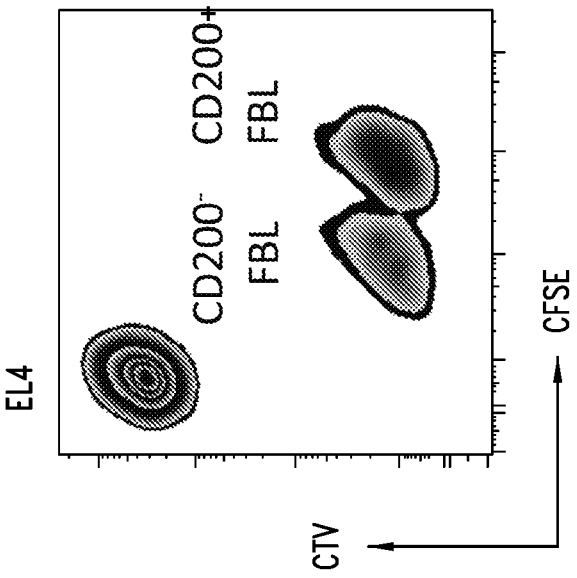


FIG. 2F

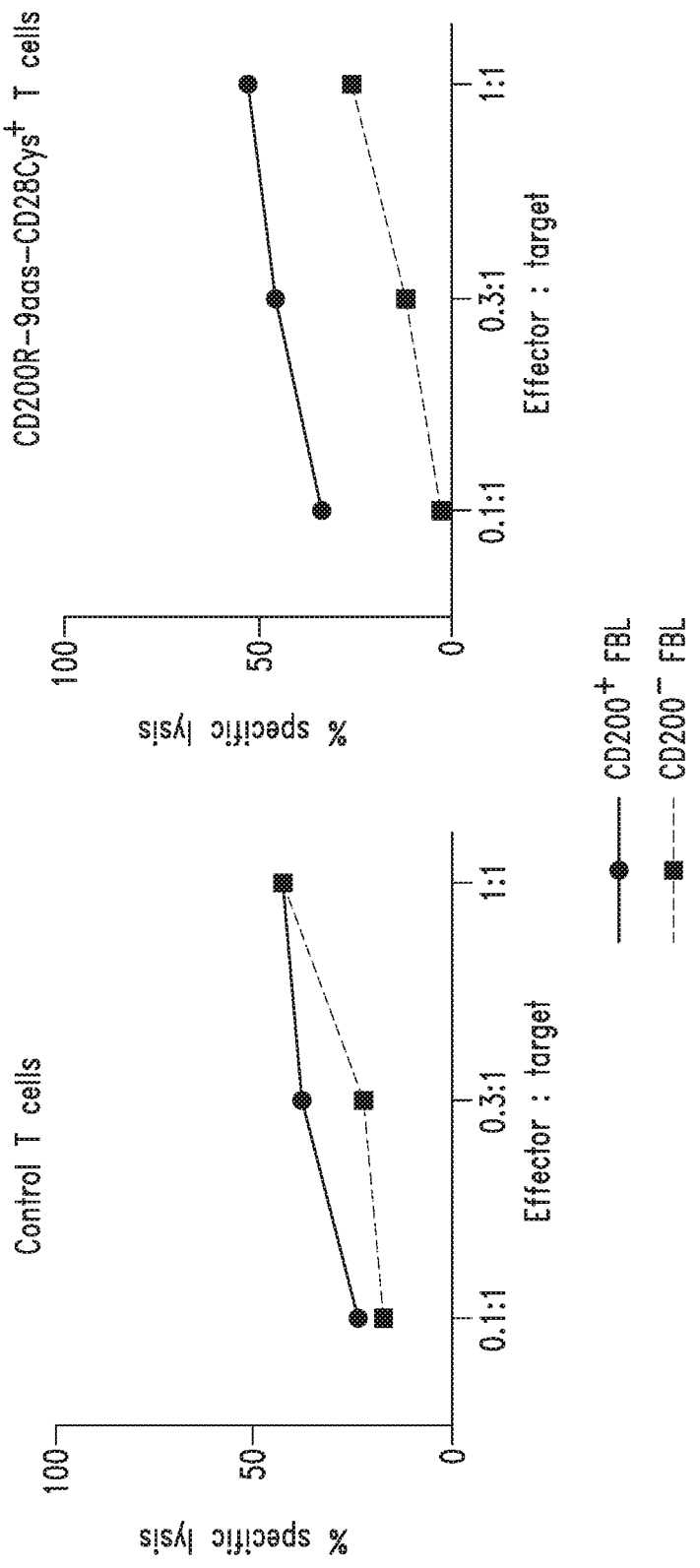

CD200R-9aas-CD28Cys+ T cells
Control T cells
% specific lysis
100
50
0
0.1:1
0.3:1
1:1
Effector : target
CD200+ FBL
CD200− FBL

FIG. 2G

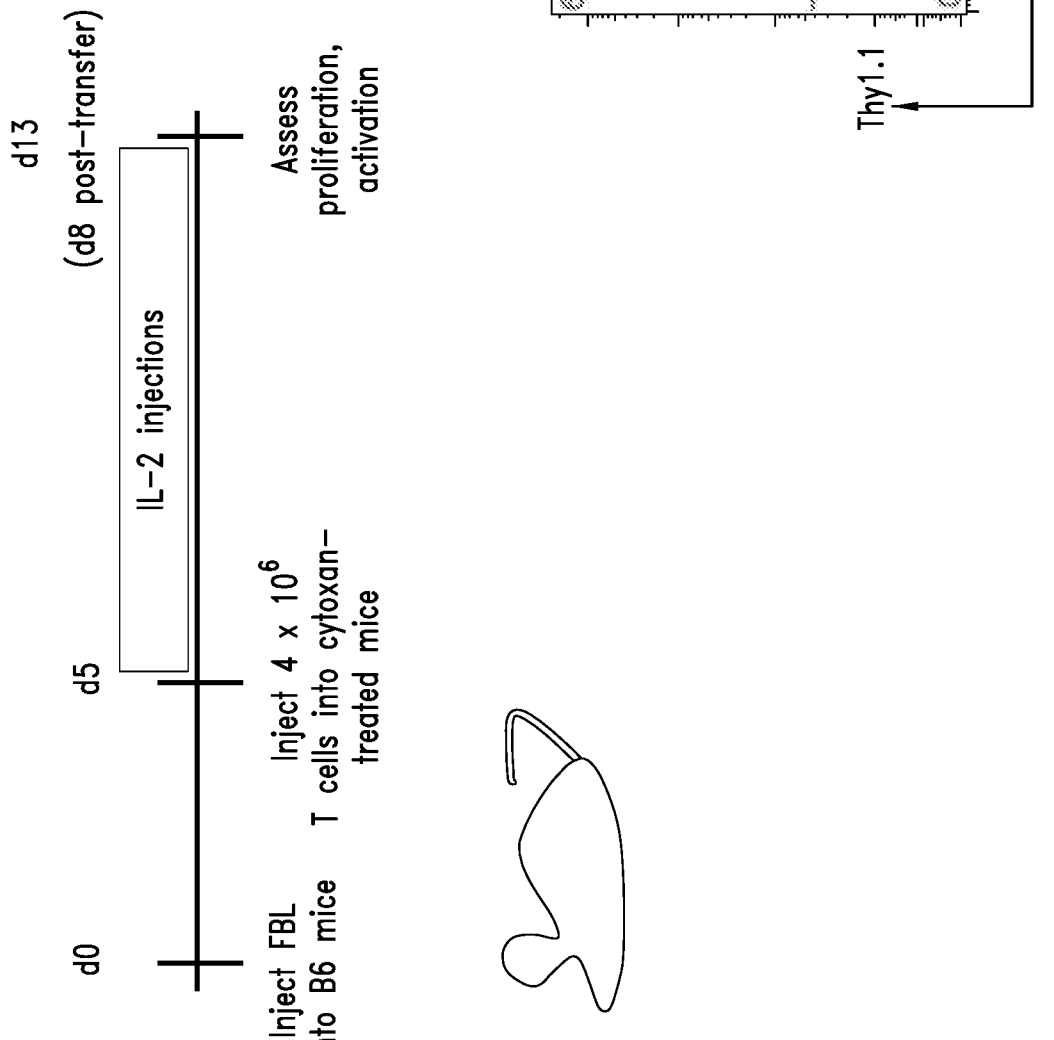

d0
d5
d13
(d8 post-transfer)
IL-2 injections
Inject FBL into B6 mice
Inject 4 x 10^6 T cells into cytoxan-treated mice
Assess proliferation, activation
Injected:
CD200R construct
Empty vector
23.7
36.3
0
0
Thy1.1
Thy1.2

FIG. 3A

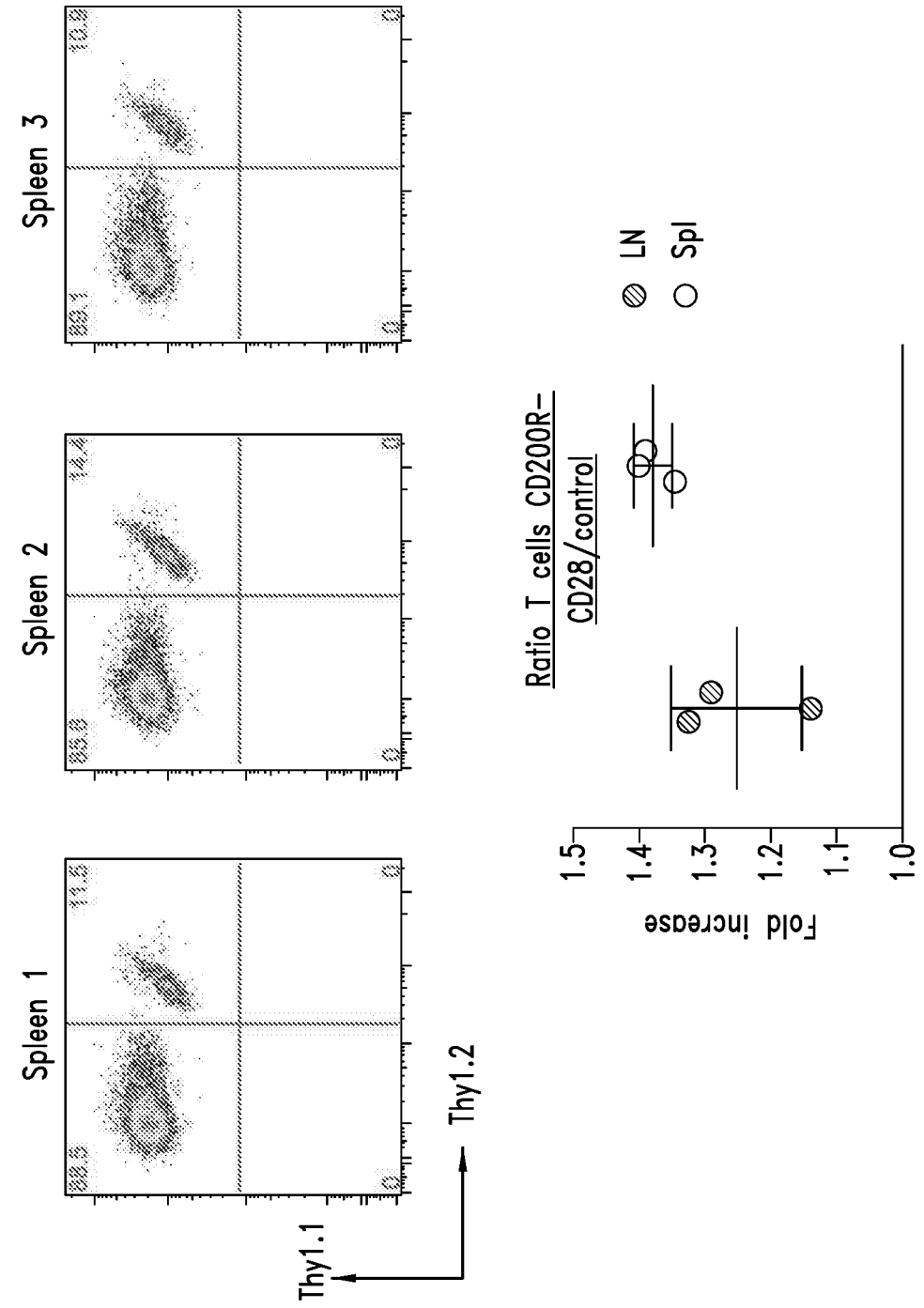

Spleen 1
Spleen 2
Spleen 3
Thy1.1
Thy1.2
Ratio T cells CD200R-
CD28/control
LN
Spl
Fold increase
1.5
1.4
1.3
1.2
1.1
1.0

FIG. 3B

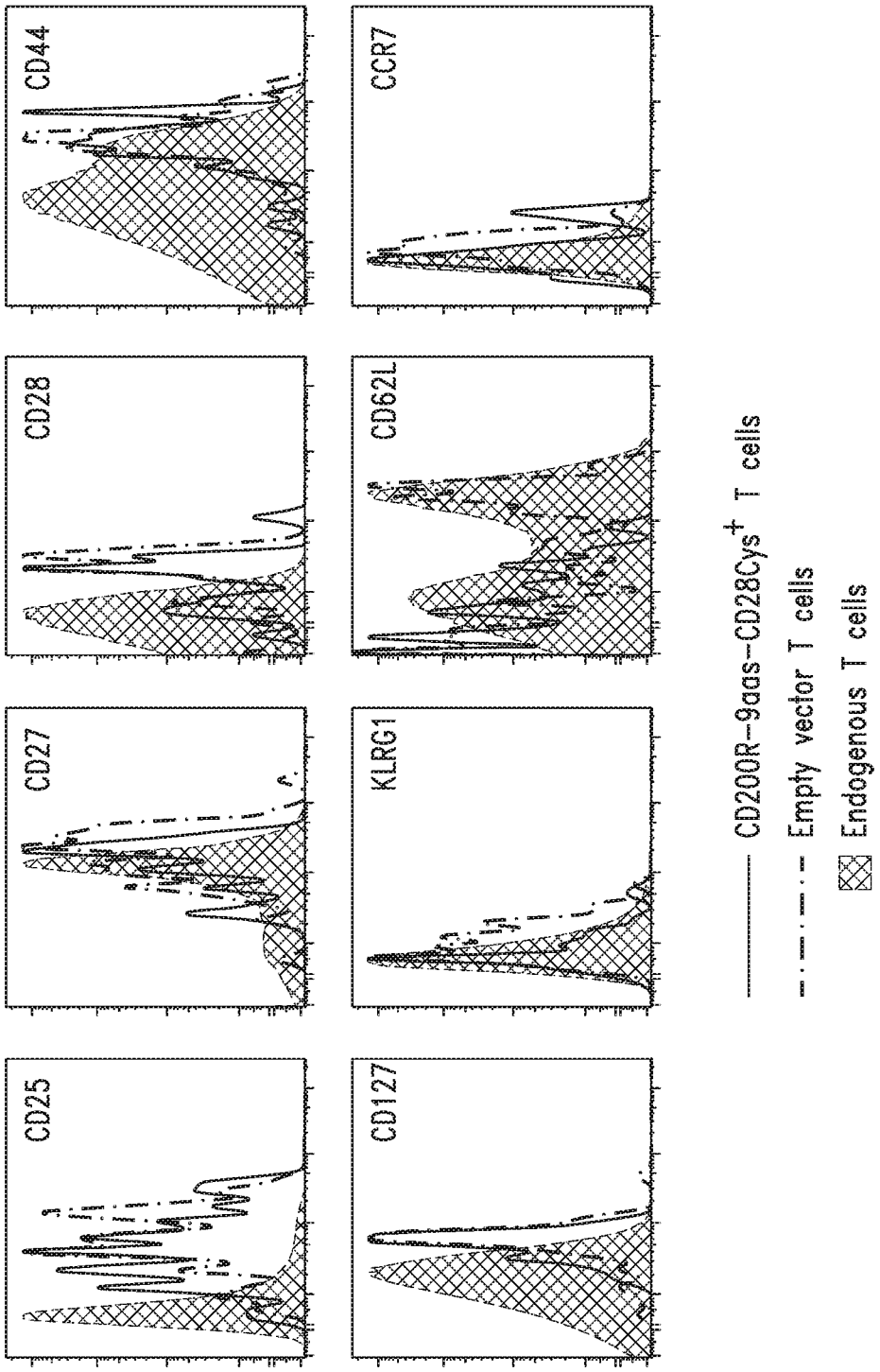

CD44
CCR7
CD28
CD62L
CD27
KLRG1
CD25
CD127
CD200R-9aas-CD28Cys+ T cells
Empty vector T cells
Endogenous T cells

FIG. 3C

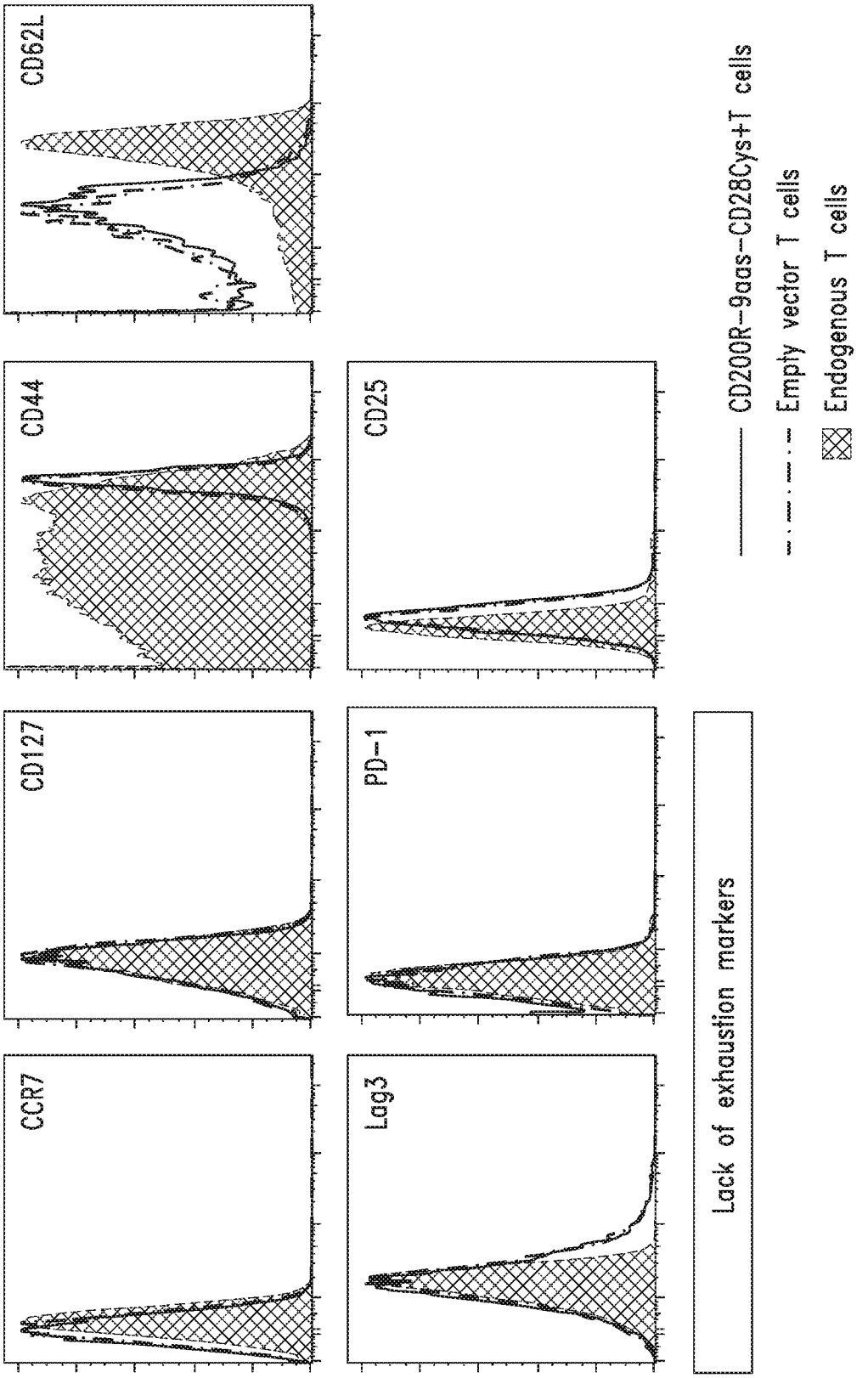

CD62L
CD44
CD127
CCR7
CD25
PD-1
Lag3
Lack of exhaustion markers
CD200R-9aas-CD28Cys+T cells
Empty vector T cells
Endogenous T cells

FIG. 3D

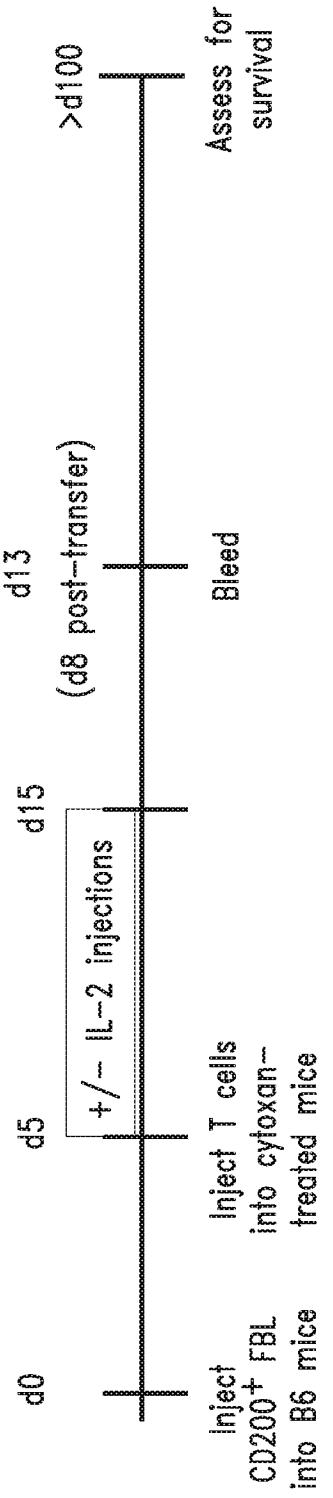

d0
d5
d15
d13
(d8 post-transfer)
>d100
+/- IL-2 injections
Inject CD200+ FBL into B6 mice
Inject T cells into cytoxan-treated mice
Bleed
Assess for survival

FIG. 4A

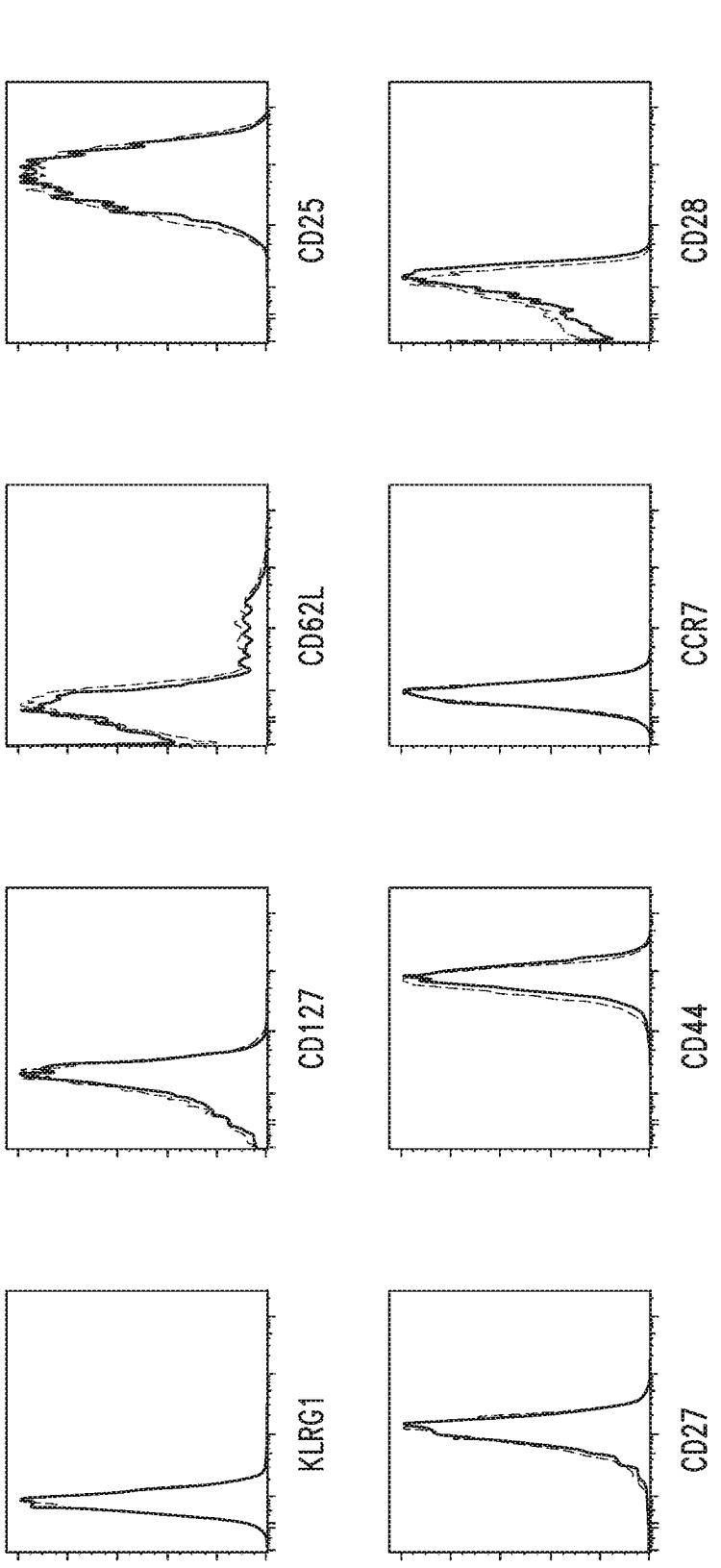

KLRG1
CD127
CD62L
CD25
CD27
CD44
CCR7
CD28
d5 post-stim 3
CD200R-CD28+
CD200R-CD28-

FIG. 4B

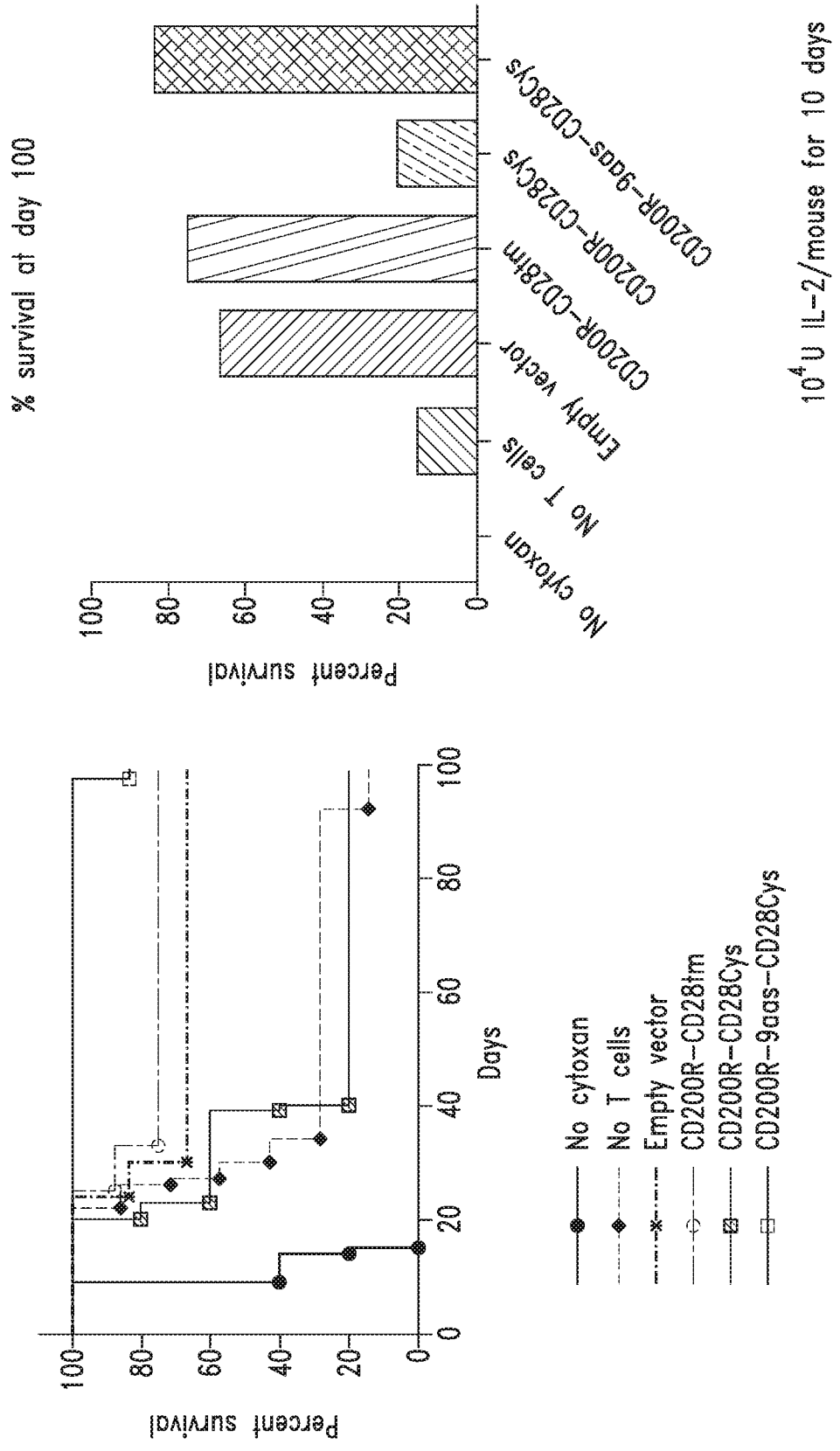

% survival at day 100
Percent survival
100
80
60
40
20
0
No cytoxan
No T cells
Empty vector
CD200R-CD28tm
CD200R-CD28Cys
CD200R-9aas-CD28Cys
10^4 U IL-2/mouse for 10 days
Percent survival
100
80
60
40
20
0
0
20
40
60
80
100
Days
No cytoxan
No T cells
Empty vector
CD200R-CD28tm
CD200R-CD28Cys
CD200R-9aas-CD28Cys

FIG. 4C

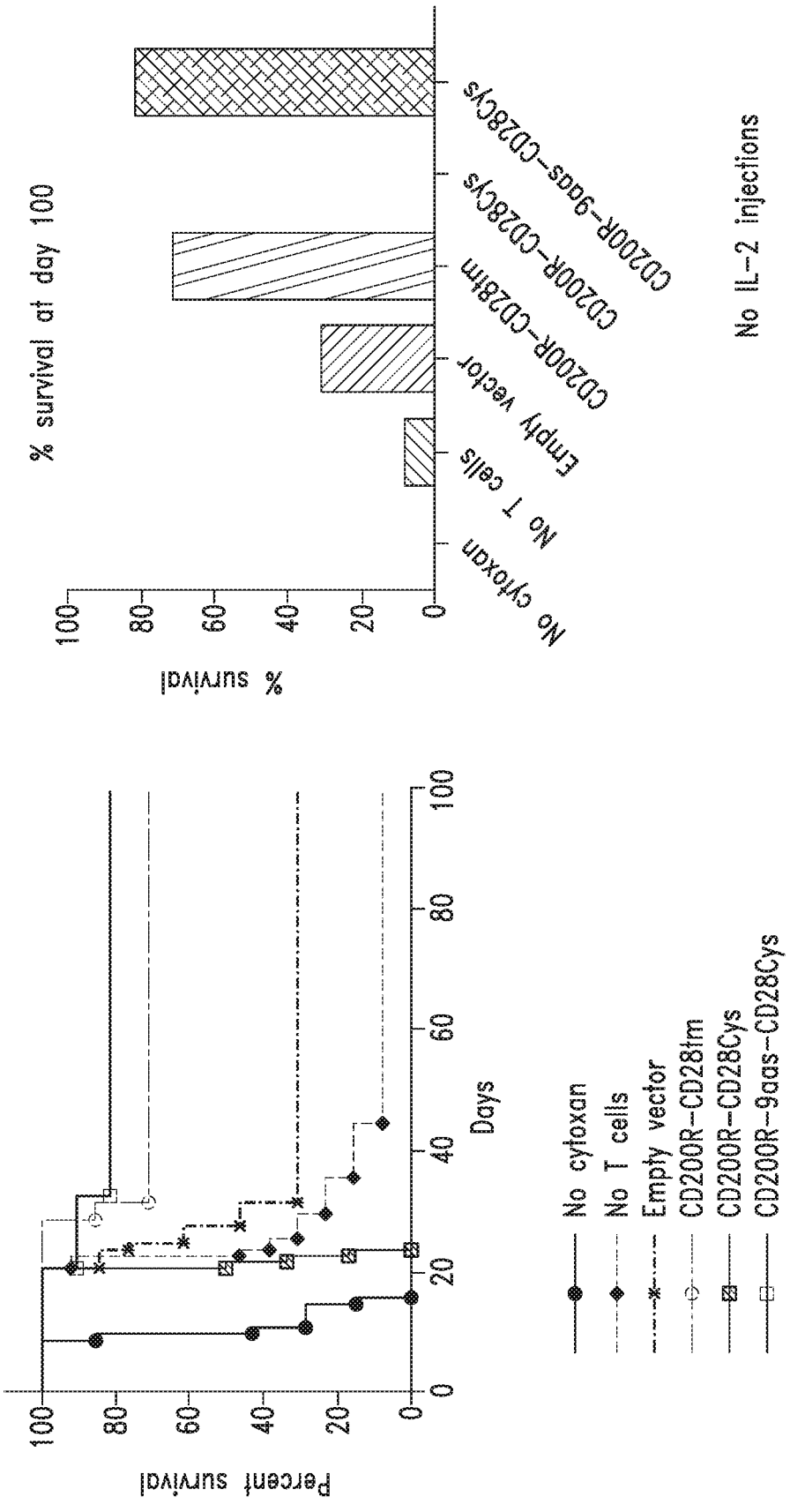

Percent survival
100
80
60
40
20
0
0
20
40
60
80
100
Days
No cytoxan
No T cells
Empty vector
CD200R-CD28tm
CD200R-CD28Cys
CD200R-9aas-CD28Cys
% survival
100
80
60
40
20
0
% survival at day 100
No cytoxan
No T cells
Empty vector
CD200R-CD28tm
CD200R-CD28Cys
CD200R-9aas-CD28Cys
No IL-2 injections

FIG. 4D

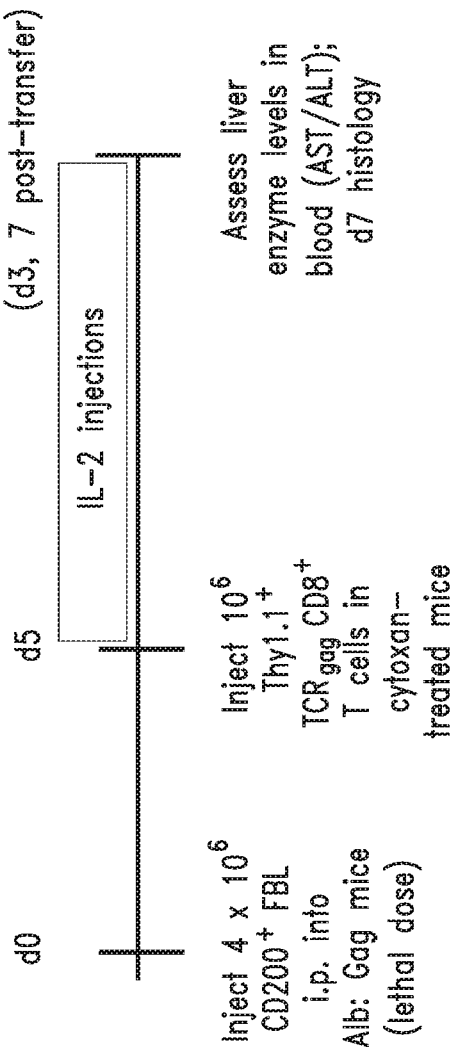

d0
d5
d8, 12
(d3, 7 post-transfer)
IL-2 injections
Inject 4 x 10^6 CD200+ FBL i.p. into Alb: Gag mice (lethal dose)
Inject 10^6 Thy1.1+ TCRgag CD8+ T cells in cytoxan-treated mice
Assess liver enzyme levels in blood (AST/ALT); d7 histology

FIG. 5A

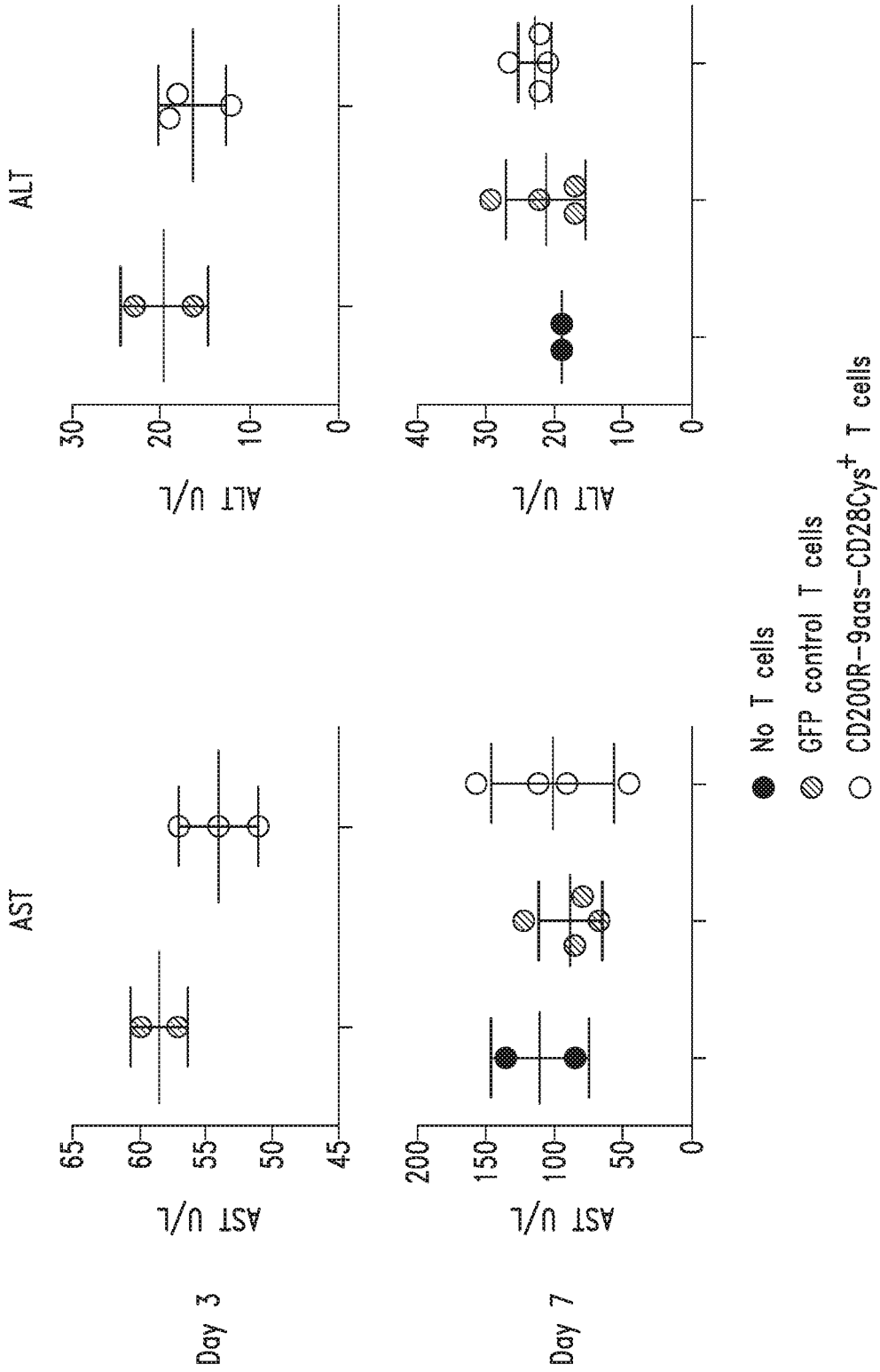

Day 3
Day 7
AST
ALT
AST U/L
ALT U/L
65
60
55
50
45
200
150
100
50
0
40
30
20
10
No T cells
GFP control T cells
CD200R-9aas-CD28Cys+ T cells

FIG. 5B

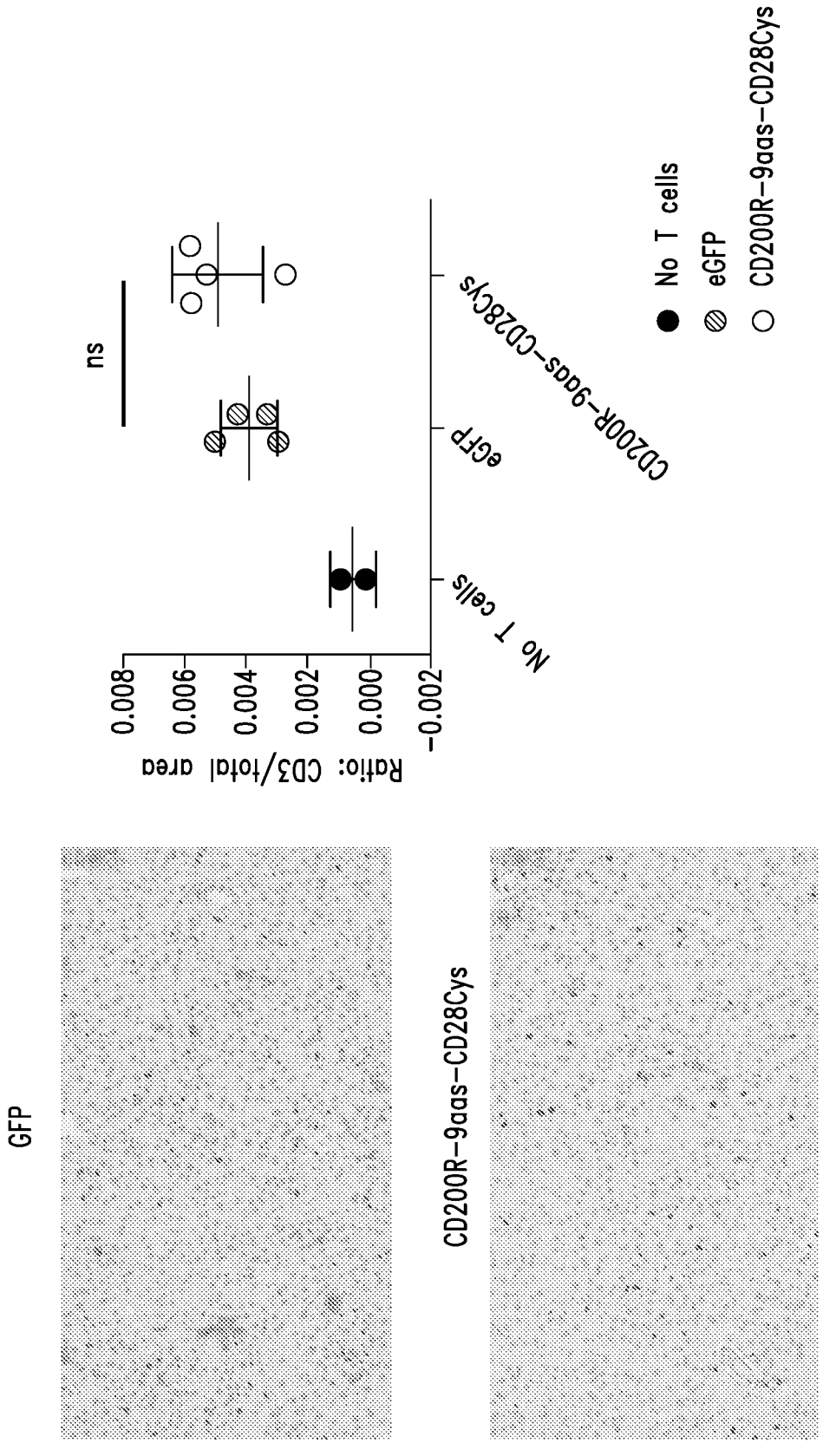

GFP
CD200R-9aas-CD28Cys
Ratio: CD3/total area
0.008
0.006
0.004
0.002
0.000
−0.002
ns
No T cells
eGFP
CD200R-9aas-CD28Cys
No T cells
eGFP
CD200R-9aas-CD28Cys

FIG. 5C

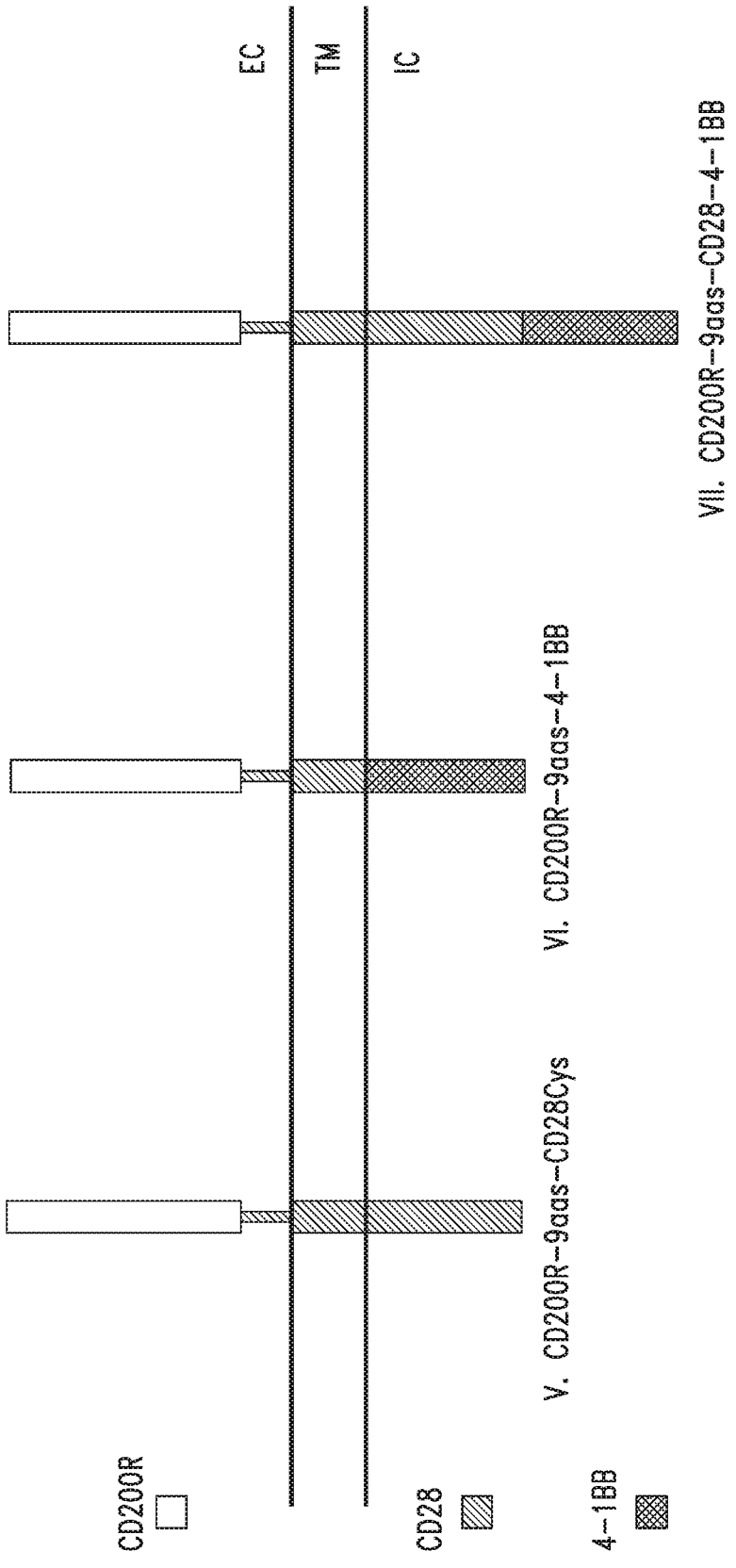

EC
TM
IC
VII. CD200R-9aas-CD28-4-1BB
VI. CD200R-9aas-4-1BB
V. CD200R-9aas-CD28Cys
CD200R
CD28
4-1BB

FIG. 6A

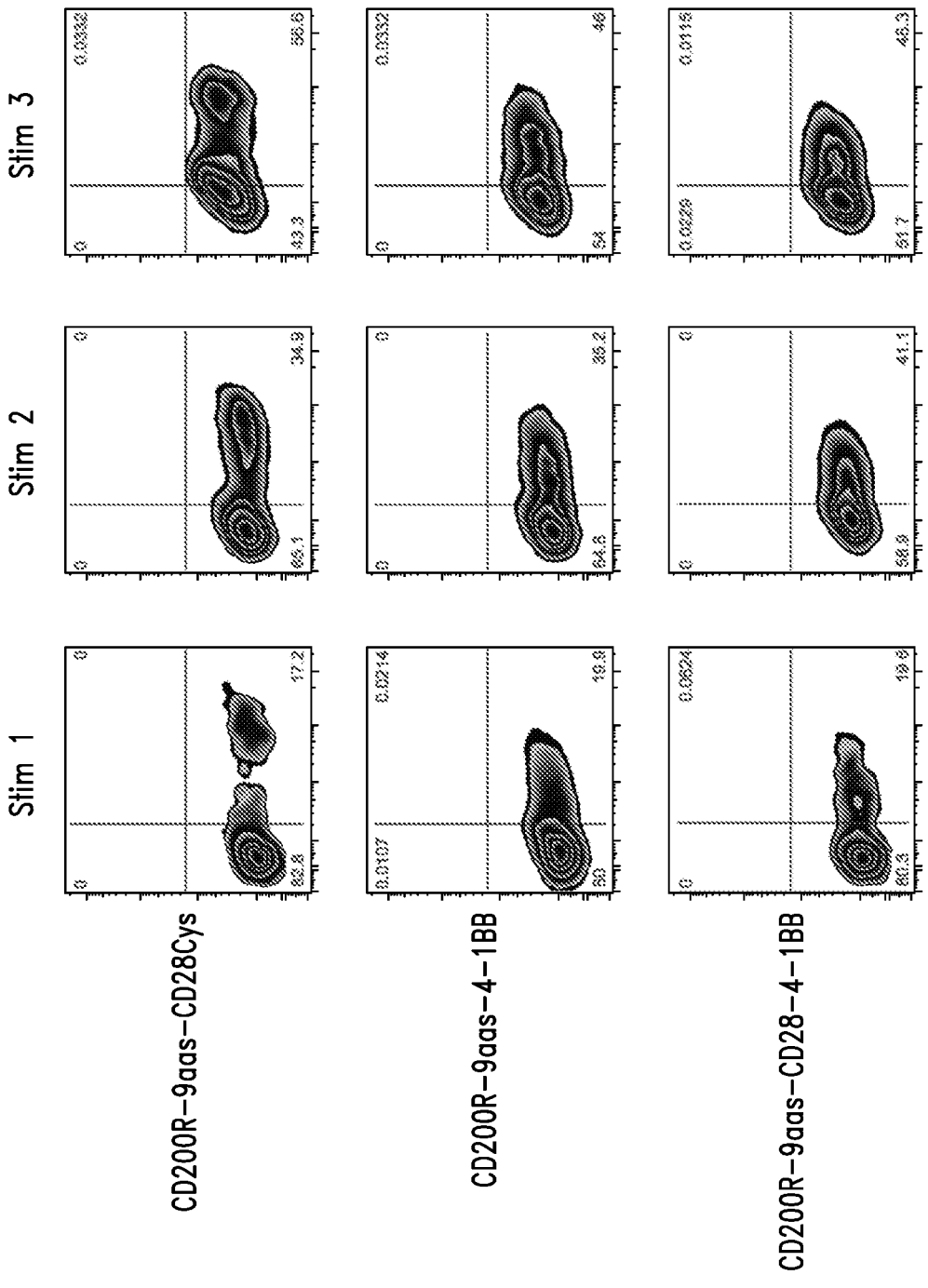

Stim 1
Stim 2
Stim 3
CD200R-9aas-CD28Cys
CD200R-9aas-4-1BB
CD200R-9aas-CD28-4-1BB
CD200R

FIG. 6B

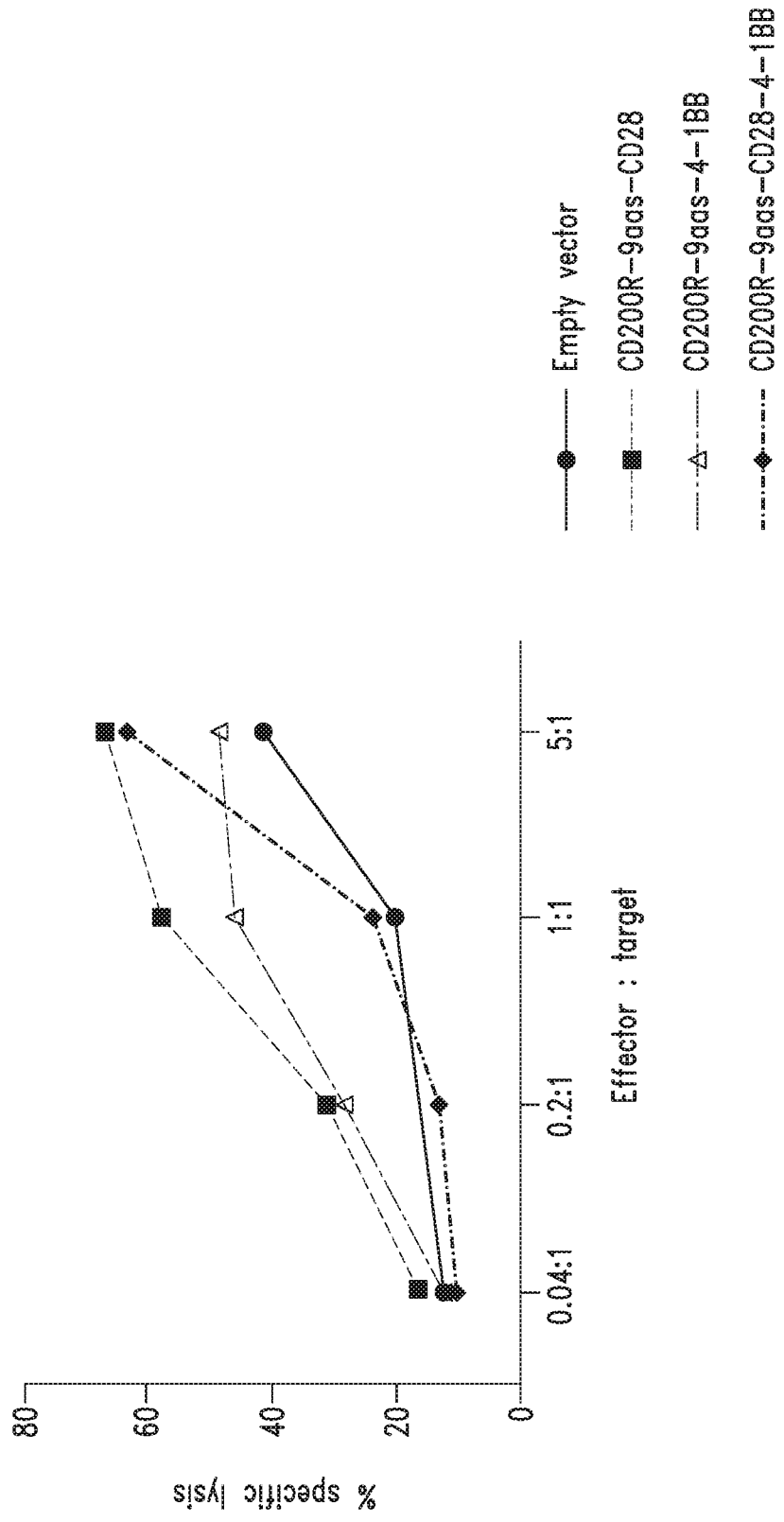

% specific lysis
80
60
40
20
0
0.04:1
0.2:1
1:1
5:1
Effector : target
Empty vector
CD200R-9aas-CD28
CD200R-9aas-4-1BB
CD200R-9aas-CD28-4-1BB

FIG. 6C

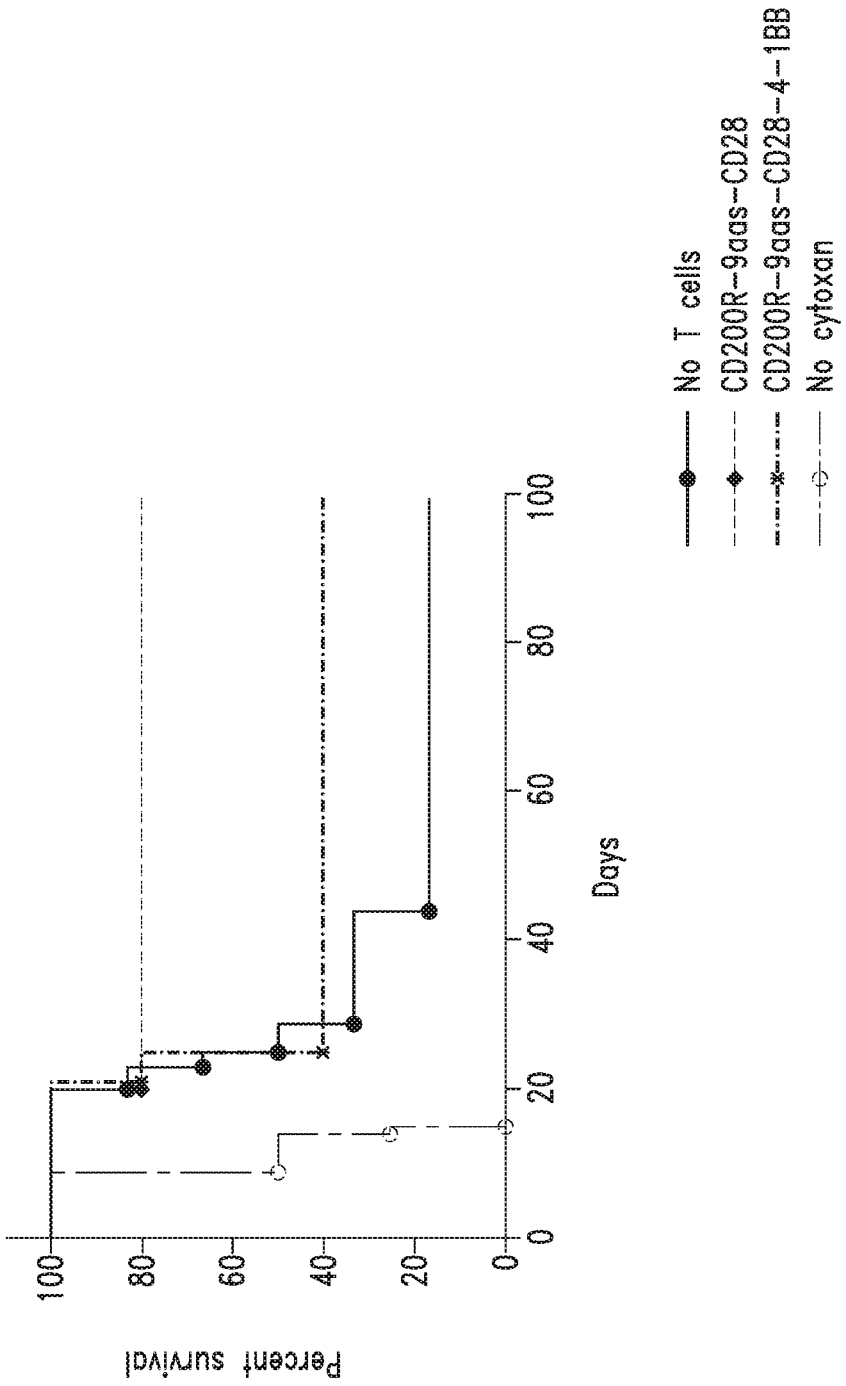

Percent survival
100
80
60
40
20
0
0
20
40
60
80
100
Days
No T cells
CD200R-9aas-CD28
CD200R-9aas-CD28-4-1BB
No cytoxan

FIG. 6D

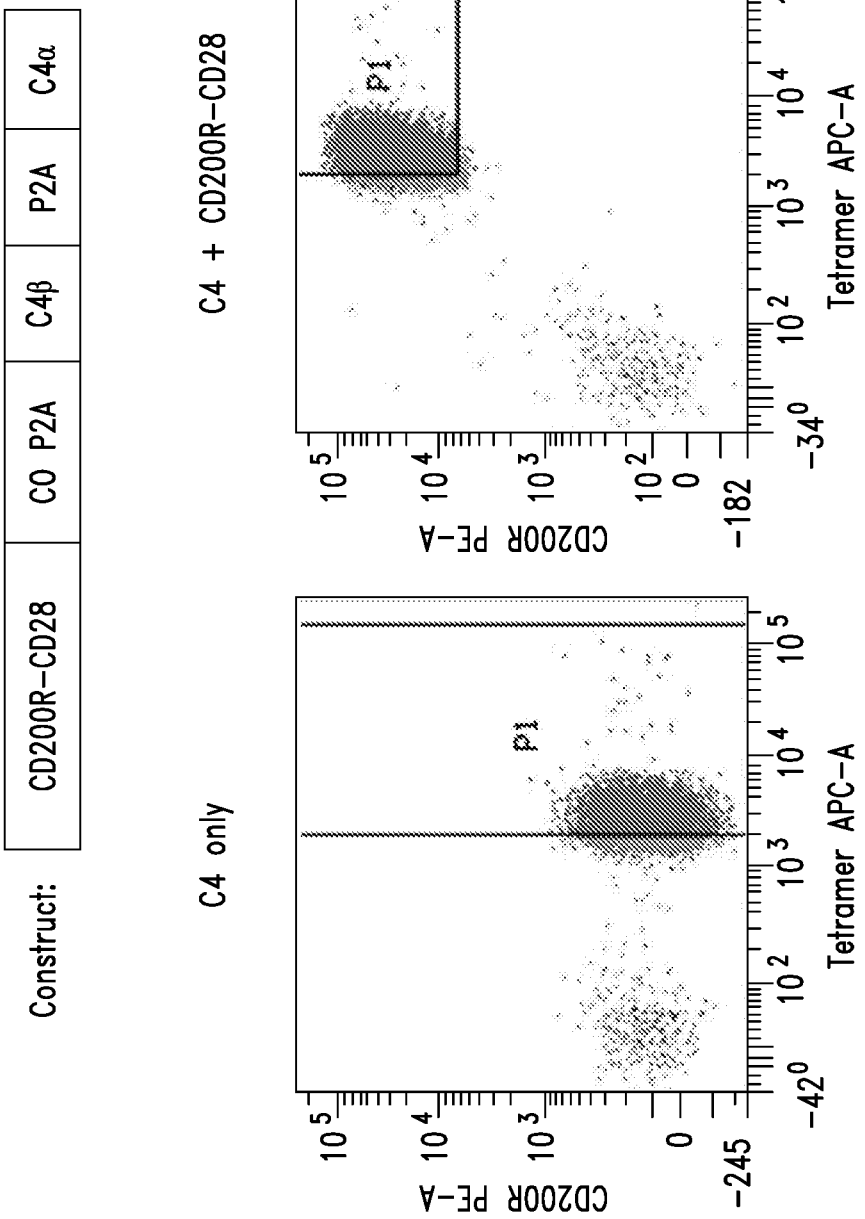

Construct:
CD200R-CD28
CO P2A
Cβ
P2A
Cα
C4 only
C4 + CD200R-CD28
P1
CD200R PE-A
Tetramer APC-A
-245
-42
-182
-34

FIG. 7A

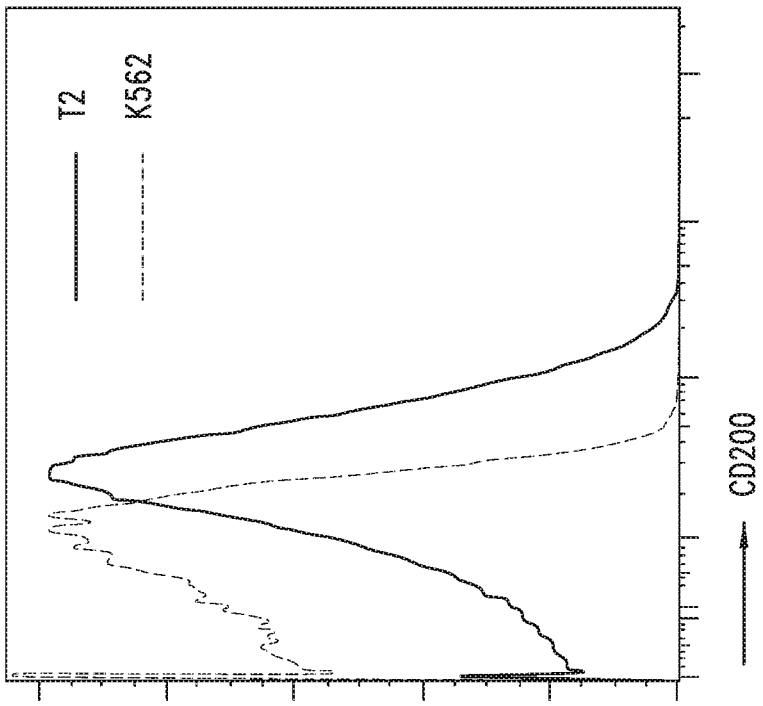


FIG. 7B

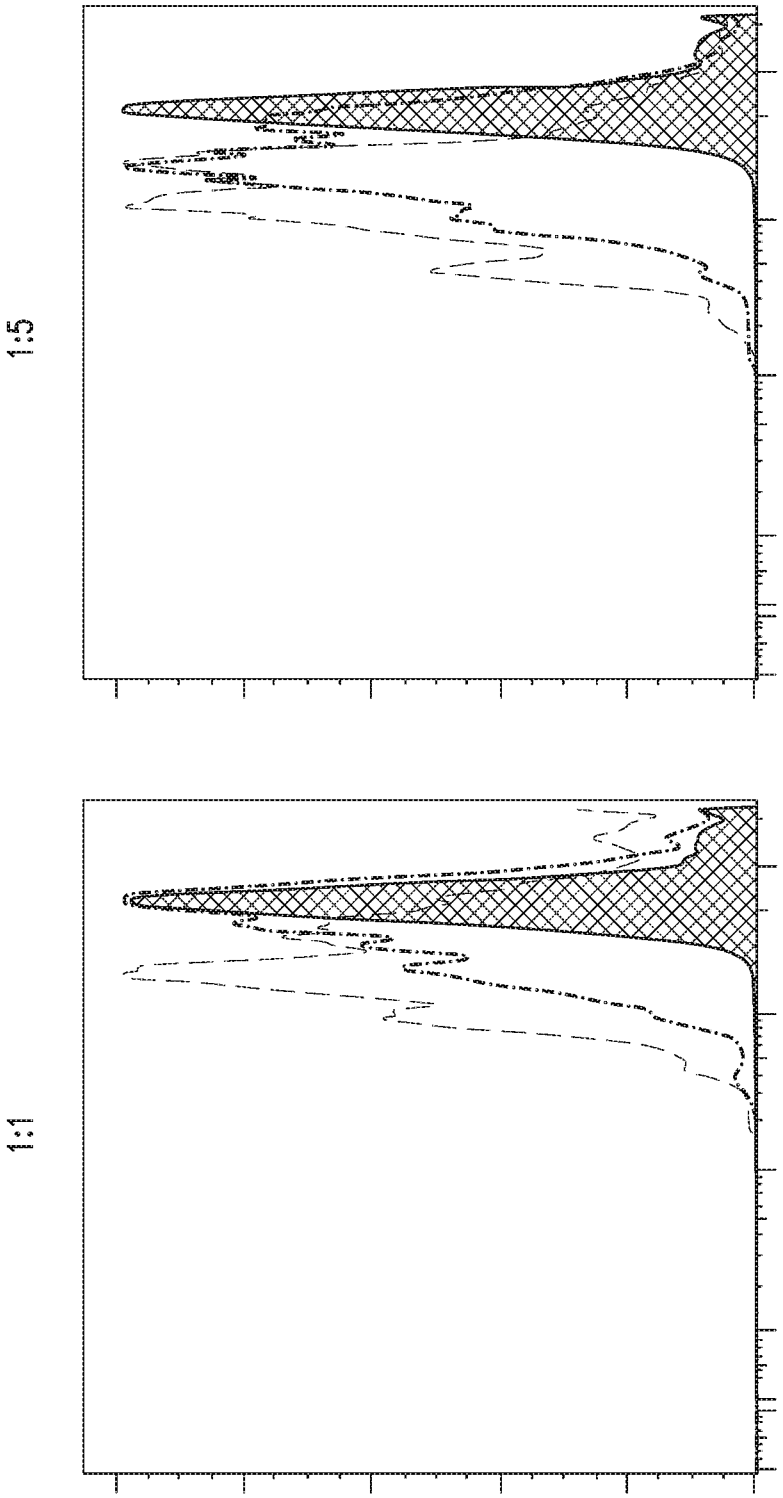


FIG. 7C

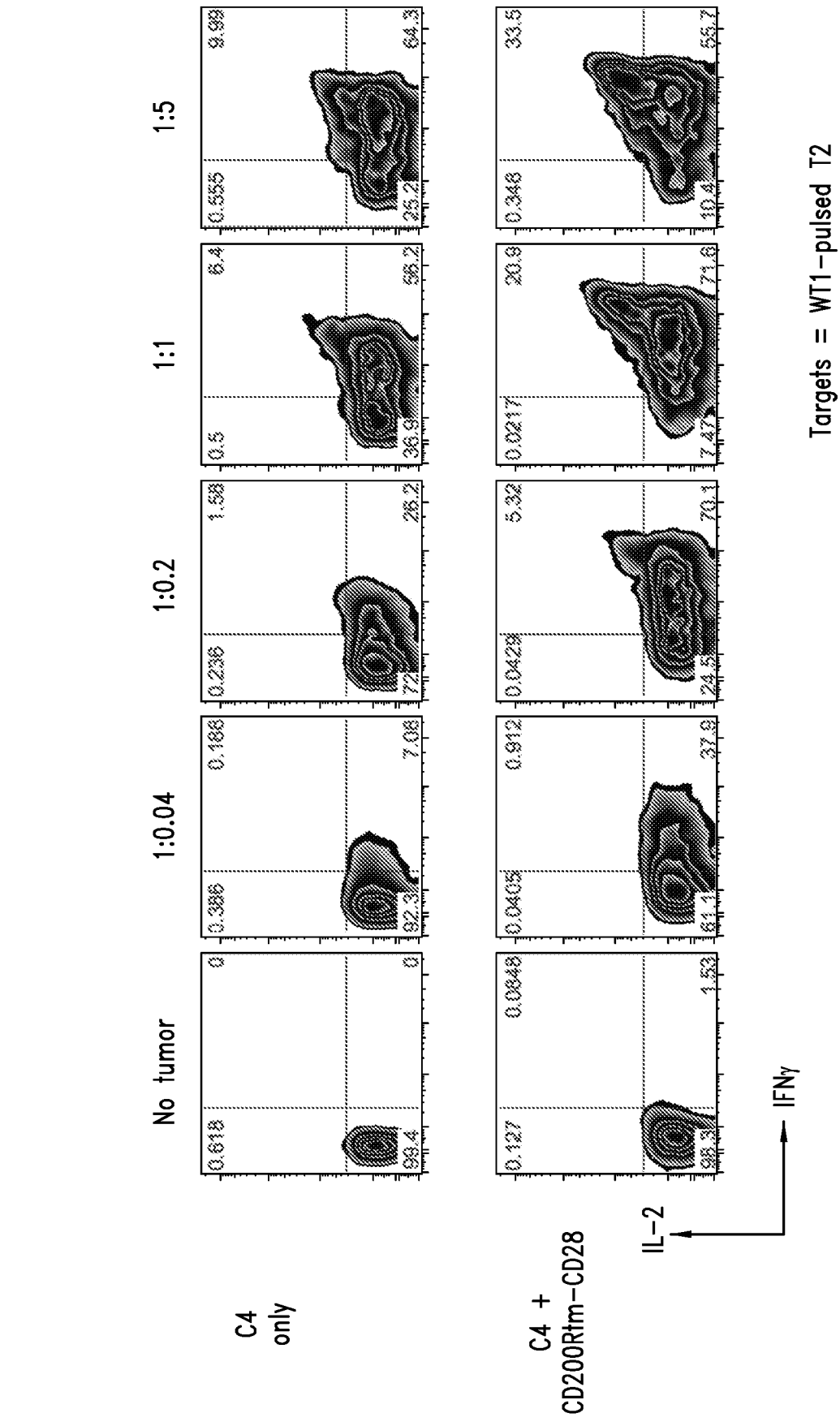

No tumor
1:0.04
1:0.2
1:1
1:5
C4 only
C4 + CD200Rtm-CD28
IL-2
IFNγ
Targets = WT1-pulsed T2

FIG. 7D

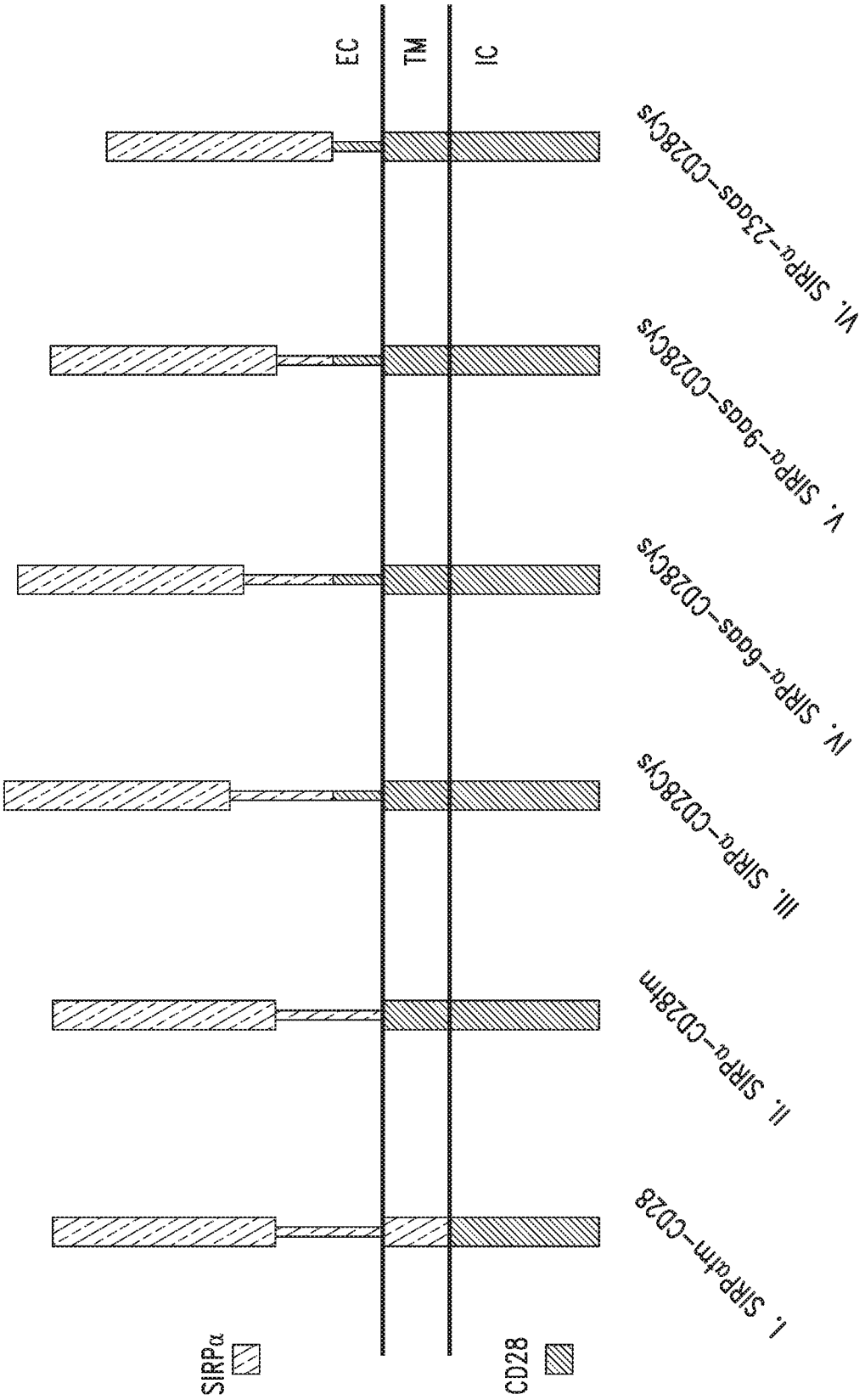

EC
TM
IC
VI. SIRPα-23aas-CD28Cys
V. SIRPα-9aas-CD28Cys
IV. SIRPα-6aas-CD28Cys
III. SIRPα-CD28Cys
II. SIRPα-CD28tm
I. SIRPatm-CD28
SIRPα
CD28

FIG. 8A

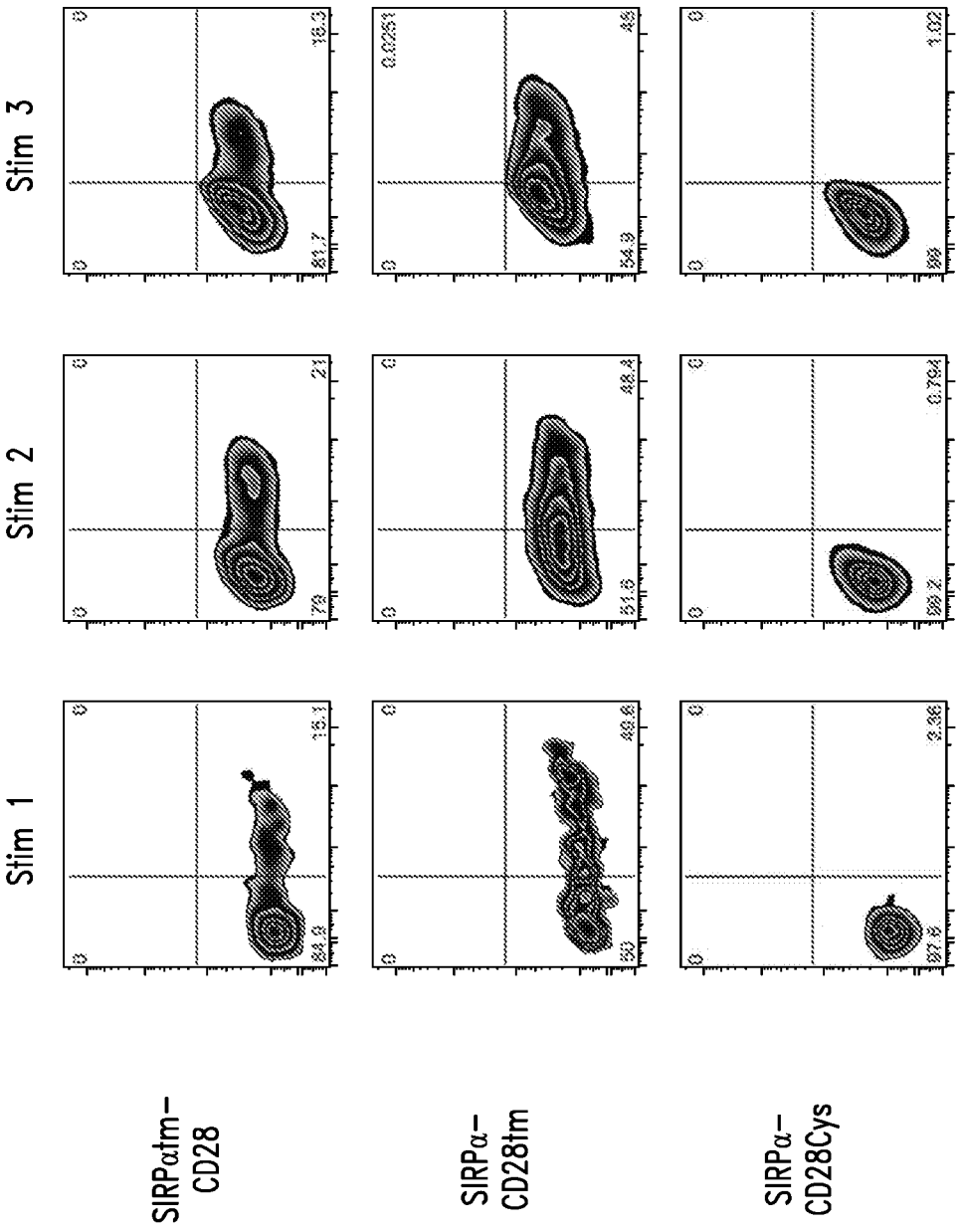

Stim 1
Stim 2
Stim 3
SIRPαtm–CD28
SIRPα–CD28tm
SIRPα–CD28Cys
SIRPα

FIG. 8B

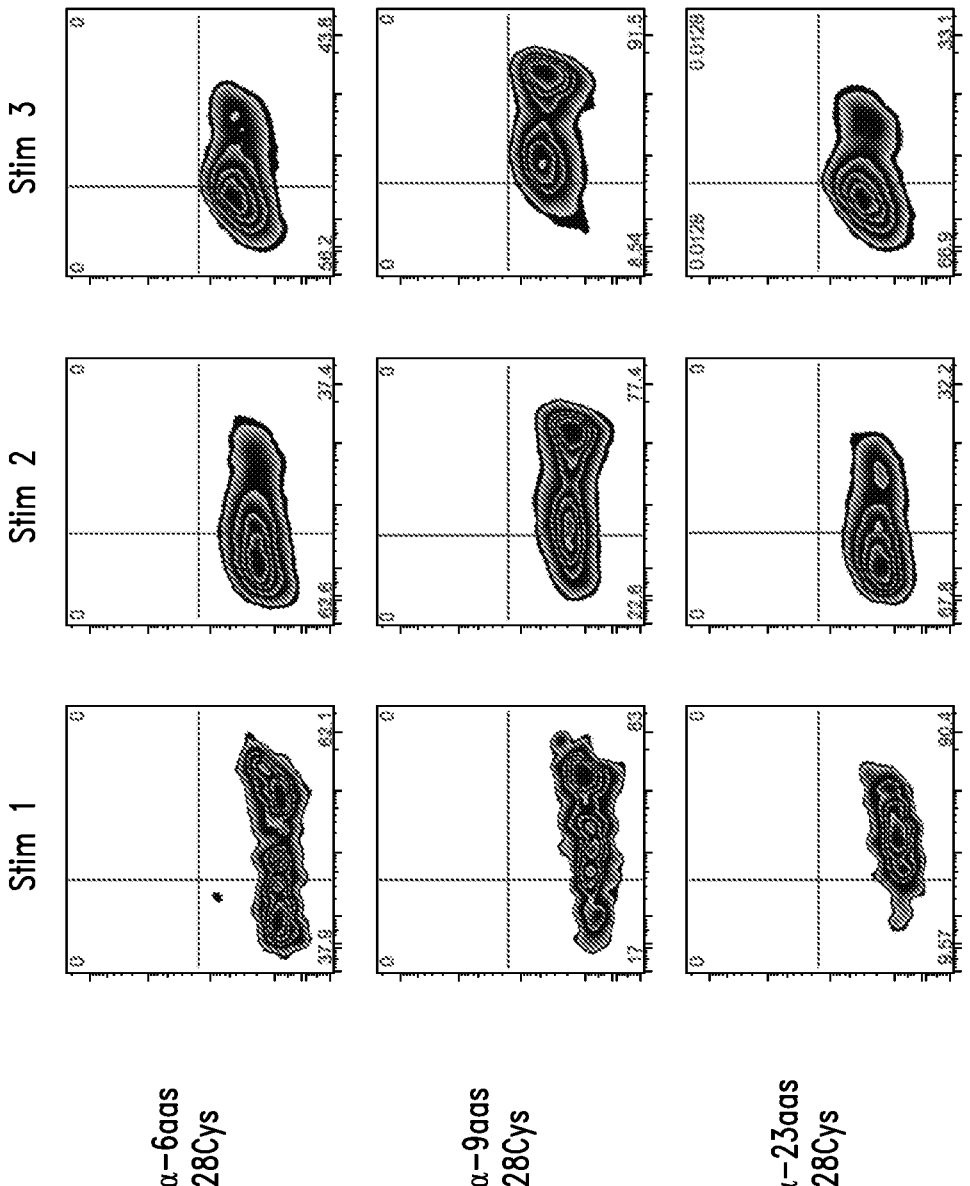

Stim 1
Stim 2
Stim 3
SIRPα−6aas CD28Cys
SIRPα−9aas CD28Cys
SIRPα−23aas CD28Cys
SIRPα

FIG. 8B (Cont.)

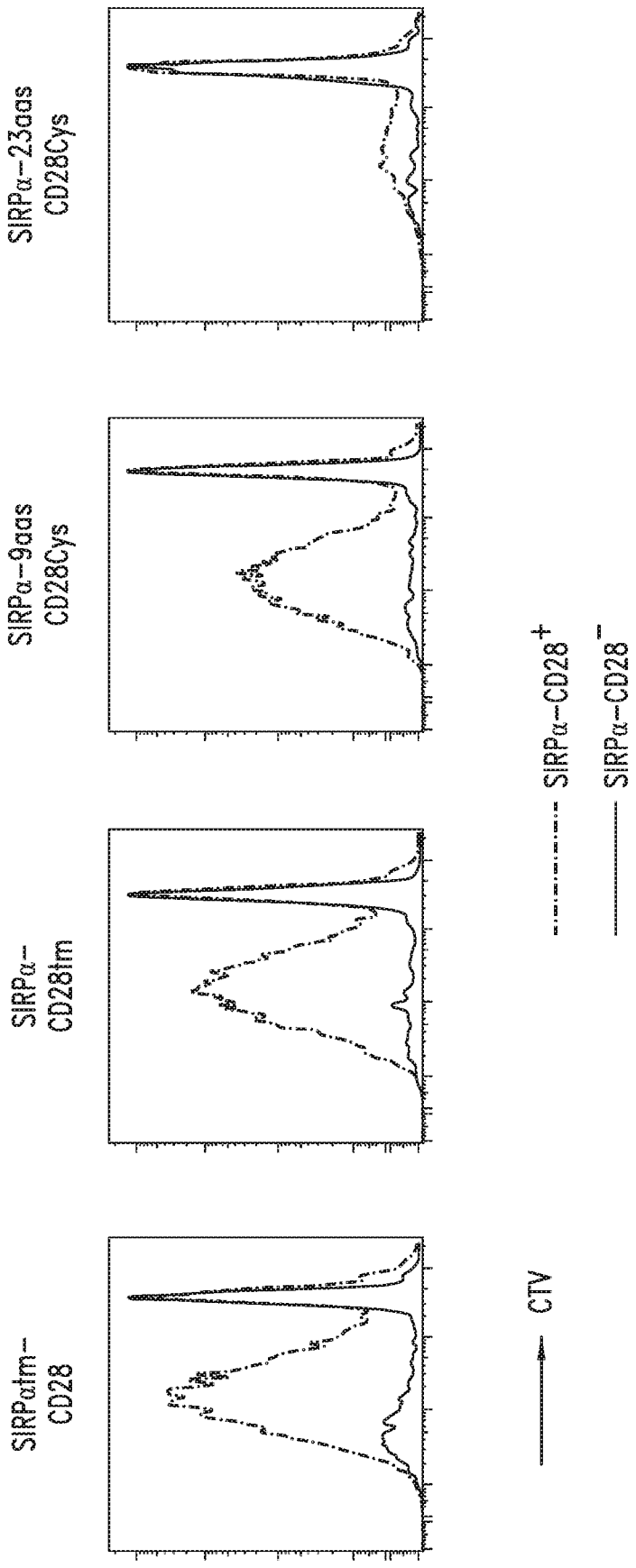

SIRPαtm–CD28
SIRPα–CD28tm
SIRPα–9aas CD28Cys
SIRPα–23aas CD28Cys
CTV
SIRPα–CD28+
SIRPα–CD28−

FIG. 8C

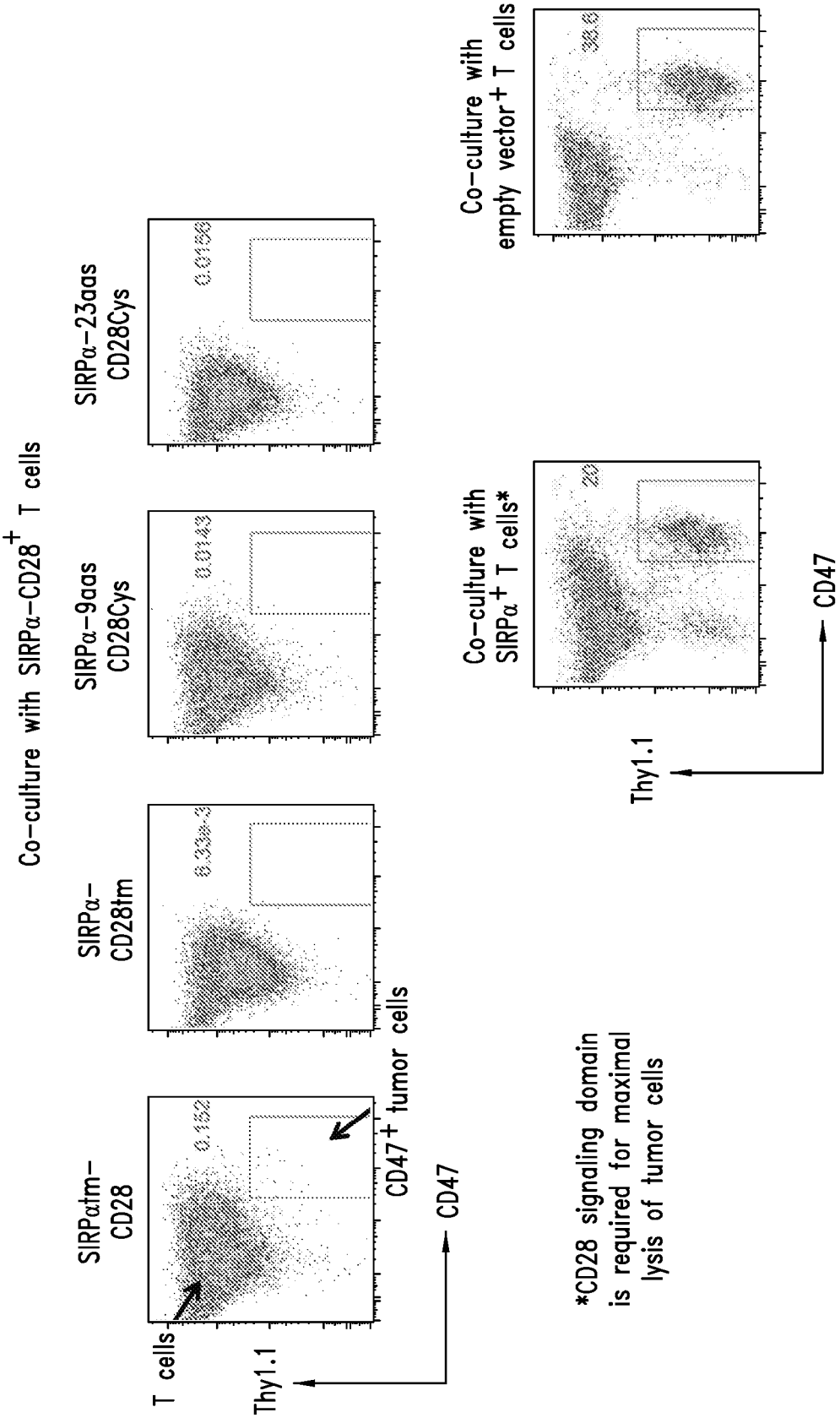

Co-culture with SIRPα-CD28+ T cells
SIRPαtm-CD28
SIRPα-CD28tm
SIRPα-9aas CD28Cys
SIRPα-23aas CD28Cys
0.152
0.0143
0.0158
T cells
Thy1.1
CD47+ tumor cells
CD47
Co-culture with SIRPα+ T cells*
Co-culture with empty vector+ T cells
*CD28 signaling domain is required for maximal lysis of tumor cells

FIG. 8D

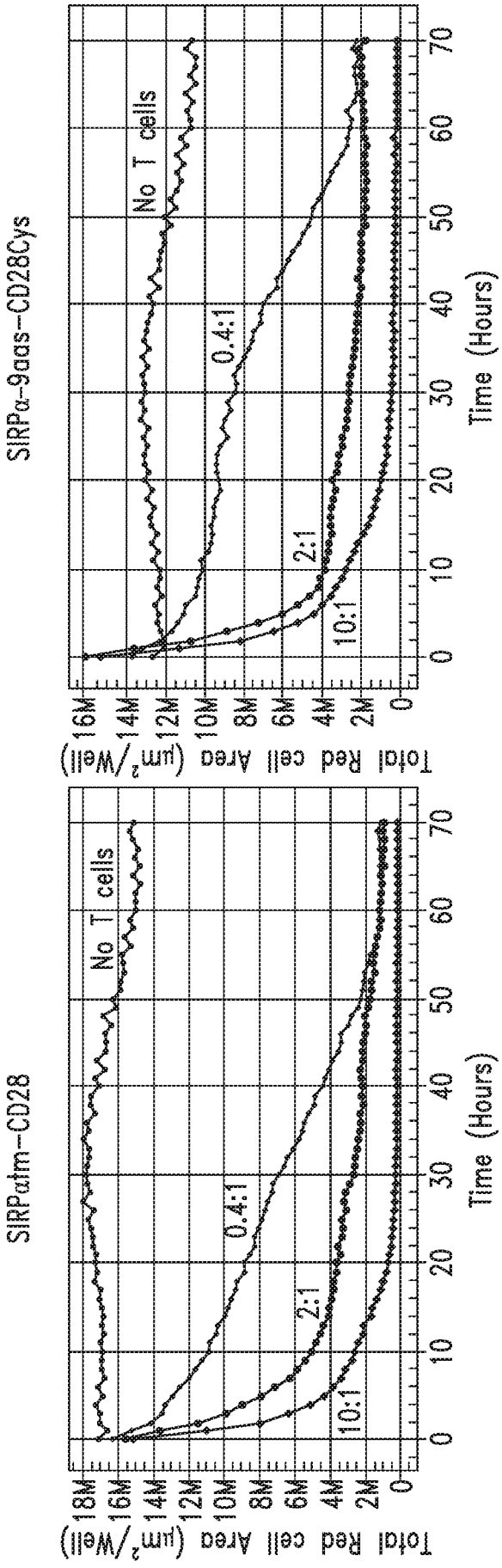

SIRPα-9aas-CD28Cys
SIRPαtm-CD28
No T cells
0.4:1
2:1
10:1
Total Red cell Area (μm²/Well)
Time (Hours)
0
2M
4M
6M
8M
10M
12M
14M
16M
18M
0
10
20
30
40
50
60
70

FIG. 8E

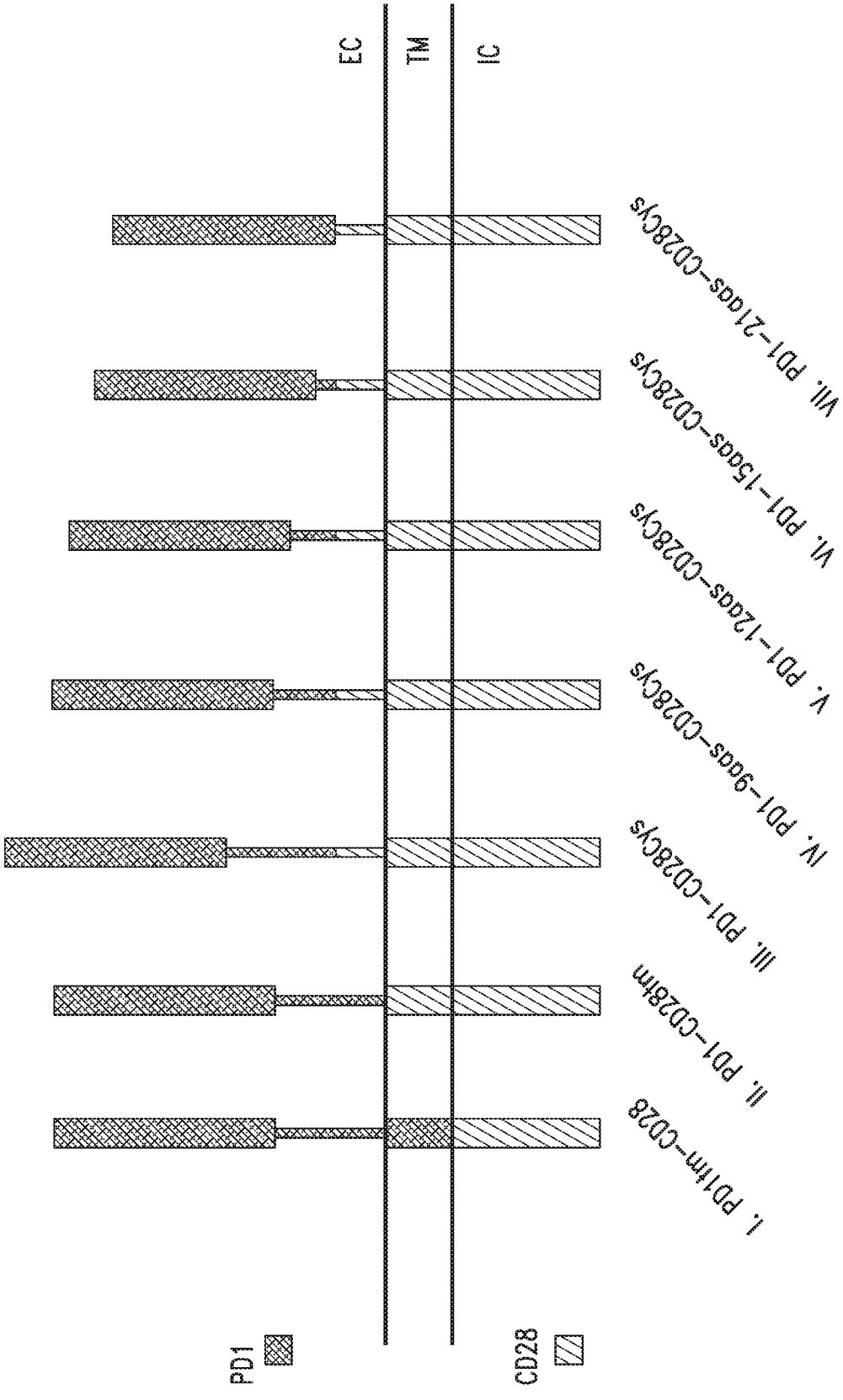

EC
TM
IC
I. PD1tm-CD28
II. PD1-CD28tm
III. PD1-CD28cys
IV. PD1-9aas-CD28cys
V. PD1-12aas-CD28cys
VI. PD1-15aas-CD28cys
VII. PD1-21aas-CD28cys
PD1
CD28

FIG. 9A

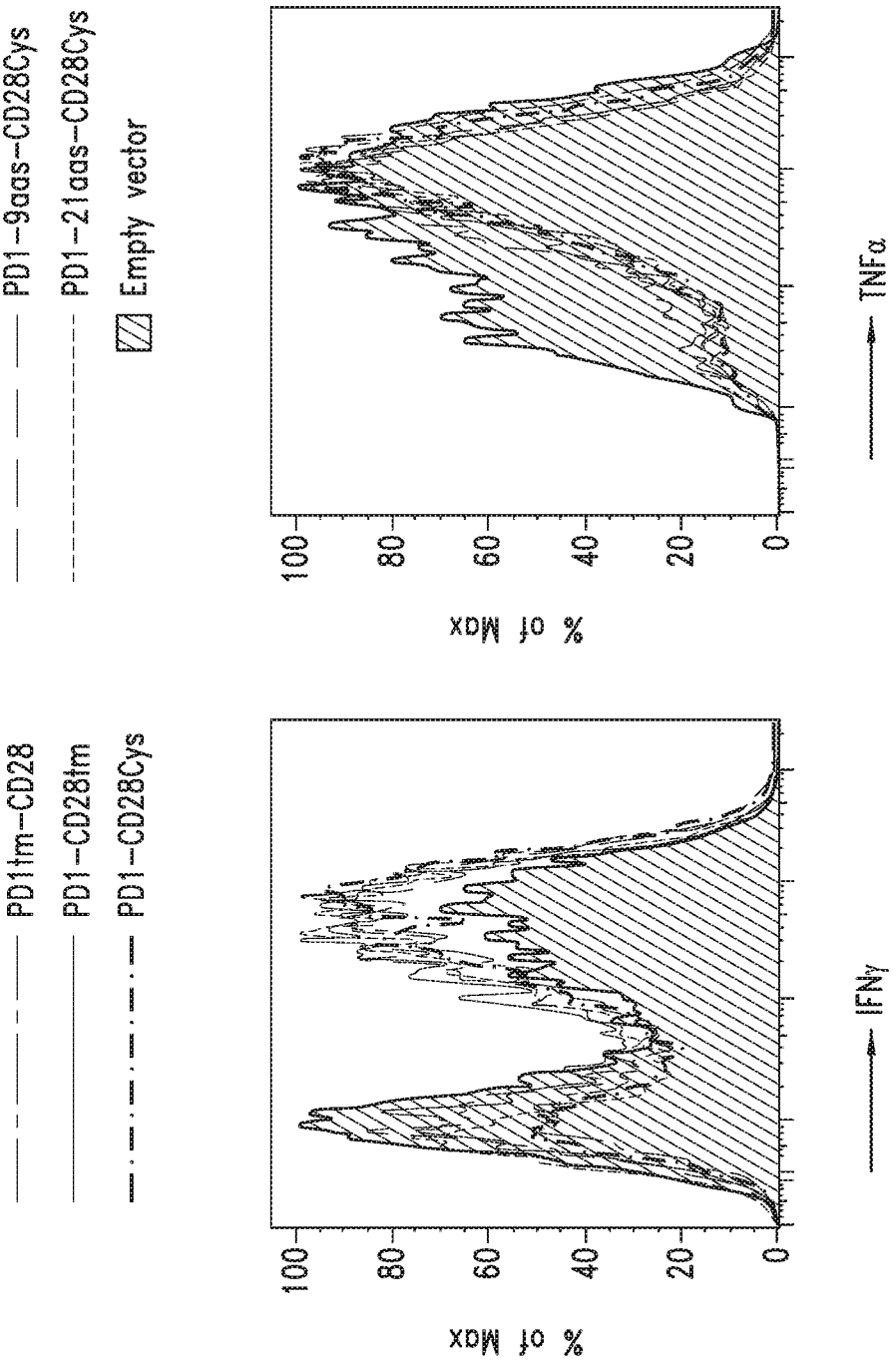

PD1tm-CD28
PD1-CD28tm
PD1-CD28Cys
PD1-9aas-CD28Cys
PD1-21aas-CD28Cys
Empty vector
% of MAX
100
80
60
40
20
0
IFNγ
TNFα

FIG. 9B

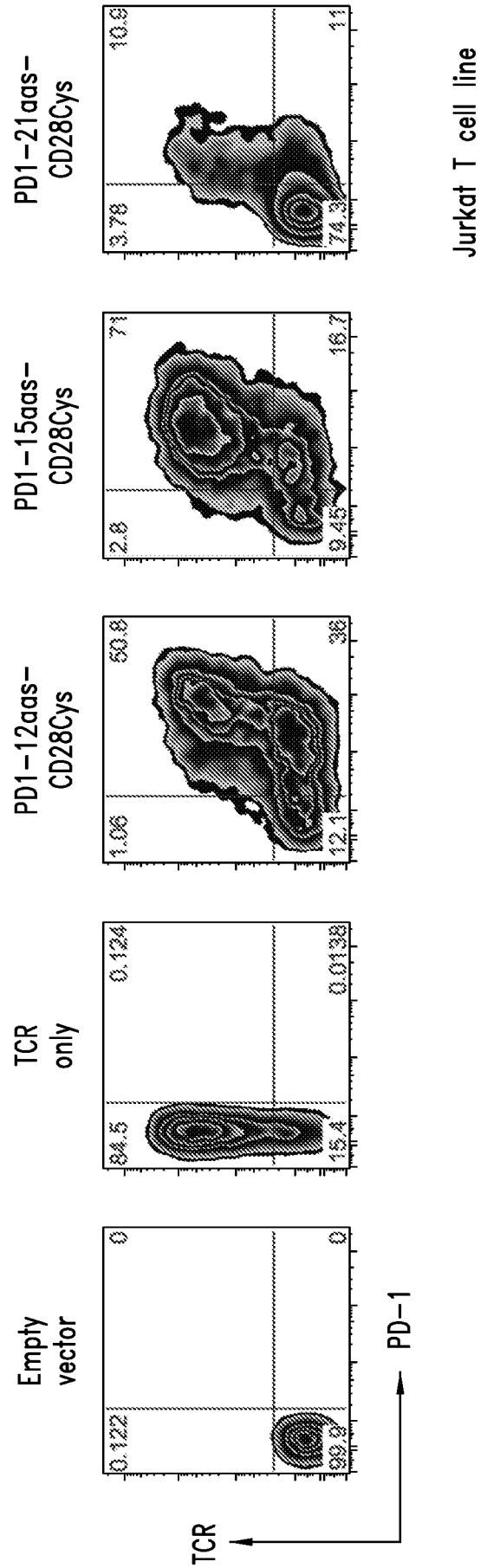

Empty vector
TCR only
PD1-12aas-CD28Cys
PD1-15aas-CD28Cys
PD1-21aas-CD28Cys
Jurkat T cell line
TCR
PD-1
0.122
0
99.9
0
84.5
0.124
15.4
0.0138
1.06
60.8
12.1
38
2.8
71
0.45
16.7
3.78
10.8
74.3
11

FIG. 10

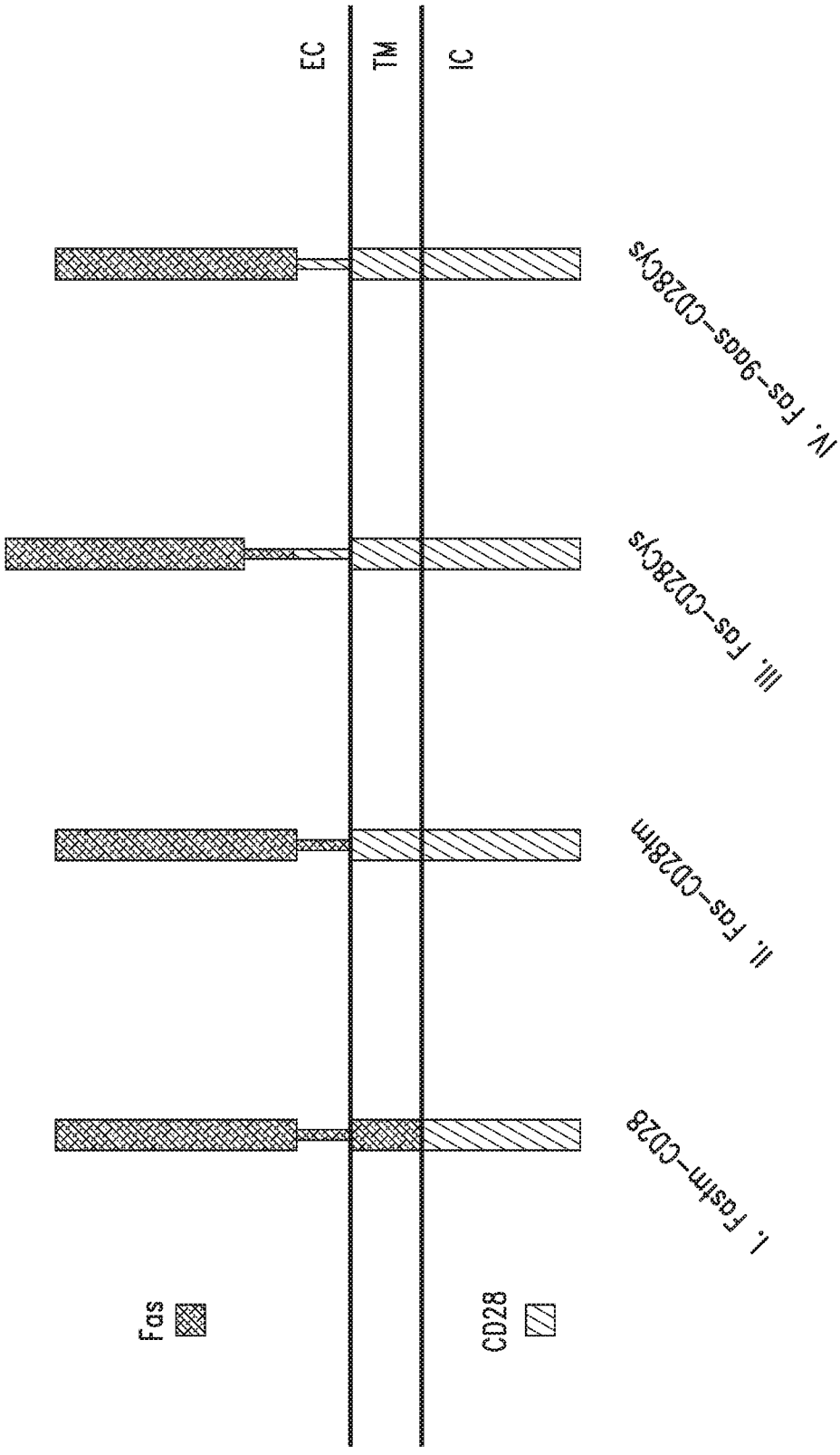
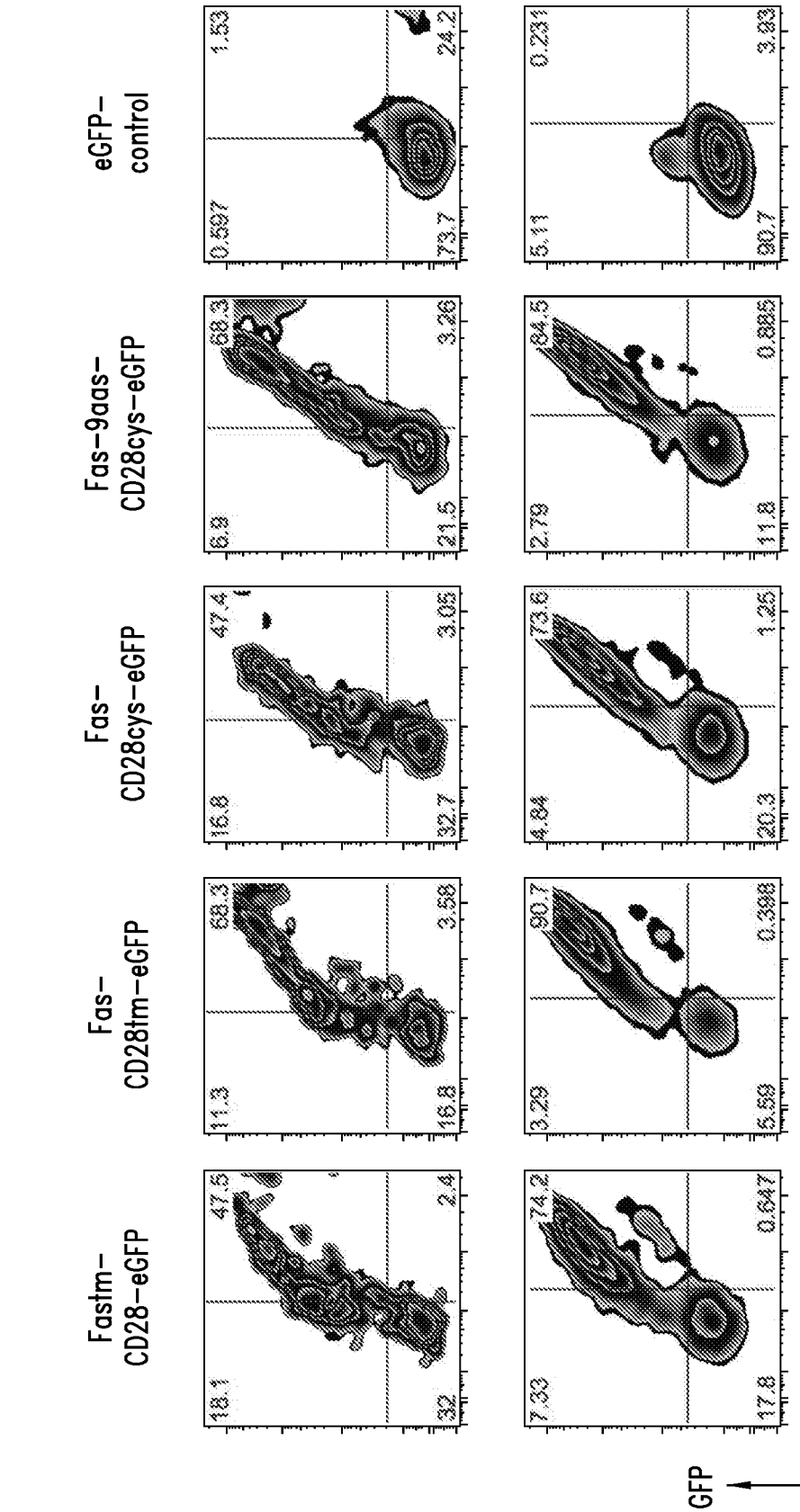

EC
TM
IC
I. Fastm-CD28
II. Fas-CD28tm
III. Fas-CD28Cys
IV. Fas-9aas-CD28Cys
Fas
CD28

FIG. 11A



Transduced TCR_{gag} T cells, 5 days post-stimulation

FIG. 11B

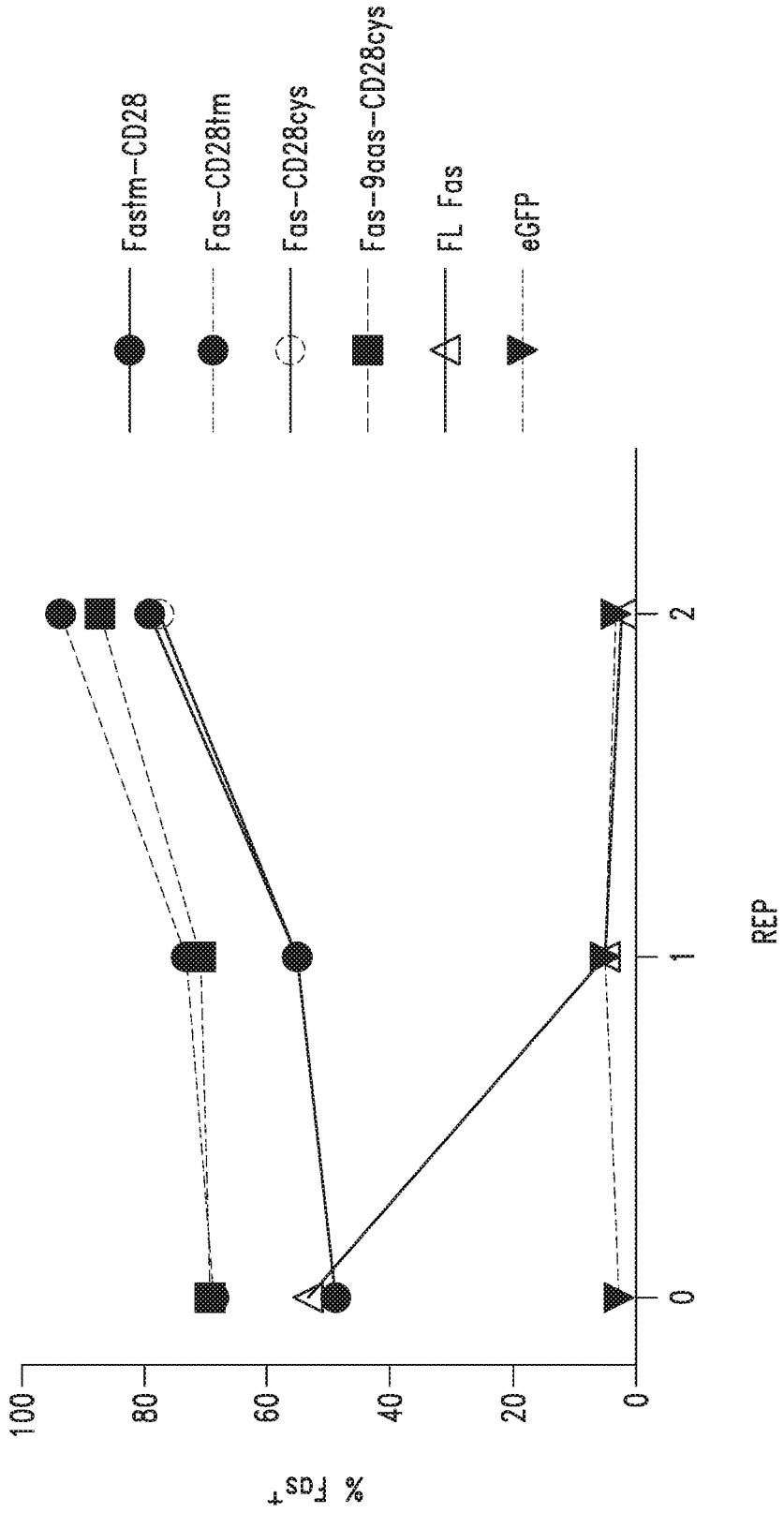

Fastm-CD28
Fas-CD28tm
Fas-CD28cys
Fas-9aas-CD28cys
FL Fas
eGFP
% FAS+
100
80
60
40
20
0
0
1
2
REP

FIG. 11C

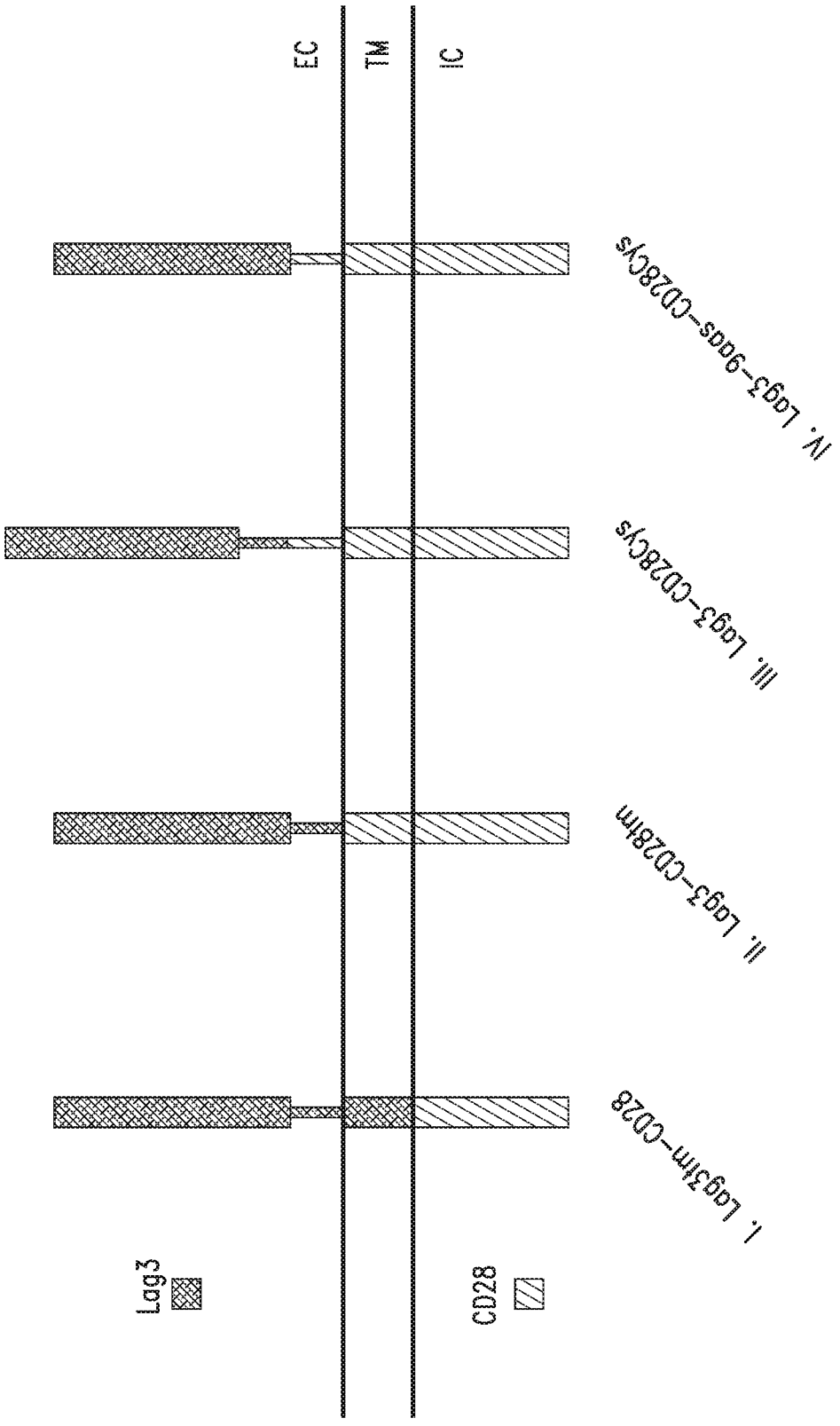

EC
TM
IC
I. Lag3tm-CD28
II. Lag3-CD28tm
III. Lag3-CD28cys
IV. Lag3-9aas-CD28cys
Lag3
CD28

FIG. 12A

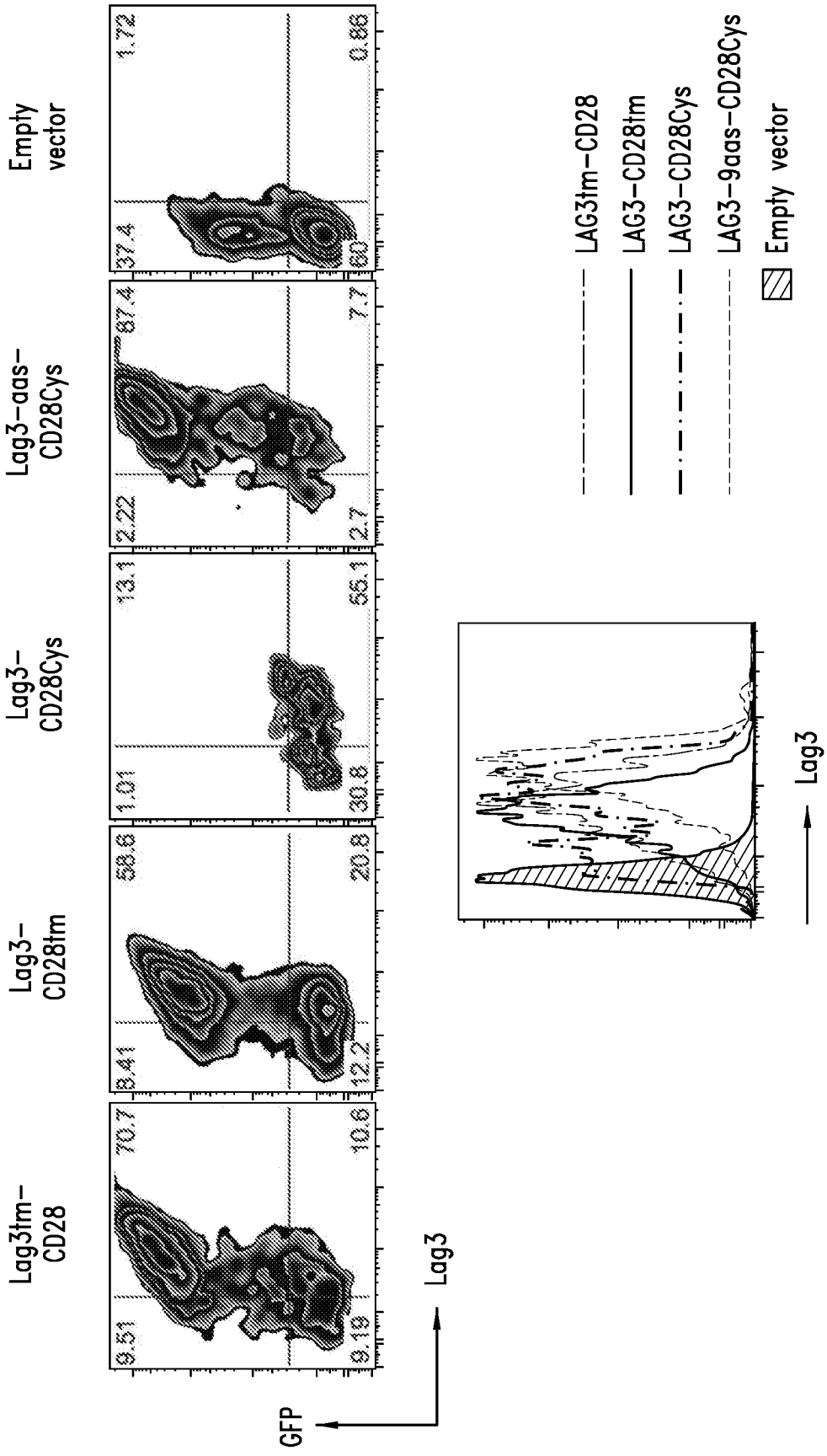

Lag3tm-CD28
Lag3-CD28tm
Lag3-CD28Cys
Lag3-aas-CD28Cys
Empty vector
GFP
Lag3
9.51
9.19
70.7
10.6
8.41
12.2
58.8
20.8
1.01
30.8
13.1
55.1
2.22
2.7
87.4
7.7
37.4
60
1.72
0.86
LAG3tm-CD28
LAG3-CD28tm
LAG3-CD28Cys
LAG3-9aas-CD28Cys
Empty vector
Lag3

FIG. 12B

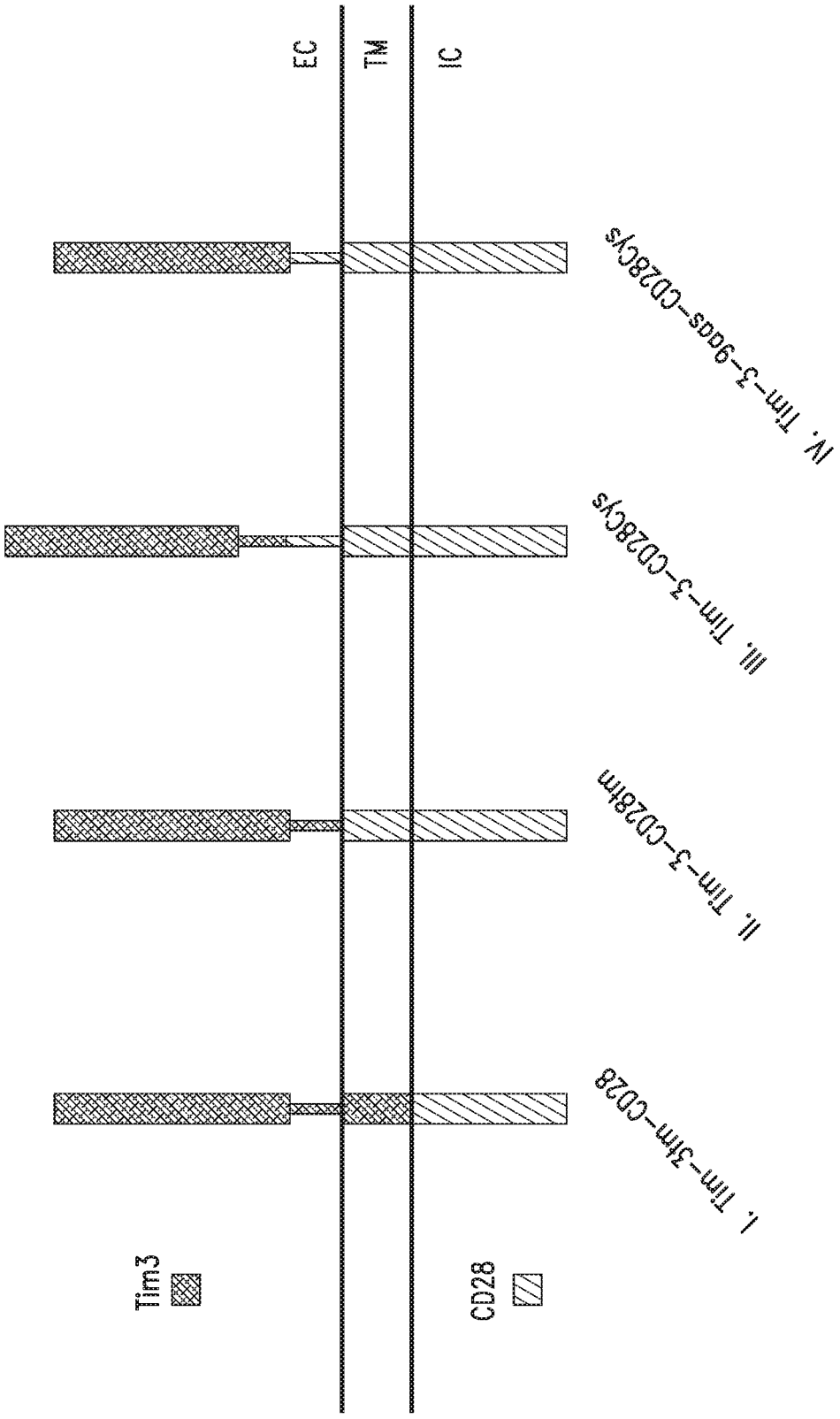

EC
TM
IC
I. Tim-3m-CD28
II. Tim-3-CD28m
III. Tim-3-CD28cys
IV. Tim-3-gas-CD28cys
Tim3
CD28

FIG. 13A

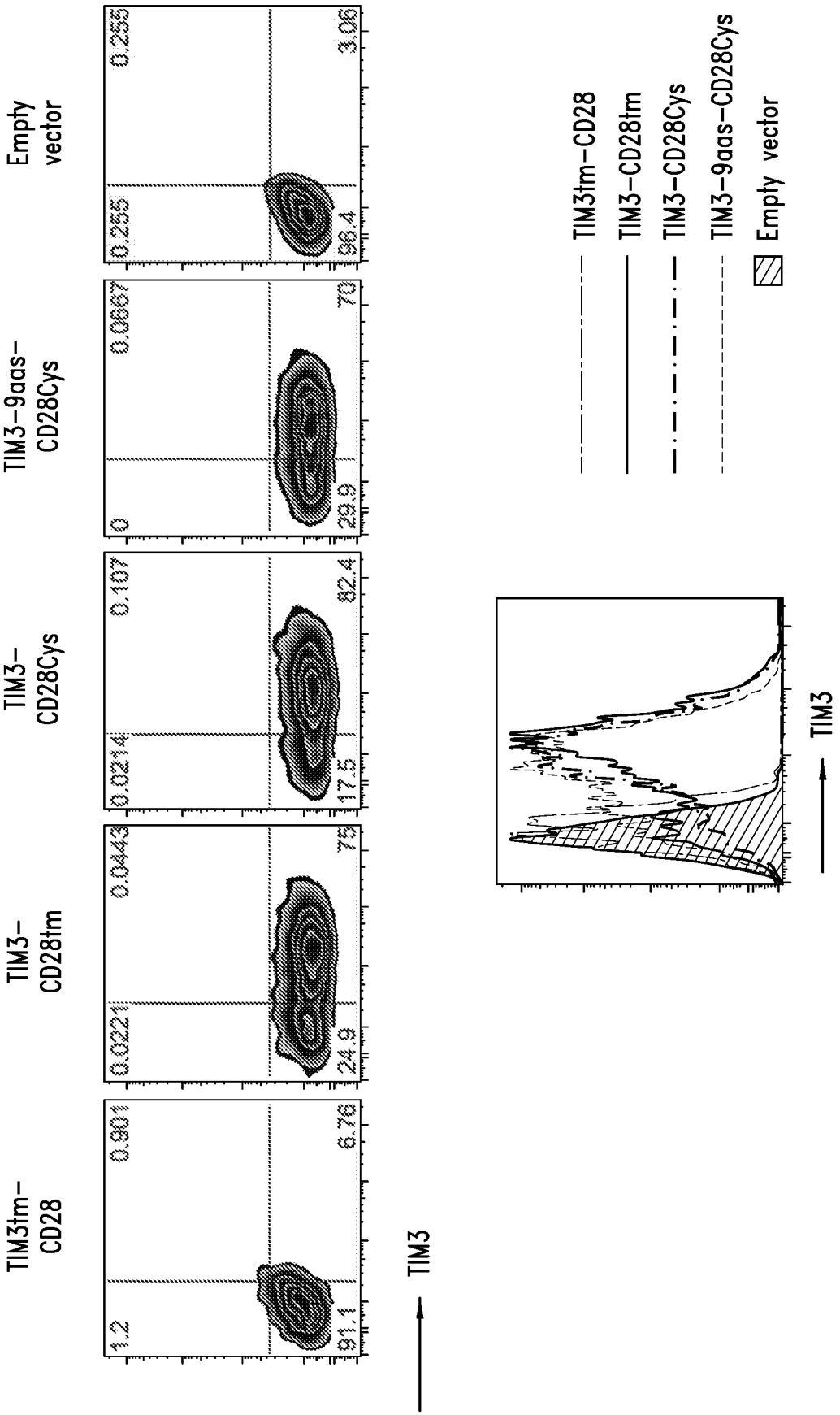

TIM3tm–CD28
TIM3–CD28tm
TIM3–CD28Cys
TIM3–9aas–CD28Cys
Empty vector
1.2
91.1
0.901
6.76
0.0221
24.9
0.0443
75
0.0214
17.5
0.107
82.4
0
29.9
0.0667
70
0.255
96.4
0.255
3.06
TIM3
TIM3tm–CD28
TIM3–CD28tm
TIM3–CD28Cys
TIM3–9aas–CD28Cys
Empty vector
TIM3

FIG. 13B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/021064

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/705 A61K35/17 C12N5/0783
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/123061 A1 (SEATTLE CHILDREN S HOSPITAL D B A SEATTLE CHILDREN S RES INST [US]) 22 August 2013 (2013-08-22)	9-11, 13-18, 20-34, 62-66, 98-104, 115-119, 130-172
Y	page 20; claims 1-104	35-61

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 June 2016	24/08/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Vollbach, Silke

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/021064

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M A KHARFAN-DABAJA ET AL: "Immunotherapy for chronic lymphocytic leukemia in the era of BTK inhibitors", LEUKEMIA., vol. 28, no. 3, 25 October 2013 (2013-10-25), pages 507-517, XP055279926, US ISSN: 0887-6924, DOI: 10.1038/leu.2013.311	9-11, 13-18, 20-34, 62-66, 98-104, 115-119, 130-172
Y	page 508	35-61
A	Koji Kono: "Copyright Journal of Stem Cells and Regenerative Medicine. All rights reserved", Journal of stem cells & regenerative medicine, 30 April 2014 (2014-04-30), pages 8-13, XP055279987, Retrieved from the Internet: URL:http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4112273/pdf/jsrm-10-008.pdf [retrieved on 2016-06-13] the whole document	9-11, 13-18, 20-66, 98-104, 115-119, 130-172
A	Marcela V Maus ET AL: "Review Series ANTIBODY DERIVATIVES AS NEW THERAPEUTICS FOR HEMATOLOGIC MALIGNANCIES Antibody-modified T cells: CARs take the front seat for hematologic malignancies", , 24 April 2014 (2014-04-24), XP055278960, DOI: 10.1182/blood- Retrieved from the Internet: URL:http://www.bloodjournal.org/content/bloodjournal/123/17/2625.full.pdf [retrieved on 2016-06-08] table 1	9-11, 13-18, 20-66, 98-104, 115-119, 130-172

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/021064

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-8
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

11, 18, 35-44, 46-66, 98-104, 115-119(completely); 1-10, 13-17, 20-34, 45 130-172(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 1-8

Present claims 1-8 are devoid of any characterizing technical feature, which would allow a comparison to be made with the prior art. Therefore a search can be performed only on the basis of structurally defining the different parts of the fusion protein.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guidelines C-IV, 7.2), should the problems which led to the Article 17(2) declaration be overcome.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 11, 18, 35-44, 46-66, 98-104, 115-119(completely); 1-10, 13-17, 20-34, 45, 130-172(partially)

Fusion protein, comprising (A) an extracellular component comprised of a binding domain that specifically binds CD200, (B) a hydrophobic component connecting the extracellular and intracellular components and (C) an intracellular component comprised of an intracellular signaling domain,

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2-14. claims: 1-10, 12-17, 19-34, 45, 67-97, 105-114, 120-172(all partially)

Fusion protein, comprising (A) an extracellular component comprised of a binding domain listed in present claims 10 and 14, (B) a hydrophobic component connecting the extracellular and intracellular components and (C) an intracellular component comprised of an intracellular signaling domain,

---

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/021064

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013123061 A1	22-08-2013	AU 2013221672 A1	07-08-2014
		CA 2861491 A1	22-08-2013
		EP 2814846 A1	24-12-2014
		HK 1205144 A1	11-12-2015
		JP 2015513394 A	14-05-2015
		US 2015038684 A1	05-02-2015
		WO 2013123061 A1	22-08-2013

摘要

本公開涉及含有細胞外結合結構域和細胞內信號傳導結構域的免疫調節融合蛋白，其中靶標的結合能夠在宿主細胞例如T細胞中產生調節信號 。本公開還涉及表達該免疫調節融合蛋白的免疫細胞在治療某些疾病例如癌症或傳染病中的用途 。