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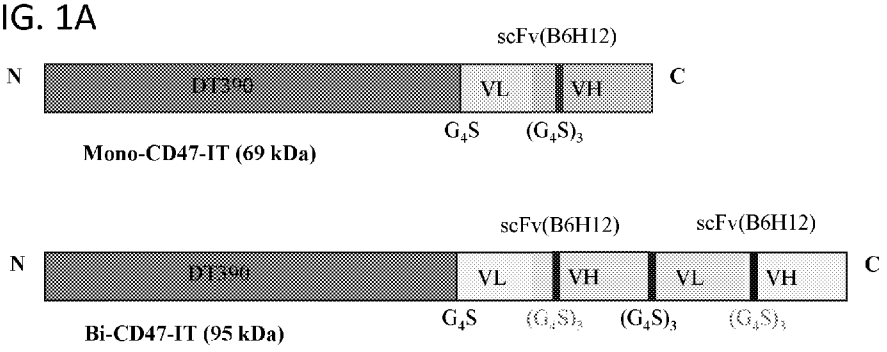
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(54) Title: BIVALENT ANTI-CD47 IMMUNOTOXIN COMPOSITIONS AND METHODS OF USING SAME IN TARGETED THERAPY

FIG. 1A



(57) Abstract: Methods of treating CD47+ cancer in a subject involving administering an immunotoxin to the subject. The immunotoxin may be a bivalent anti-human CD47 immunotoxin that includes a portion of a diphtheria toxin. The disclosed immunotoxin treats CD47+ cancers via targeted therapy.

WO 2025/019481 A1

BIVALENT ANTI-CD47 IMMUNOTOXIN COMPOSITIONS AND METHODS OF USING SAME IN TARGETED THERAPY

TECHNICAL FIELD

The present disclosure relates generally to compositions and methods for treating $CD47^+$ cancers. Specific implementations include administration of a bivalent CD47 diphtheria immunotoxin to treat $CD47^+$ tumors.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority pursuant to 35 U.S.C. § 119(e) of U.S. provisional patent application No. 63/527,124 entitled "ANTI-CD47 IMMUNOTOXINS AND METHODS OF USING SAME," filed on 17 July 2024, which is hereby incorporated by reference in its entirety.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been submitted electronically in xml format and is hereby incorporated by reference in its entirety. Said xml file, created on 16 July 2024, is named P309906WO01.xml and is 34,298 bytes in size.

BACKGROUND

Acute lymphoblastic leukemia (ALL) is the most common pediatric malignancy. It can be divided into two subtypes, B-cell ALL (B-ALL) and T-cell ALL (T-ALL). T-ALL accounts for about 15% of newly diagnosed ALL cases and has been associated with poor prognosis. The standard of care often includes multi-agent chemotherapy regimens with or without cranial radiation therapy. Up to 20% of children with T-ALL experience refractory disease, relapse, or treatment-related mortality.

Cluster of differentiation 47 (CD47) is a transmembrane protein overexpressed on the surface of many types of cancer cells (i.e., "$CD47^+$ cancer"), including T-ALL. CD47 is the ligand of signal regulatory protein alpha (SIRPα), which is expressed on macrophages and dendritic cells. The CD47-SIRPα axis is an innate immune checkpoint that serves as a "do not eat me" signal during the engulfment of macrophages and dendritic cells. Monotherapies with CD47 blockade have been ineffective in human clinical trials of many tumor types. Accordingly, compositions and methods for treating $CD47^+$ cancers are needed.

SUMMARY

Embodiments disclosed herein relate to compositions and methods for treating $CD47^+$ cancers by administering at least one anti-human CD47 diphtheria-based immunotoxin to reduce or eliminate cancerous cells.

In accordance with embodiments of the present disclosure, an anti-human CD47 immunotoxin may include a toxin portion including a diphtheria toxin or a fragment thereof, and a targeting portion linked to the toxin portion and including at least a first anti-human CD47 antibody or fragment thereof. In some examples, the diphtheria toxin or fragment thereof includes about 390 amino acids from the diphtheria toxin. In some examples, the toxin portion is located at or near an N terminus of the anti-human CD47 immunotoxin. In some examples, the targeting portion further includes a second anti-human CD47 antibody or fragment thereof. In some examples, the anti-human CD47 antibody or fragment thereof includes an antigen-binding portion of the anti-human CD47 antibody or fragment thereof. In some examples, the antigen-binding portion includes VH and VL regions from an anti-human CD47 antibody. In some examples, the targeting portion is located at or near a C terminus of the anti-human CD47 immunotoxin. the toxin portion is linked to the targeting portion by at least one linker, and the linker comprises four glycine residues and one serine residue.

In some examples, a nucleic acid molecule encodes the anti-human CD47 immunotoxin. In some examples, the nucleic acid molecule is codon-optimized for expression in a methylotropic yeast. In some examples, a vector includes the nucleic acid molecule. In some examples, a host cell expresses the nucleic acid molecule. In some examples, the host cell is a cell of *Pichia pastoris*. In some examples, a pharmaceutical composition includes the anti-human CD47 immunotoxin and a pharmaceutically acceptable carrier.

In accordance with embodiments of the present disclosure, a method of treating a subject having a $CD47^+$ cancer may involve administering a therapeutically effective amount of an anti-human CD47 immunotoxin, and the immunotoxin may include a toxin portion including a diphtheria toxin or a fragment thereof, and a targeting portion linked to the toxin portion and including at least a first anti-human CD47 antibody or fragment thereof. The subject may be a human and may be suffering from one or more of cutaneous T-cell lymphoma (CTCL) (including human CD47+CD25+CCR4+CD30+ CTCL), peripheral T-cell lymphoma (PTCL), lung cancer (including human CD47+ lung cancer), triple-negative breast cancer, head and neck cancer, melanoma, and bladder cancer.

In accordance with embodiments of the present disclosure, a method of reducing a population of $CD47^+$ cells may involve adding an anti-human CD47 immunotoxin to the population, and the immunotoxin may include a toxin portion including a diphtheria toxin or a

fragment thereof, and a targeting portion linked to the toxin portion and including at least a first anti-human CD47 antibody or fragment thereof. Addition of the anti-human CD47 immunotoxin may reduce the number of $CD47^+$ cells compared to no addition of the anti-human CD47 immunotoxin or addition of an anti-human CD47 targeting portion alone. In many embodiments, the reducing may be in a population of cells derived from peripheral blood, the spleen, the liver, bone marrow, brain, spinal cord, and other tissues and organs.

This Summary is neither intended to be, nor should it be, construed as being representative of the full extent and scope of the present disclosure. Moreover, references made herein to "the present disclosure," or aspects thereof, should be understood to mean certain embodiments of the present disclosure and should not be construed as limiting all embodiments to a particular description. The present disclosure is set forth in various levels of detail in this Summary as well as in the attached drawings and Detailed Description and no limitation as to the scope of the present disclosure is intended by either the inclusion or non-inclusion of elements, components, etc. in this Summary. Features from any of the disclosed embodiments may be used in combination with one another without limitation. In addition, other features and advantages of the present disclosure will become apparent to those of ordinary skill in the art through consideration of the following Detailed Description and the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

FIGs 1A-1G show Bi-CD47-IT generation using a unique diphtheria toxin resistant yeast Pichia pastoris expression system. FIG. 1A presents schematic diagrams of mono-CD47-IT and bi-CD47-IT. DT390, first 390 amino acids of diphtheria toxin; mono-CD47-IT, monovalent anti-human CD47 immunotoxin; bi-CD47-IT, bivalent anti-human CD47 immunotoxin; scFv, single-chain variable fragment; G_4S (SEQ ID NO: 26), four glycine residues and one serine residue; N, N-terminal; C, C-terminal. **FIG. 1B** presents an SDS-PAGE (4–12% NuPAGE) analysis of mono-CD47-IT and bi-CD47-IT. **FIG. 1C** shows Western blot analysis using a mouse anti-diphtheria toxin (DT) mAb. **FIG. 1D** shows Western blot analysis using a mouse anti-His mAb. In FIGs. 1B-1D, Lane 1: Protein marker; Lane 2-3: mono-CD47-IT (69 kDa); Lane 4- 5: bi-CD47-IT (95 kDa). The weak low molecular-weight bands in both SDS gel and Western blot analyses are the broken-down products of mono-CD47-IT or bi-CD47-IT. FIG. 1E shows data from in vitro binding avidity analysis of the Alexa Flour 488-labeled mono-CD47-IT and bi-CD47-IT to human CD47+ T-ALL CCRF-CEM cells by flow cytometry. FITC-

anti-human CD47 mAb (B6H12) was used as a positive control, and Alexa Flour 488-labeled isotype mouse IgG1 served as a negative control. The data are representative of three individual experiments. FIG. 1F is data of K_D determination of mono-CD47- IT and bi-CD47-IT to human CD47+ T-ALL CCRF-CEM cells using flow cytometry and nonlinear least-squares fitting. The mean fluorescence intensity (MFI) was plotted over a wide range of Alexa Flour 488-labeled mono-CD47-IT or bi-CD47-IT concentrations. Nonlinear regression was based on the equation $Y = Bmax \times X/ (K_D + X)$, where Y = MFI at the given Alexa Flour 488- labeled mono-CD47-IT or bi-CD47-IT after subtracting the background, X = Alexa Flour 488- labeled mono-CD47-IT or bi-CD47-IT concentration, and Bmax = the maximum specific binding in the same units as Y. (G) In vitro efficacy of mono-CD47-IT and bi-CD47-IT to human CD47+ T-ALL CCRF-CEM cells determined by the CellTiter-Glo® Luminescent Cell Viability Assay (blue line: mono-CD47-IT group; green line: bi-CD47-IT group; black line: pCD3-IT group as negative control). Y-axis: percent inhibition of cell viability determined by the number of viable cells based on the quantification of ATP. X-axis: immunotoxin concentration. Cycloheximide (1.25 mg/mL) was used as a positive control. The negative control wells contained cells without immunotoxin. Data are from three individual experiments. Error bars indicate SD.

FIGs 2A-E show that Bi-CD47-IT was highly potent against T-ALL in a T-ALL CCRF-CEM CDX mouse model. Briefly, human $CD47^+CCR4^+$ CCRF-CEM cells were intravenously injected into the *NSG* mice that were then administered via intraperitoneal injection of C21-IT (negative control, n=7), mono-CD47-IT (n=7), bi-CD47-IT (n=7), or CCR4-IT (positive immunotoxin control, n=7) once daily at 8.43×10^{-10} mol/kg for 10 consecutive days starting on day 4 after the tumor cell injection. On day 23, two tumor-bearing mice from each group were euthanized to collect the peripheral blood, spleen, and liver. **(FIG. 2A)** Kaplan-Meier survival curves were recorded for C21-IT (black line) with median survival of 24 days, mono-CD47-IT (blue line) with median survival of 28 days, and bi-CD47-IT (purple line) with median survival of 43 days, CCR4-IT (green line) with median survival days of 30 days. The *p*-values were calculated using the Mantel-Cox log-rank test (GraphPad Prism 9.4.1.) **(FIG. 2B)** Flow cytometry analysis to monitor the *in vivo* depletion of bi-CD47- IT to human $CD47^+CCR4^+$ T-ALL CCRF-CEM cells. Upper panel: Flow cytometry data. X-axis: PerCP/Cy5.5 anti-mouse CD45 mAb. Y-axis: PE-anti-human CD47 mAb (B6H12). Lower panel: Ratio of the human $CD47^+$ cells versus murine $CD45^+$ cells in the peripheral blood. *P*-values were calculated using the two-tailed Student t-test (GraphPad Prism 9.4.1.) **(FIG. 2C)** Upper panel: Spleen gross necropsy examination. Lower panel: Spleen weight as a percentage of the corresponding body weight. *P*-values were calculated using the two-tailed Student t-test (GraphPad Prism 9.4.1). **(FIG. 2D)** Spleen immunohistochemistry. Human $CD47^+$ T-ALL CCRF-CEM cells were stained

using red fluorescence and the cell nuclei was stained using DAPI (4',6-diamidino-2-phenylindole). Immunofluorescent microscope: 400× magnification, scale bar: 50 μM. **(FIG. 2E)** Liver pathology analysis. Upper panels: 40× magnification, scale bars: 500 μM; and Lower panels: 200× magnification, scale bars: 100 μM under light microscope.

FIGs 3A-3G present results for a serial study of bi-CD47-IT efficacy in a T-ALL CCRF-CEM CDX mouse model. On day 4, 14, and 21 post the tumor cell injection, two tumor-bearing mice from each group were euthanized to collect the peripheral blood, spleen, liver, bone marrow, brain, spinal cord to monitor the depletion of the T-ALL blast cells. C21-IT group (n=12). Bi-CD47-IT group (n=12). **(FIG. 3A)** Kaplan-Meier survival curves were recorded for C21-IT (black line) with median survival of 24 days and bi-CD47-IT (purple line) with median survival of 43 days. The *p*-value was calculated using the Mantel-Cox log-rank test (GraphPad Prism 9.4.1). **(FIG. 3B)** Flow cytometry analysis to monitor the depletion of the blast cells (human HLA/ABC$^+$CD7$^+$CD5$^+$CD8$^-$) in the peripheral blood on day 4, 14, 21 and 28 post the tumor cell injection. **(FIG. 3C)** Left panel: spleen gross necropsy examination. Right panel: spleen weight as a percentage of the corresponding body weight. Flow cytometry analysis to monitor the depletion of the T-ALL blast cells (human HLA/ABC$^+$CD7$^+$CD5$^+$CD8$^-$) **(FIG. 3D)** in the spleen on day 21, **(FIG. 3E)** bone marrow on day 21, **(FIG. 3F)** brain and spinal cord on day 21. *P*-values were calculated using the two-tailed Student t-test (GraphPad Prism 9.4.1.). $P<0.05$ was considered as statistically significant and "ns" as statistically not significant. **(FIG. 3G)** Liver pathology analysis on day 21 post the tumor cell injection. T-ALL cells were H&E stained as blue. Upper panels: 40× magnification, scale bars: 500 μM; and Lower panels: 200× magnification, scale bars: 100 μM under light microscope.

FIGs 4A-4F show that Bi-CD47-IT was highly potent against T-ALL in an overt T-ALL CCRF-CEM CDX mouse model. Human CD47+ T-ALL CCRF-CEM cells were IV injected into the NSG mice on day 0 and started with the immunotoxin treatment (bi-CD47-IT or C21-IT) on day 7 when 6% T-ALL blast cells were detected in the peripheral blood. On day 21 post the tumor cell injection, two tumor-bearing mice from each group were euthanized to collect the peripheral blood, spleen, liver, bone marrow, brain, and spinal cord to monitor the depletion of the T-ALL blast cells. C21- IT group (n=7). Bi-CD47-IT group (n=7). **(FIG. 4A)** Kaplan-Meier survival curves were recorded for C21-IT (black line) with median survival of 23 days and for bi-CD47-IT (purple line) with median survival of 35 days. P-values for the survival curves were calculated using the Mantel-Cox log- rank test (GraphPad Prism 9.4.1). Flow cytometry analysis to monitor the depletion of T-ALL blast cells (human HLA/ABC+CD7+CD5+CD8-) **(FIG. 4B)** in the peripheral blood, **(FIG. 4C, lower panel)** spleen, **(FIG. 4D, lower panel)** bone marrow, **(FIG. 4E)** brain and spinal cord. **(FIG. 4C and FIG. 4D, upper panel)** Spleen and

bone gross necropsy examination. **(FIG. 4F)** Liver pathology analysis on day 21 post the tumor cell injection. T-ALL cells were H&E stained as blue. Upper panels: 40× magnification, scale bars: 500 μM; and Lower panels: 200× magnification, scale bars: 100 μM under light microscope.

FIGs 5A-5E show that Bi-CD47-IT was even more potent against T-ALL in an overt T-ALL Molt-4 CDX mouse model. Human CD47+ T-ALL Molt-4 cells were IV injected into NSG mice on day 0 and started with the immunotoxin treatment (bi-CD47-IT or C21-IT) on day 4, when 1.3-2.2 %T-ALL blast cells were detected in the peripheral blood. On day 14 and 23 post the tumor cell injection, two mice from each group were euthanized to collect the peripheral blood, spleen, liver, and bone marrow to monitor the depletion of the T-ALL blast cells. C21-IT group (n=7). Bi-CD47-IT group (n=7). **(FIG. 5A)** Kaplan-Meier survival curves were recorded for C21-IT (black line) with median survival of 22 days and bi-CD47-IT (purple line) with 60% cure rate (3 of 5 mice). P-values were calculated using the two-tailed Student t-test (GraphPad Prism 9.4.1). $P<0.05$ was considered statistically significant and "ns" as statistically not significant. Flow cytometry analysis to monitor the depletion of T-ALL blast cells (human HLA/ABC+CD7+CD5+CD8-) **(FIG. 5B)** in the peripheral blood, **(FIG. 5C, bottom panel)** spleen, **(FIG. 5D, lower panel)** bone marrow. **(FIG. 5C, top panel)** spleen gross necropsy. **(FIG. 5D, upper panel)** bone gross necropsy. **(FIG. 5E)** Liver pathology analysis on day 14 and 23 post the tumor cell injection. T-ALL cells were H&E stained as blue. Upper panels (day 14 and 23): 40× magnification, scale bars: 500 μM; and Lower panels (day 14 and 23): 200× magnification, scale bars: 100 μM under light microscope.

FIGs 6A-6N present experimental data showing that Bi-CD47 IT was highly potent against T-ALL in two T-ALL PDX mouse models. **(FIGs 6A-6G)** Overt T-ALL PDX sample #1 mouse model. Human CD47+ T-ALL PDX sample #1 tumor cells (5×10^6 per mouse) were intravenously injected into the NSG mice on day 0. The immunotoxin treatment started on day 7 when >30% of T-ALL blast cells were detected in the peripheral blood. On day 28, two tumor-bearing mice were euthanized to collect peripheral blood, spleen, liver, bone marrow, brain, and spinal cord to monitor the depletion of the T-ALL blast cells. C21-IT group (n=7). Bi-CD47-IT group (n=8). **(FIG. 6A)** Kaplan-Meier survival curves were recorded for C21-IT (black line) with median survival of 33 days and bi-CD47-IT (purple line) with median survival of 54 days. Flow cytometry analysis to monitor the depletion of bi-CD47-IT to the T-ALL blast cells (HLA/ABC+CD7+CD5+CD8-) **(FIG. 6B)** in the peripheral blood, **(FIG. 6D, lower panel)** spleen, **(FIG. 6E, lower panel)** bone marrow, **(FIG. 6F)** brain and spinal cord. **(FIG. 6D, left panel)** spleen gross necropsy. **(FIG. 6E, upper panel)** bone gross necropsy. **(FIG. 6C)** On day 28, the T-ALL blast cells were stained with black color in the blood smear using Sudan Black B

Staining Kit. Upper panels: 200× magnification, scale bars: 100 μM; lower panels: 400× magnification, scale bars: 50 μM under light microscope. **(FIG. 6G)** Liver pathology analysis. The T-ALL cells were H&E stained as blue. Upper panels: 40× magnification, scale bars: 500 μM; and Lower panels: 200× magnification, scale bars: 100 μM under light microscope. **(FIGs 6H-6N)** Overt T-ALL PDX sample #2 mouse model. Human CD47+ T- ALL PDX sample #2 tumor cells (5×10^6 per mouse) were intravenously injected into the NSG mice on day 0. The immunotoxin treatment started on day 7 when 5% of T-ALL blast cells were detected in the peripheral blood. On day 33, two tumor-bearing mice from each group were euthanized to collect the peripheral blood, spleen, liver, bone marrow, brain, spinal cord to monitor the depletion of the T-ALL blast cells. C21-IT group (n=7). Bi-CD47-IT group (n=7). **(FIG. 6H)** Kaplan- Meier survival curves were recorded for C21-IT (black line) with median survival of 53 days and bi-CD47-IT (purple line) with median survival of 85 days. Flow cytometry analysis to monitor the depletion of the T-ALL blast cells (HLA/ABC+CD7+CD5+CD8-) **(FIG. 6I)** in the peripheral blood, **(FIG. 6K, lower panel)** spleen, **(FIG. 6L, lower panel)** bone marrow, **(FIG. 6M)** brain and spinal cord. **(FIG. 6K, upper panel)** spleen gross necropsy. **(FIG. 6L, upper panel)** bone gross necropsy. **(FIG. 6J)** On day 33, the T-ALL blast cells were stained with black color in the blood smear using Sudan Black B Staining Kit. Upper panels: 200× magnification, scale bars: 100 μM; lower panels: 400× magnification, scale bars: 50 μM under light microscope. **(FIG. 6N)** Liver pathology analysis. T-ALL cells were H&E stained as blue. Upper panels: 40× magnification, scale bars: 500 μM; and Lower panels: 200× magnification, scale bars: 100 μM under light microscope.

FIGs 7A-7E present experiments confirming that Bi-CD47-IT showed no toxicity to normal human tissues. (FIG. 7A) *In vitro* binding avidity of bi-CD47-IT to human red blood cells. PE-anti-human CD47 mAb (B6H12) was used as a positive control, Alexa Flour 488-labeled isotype mouse IgG1 served as a negative control. The data are representative of three individual experiments. **Left two panels:** Flow cytometry binding avidity analysis of bi-CD47-IT to donor #1 and donor #2 human red blood cells. **Right two panels:** K_D determination of bi-CD47-IT to donor #1 and donor #2 human red blood cells. **(FIG. 7B)** Binding avidity comparison of FITC-labelled Magrolimab with Alexa Flour 488-labeled bi-CD47-IT to human red blood cells. **(FIG. 7C)** Binding avidity comparison of Alexa Flour 488-labeled bi-CD47-IT to T-ALL CCRF-CEM cells versus human lymphocytes, human monocytes, and human red blood cells. **(FIG. 7D)** Hemagglutination activity of bi-CD47-IT to human red blood cells. pCD3-IT was included as negative immunotoxin control. Magrolimab (anti-human CD47 mAb, clone Hu5F9- G4) served as positive hemagglutination control. Bi-CD47-IT, pCD3-IT, Magrolimab were incubated with the human blood samples for 20 hours at 37°C. Data are representative of

three independent experiments. **(FIG. 7E)** Human lymphocytes were transiently depleted by bi-CD47-IT treatment in humanized mice. Bi-CD47-IT was IP injected at 8.43×10^{-10} moles/kg for 10 consecutive days into the humanized mice carrying >30% of human chimerism (n=3). PBS treatment served as negative control group (n=3). The peripheral blood samples were collected pre-treatment and weekly via tail vein for flow cytometry analysis to monitor the depletion of the human lymphocytes (human $CD45^+$ cells). *P*-values were calculated using the two-tailed Student t-test (GraphPad Prism 9.4.1). $P<0.05$ was considered as statistically significant and "ns" as statistically not significant.

Figure 8A-I. Serial efficacy study of bi-CD47-IT in a T-ALL CCRF-CEM CDX mouse model. Flow cytometry analysis to monitor the depletion of human T-ALL blast cells (human HLA/ABC+CD7+CD5+CD8-) **(FIGs. 8A-B)** in the peripheral blood on day 14 and 21, **(FIGs. 8C-D)** in the spleen on day 14 and 21, **(FIG. 8F-G)** in the bone marrow on day 14 and 21, **(FIG. 8H)** in the brain on day 21, **(FIG. 8I)** in the spinal cord on day 21. **(FIG. 8E)** Bone (femurs and tibias) gross necropsy examination on day 0, 4, 14, and 21.

Figure 9A-G. Bi-CD47-IT has high efficacy against T-ALL in an overt T-ALL CCRF-CEM CDX mouse model. Flow cytometry analysis to monitor the T-ALL blast cells (human HLA/ABC+CD7+CD5+CD8-) **(FIG. 9A)** in the peripheral blood on day 6 (pre-treatment), **(FIG. 9B)** in the peripheral blood on day 21, **(FIG. 9C)** in the spleen on day 21, **(FIG. 9E)** in the bone marrow on day 21, **(FIG. 9F)** in the brain on day 21, **(FIG. 9G)** in the spinal cord on day 21. **(FIG. 9D)** Percentage of spleen weight to the corresponding body weight on day 21. P-values were calculated using the two-tailed Student t-test (GraphPad Prism 9.4.1).

Figure 10A-H. Bi-CD47-IT has even higher efficacy against T-ALL in an overt T-ALL Molt-4 CDX mouse model. Flow cytometry analysis to monitor the T-ALL blast cells (human HLA/ABC+CD7+CD5+CD8-) **(FIG. 10A)** in the peripheral blood on day 4 (pre-treatment), **(FIG. 10B)** in the peripheral blood on day 14, **(FIG. 10C)** in the peripheral blood on day 23, **(FIG. 10D)** in the spleen on day 23, **(FIG. 10E)** in the bone marrow on day 23, **(FIG. 10F)** in the brain on day 23, **(FIG. 10G)** in the spinal cord on day 23. **(FIG. 10H)** Flow cytometry analysis to detect the T-ALL blast cells in the peripheral blood on day 131 of the clinically cured mice treated with bi-CD47-IT. **(FIG. 10I)** T-ALL MRD analysis by quantitative PCR of the blood and bone marrow on day 131 for the clinically cured mice treated with bi-CD47-IT.

Figure 11A-P. Bi-CD47-IT has high efficacy against T-ALL in two overt PDX mouse models. **(FIGs. 11A-H)** Overt PDX sample #1 mouse model: Flow cytometry analysis to monitor the T-ALL blast cells (human HLA/ABC+CD7+CD5+CD8-) **(FIGs. 11A-C)** in the peripheral blood on day 7 (pre-treatment), 18, and 28, (E) in the spleen on day 28, **(FIG. 11F)** in

the bone marrow on day 28, (**FIG. 11G**) in the brain on day 28, (**FIG. 11H**) in the spinal cord on day 28. (**FIG. 11D**) Percentage of spleen weight to the corresponding body weight. (**FIGs. 11I-P**) Overt PDX sample #2 mouse model: Flow cytometry analysis to monitor the T-ALL blast cells (human HLA/ABC+CD7+CD5+CD8-) (**FIGs. 11I-K**) in the peripheral blood on day 18 and 32, (**FIG. 11M**) in the spleen on day 33, (**FIG. 11N**) in the bone marrow on day 33, (**FIG. 11O**) in the brain on day 33, (**FIG. 11P**) in the spinal cord on day 33. (**FIG. 11L**) Percentage of spleen weight to the corresponding body weight.

Figure 12. Bi-CD47-IT does not induce macrophage-mediated phagocytosis via blocking CD47/SIRPα pathway. The goal of this assay is to assess the possible immunotherapy mechanism of bi-CD47-IT. In vitro macrophage-mediated phagocytosis was measured by flow cytometry analysis. The phagocytosis rate was defined as the percentage of Green CMFDA+ cells within Deep Red+ macrophages. BiscFv(B6H12), without DT390 portion, was included as the binding domain only control of bi-CD47-IT. Magrolimab (anti-human CD47 mAb, clone Hu5F9-G4) and anti-human CD47 mAb (B6H12) were included as positive controls. Daratumumab (anti-human CD38 mAb) was added to provide elevated phagocytosis signal.

Figure 13A-C. Bi-CD47-IT demonstrated no toxicity to normal human tissues. (**FIG. 13A**) Flow cytometry binding avidity comparison of Alexa Fluor 488-labeled bi-CD47-IT with Magrolimab (FITC anti-human CD47 mAb, clone Hu5F9-G4) to human red blood cells. (**FIG. 13B**) Flow cytometry binding avidity comparison of Alexa Fluor 488-labeled bi-CD47-IT to human T-ALL CCRF-CEM cells versus human lymphocytes, human monocytes, and human red blood cells. Alexa Fluor 488-labeled isotype mouse IgG1 served as negative control. (**FIG. 13C**) Flow cytometry analysis to monitor the depletion of the human lymphocytes (human CD45+ cells) in the humanized mice treated with bi-CD47-IT for 10 consecutive days (day 0 to 9).

DETAILED DESCRIPTION

The present disclosure relates to compositions and methods for treating $CD47^+$ cancers. The compositions may be selected from one or more anti-human CD47 immunotoxins. In many embodiments the disclosed anti-human CD47 immunotoxin comprises diphtheria toxin. The methods disclosed herein involve decreasing the number and/or size of cancerous $CD47^+$ cells via administration of an immunotoxin. Administration of an immunotoxin in the manner disclosed may inhibit protein synthesis in cancerous $CD47^+$ cells or otherwise cause cell death. The particular dose of immunotoxin may vary and may depend on the specific compound, the route of administration, and other factors.

Disclosed herein is a bivalent anti-CD47 immunotoxin, bi-CD47-IT, a novel, genetically engineered recombinant immunotoxin for targeted therapy of CD47+ cancers, including T-ALL.

Applicants developed the disclosed bi-CD47-IT, by expressing the immunotoxin in diphtheria toxin-resistant yeast (*Pichia pastoris*) system. Use of this system overcomes expression and purification challenges encountered with E. coli-based expression systems and delivers high production level and excellent purification quality of bi-CD47-IT.

CD47 is overexpressed on human CD47+ cancers, including T-ALL, (26-fold more CD47 than human red blood cells). Applicants hypothesized that CD47 might be used for targeted therapy of CD47+ cancers including T-ALL. One aim was to engineer a recombinant anti-human CD47 immunotoxin with enhanced binding avidity to CD47+ T-ALL and other CD47+ cancers, with no or weak binding to CD47+ normal tissues including human red blood cells to avoid the possible toxicities to normal tissues. Applicant's initial studies demonstrated that the presently disclosed immunotoxin, bi-CD47-IT, possessed highly potent efficacy to CD47+ T- ALL, showed littler or no binding avidity and no hemagglutination in human red blood cells, and weak binding to human lymphocytes and monocytes. Not only did Bi-CD47-IT show highly potent in vivo efficacy in early T-ALL CDX mouse models, but also showed highly potent efficacy against overt T-ALL CDX and PDX mouse models.

Remarkably, bi-CD47-IT cured 60% (3 of 5 mice) of the tumor-bearing mice with only 10-day treatment in a T-ALL Molt-4 CDX mouse model, which indicates that bi-CD47-IT has potential to cure T-ALL patients clinically.

Initially, Applicants anticipated that bi-CD47-IT had the potential act through macrophages by blocking the CD47-SIRPα axis to enhance the phagocytosis of macrophage to the CD47+ tumor cells for immunotherapy. However, in vitro and in vivo data using biscFv(B6H12) demonstrated that bi-CD47-IT functions only through targeted therapy, not through macrophage-mediated immunotherapy.

CD47 is also overexpressed on other blood and solid cancer cells. Thus, bi-CD47-IT is disclosed as a broad-spectrum therapeutic for CD47+ cancers, for example peripheral T-cell lymphoma (PTCL), cutaneous T-cell lymphoma (CTCL) and lung cancer.

CD47 is also expressed on normal tissues including human red blood cells and lymphocytes with low binding avidities, making specificity/on-target toxicity concern when developing CD47-based therapies. For this reason, some clinical trials of anti-human CD47 mAbs including Magrolimab were suspended. One of the toxicities associated with CD47 mAbs is the partial depletion of human red blood cells leading to anemia. Applicants herein demonstrate that the presently disclosed immunotoxin, bi-CD47-IT, showed no binding or hemagglutination of human red blood cells. This data indicates that there is little or no anemia risk with the presently disclosed immunotoxin treatment. Applicants also performed a toxicity study of the disclosed immunotoxin in humanized mice, to study possible toxicity to human

lymphocytes. Applicants demonstrated that the disclosed immunotoxin transiently depleted human lymphocytes for ~4 weeks and no clinical adverse events were observed. Without wishing to be restricted by theory, Applicants speculate that the moderate/weak binding avidity of bi-CD47-IT (K_D=281 nM to T-ALL CCRF-CEM cells) contributes to lack of adverse events. In contrast, the above-discussed Magrolimab bound to human red blood cells very strongly with K_D value of 9.96 nM and induced in vitro hemagglutination at a very low concentration (1.2 μg/mL).

The CD47 receptor density is dramatically higher on the surface of cancer cells, including T-ALL CCRF-CEM cells, than on normal tissues, including human red blood cells. The high specificity of bi-CD47-IT contributes to its following 3 features to make full use of the CD47 expression level difference on cancer cells versus normal tissues. 1) Low binding affinity (compared to the corresponding mAb); 2) Low does (less than 20 μg/kg in patients); 3) Short half-life (~30 minutes). In addition, bi-CD47-IT does not contain the Fc region of mAbs. This lack of Fc may allow the presently disclosed immunotoxin to avoid Fc-relevant toxicity of anti-human CD47 mAbs. The short half-life of Applicants' immunotoxin, in one embodiment bi-CD47-IT (~30 min), might also contribute to the good safety profile of bi-CD47-IT – as mAbs have considerably longer half-lives (10-21 days), .

Applicants also disclose anti-murine bi-CD47-IT, which may be useful for studying toxicity of the disclosed immunotoxins in syngeneic mouse models and in the presence of murine immune system. Applicants also disclose variant anti-human bi-CD47-IT (5F9 clone) with crosses species reactivity, for example to porcine CD47.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. In the case of conflict, the present specification, including definitions, will control. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference. The references cited herein are not admitted to be prior art.

Immunotoxins

As used herein, "immunotoxin" is used to describe a conjugate (or fusion protein) of a toxin protein, or portion thereof, and at least one antigen binding portion, for one example an antibody single chain variable fragment (scFv). Single chain variable fragments are fusion proteins of the variable regions of the heavy (VH) and light (VL) chains of immunoglobulins, connected to form a single polypeptide chain with a short linker peptide (e.g., 1-50 amino acids). The linker may allow the scFv to fold into a structure resembling the antigen binding site of a full-length antibody and suitable for antigen binding. Immunotoxins may also include a linker

between the toxin and the binding portion. As used herein, the term "immunotoxin" includes both the nucleic acid encoding the fusion protein and the translated fusion protein itself.

The toxin portion of the disclosed immunotoxin may be derived from various toxins. In many embodiments, the toxin portion is derived from diphtheria toxin (DT), which is a single chain, 62 kDa protein consisting of 535 amino acid residues and produced by Corynebacterium diphtheria containing lysogenic beta phage. In one embodiment, the toxin portion includes the first approximately 390 amino acids of the diphtheria toxin (DT390), and/or the nucleic acid that encodes DT390. DT390 may be positioned at or near the N terminus of an immunotoxin disclosed herein. DT390 may have the DNA sequence of SEQ ID NO: 1 and the peptide sequence of SEQ ID NO: 2. In many embodiments, the disclosed toxin portion maybe may be a protein at least 80% identical to SEQ ID NO:2.

The targeting portion includes at least one anti-human CD47 antibody or fragment thereof. The anti-human CD47 antibody or fragment thereof may be from a humanized anti-CD47 block antibody. In many embodiments, the humanized anti-CD47 antibody may be a single chain antibody fragment, for example an scFv. In many embodiments, the scFv may comprise a variable heavy chain sequence (VH) and a variable light chain sequence (VL). In some embodiments, the scFv is referred to as B6H12, and may be coded for by the sequence SEQ ID NO: 3. In many embodiments, the targeting portion may have a sequence at least about 80% identical to the the sequence of the protein coded for by SEQ ID NO:3.

One or more linkers may be positioned between portions of the immunotoxin, such as between the toxin portion and the targeting portion. The one or more linkers may be positioned within portions of the immunotoxin, such as within an scFv, such as between a VH and a VL. The number of consecutively repeated linkers may be one, two, three, or more. Each linker may encode one or more amino acids, such as one to twenty or five to fifteen. In embodiments, the linker includes four glycine residues and a serine residue (G_4S; SEQ ID NO: 26), or the nucleic acid that encodes such peptide. The G_4S (SEQ ID NO: 26) linker may be coded by the sequence SEQ ID NO: 4.

Examples of specific immunotoxins are shown in FIG. 1A. In one example, the immunotoxin is a monovalent anti-human CD47 immunotoxin (mono-CD47-IT) comprising a DT390 toxin portion at the N terminus linked to a B6H12 scFv at or near the C terminus by at least one G_4S (SEQ ID NO: 26) linker. The B6H12 scFv includes at least one, such as three, G_4S (SEQ ID NO: 26) linkers positioned between the VL and VH domains. The mono-CD47-IT may have a molecular weight of about 69 kDa when translated into a protein. The mono-CD47-IT may include one or more peptide sequences that may be useful for identification and/or purification. In some embodiments, the peptide sequence is a C-terminal histidine ("his") tag,

which may aid in purification. The mono-CD47-IT, with a his tag, may have the DNA sequence of SEQ ID NO: 5 and protein sequence of SEQ ID NO: 6. In many embodiments, the immunotoxin may be a protein at least about 80% or more identical to the protein of SEQ ID NO:6

With continued reference to FIG. 1A, in another example, the immunotoxin may be a bivalent anti-human CD47 immunotoxin (bi-CD47-IT) comprising a DT390 toxin portion at the N-terminus linked to two tandem B6H12 scFv's, each including a VL and a VH domain. The DT390 portion is linked to a first scFv by at least one G_4S (SEQ ID NO: 26) linker, which in turn is linked to the second B6H12 scFv at or near the C terminus by at least one, such as three, G_4S (SEQ ID NO: 26) linkers. Each B6H12 scFv includes at least one, such as three, G_4S (SEQ ID NO: 26) linkers positioned between the VL and VH domains. The bi-CD47-IT may have a molecular weight of about 95 kDa when translated into a protein. The bi-CD47-IT may include a C-terminal his tag, which may aid in purification. The bi-CD47-IT, with a his tag, may have the DNA sequence of SEQ ID NO: 7 and peptide sequence of SEQ ID NO: 8.

Without being limited to any mechanism or mode of action, the immunotoxins of the present disclosure may bind to a cell surface via interaction between a single targeting portion or two targeting portions. In the case of bi-CD47-IT, the cell may be bound by an interaction between either, or both, of the anti-human CD47 scFv regions and the extracellular domain of CD47 on the cell's surface. The toxin, e.g., the DT390 domain, is internalized by the cell where it inhibits protein synthesis and thereby causes cell death.

Immunotoxins may be prepared according to the disclosure of DNA constructs of immunotoxins in the Examples section below. The DNA constructs may be incorporated in a vector. The immunotoxin DNA constructs may be codon-optimized nucleic acid molecules optimized for expression in a methylotropic yeast. The methylotropic yeast may be *Pichia pastoris*. A host cell, such as a methylotropic yeast cell, may express the nucleic acid molecule.

The immunotoxins disclosed herein may have strong and/or selective binding affinity and/or avidity for CD47+ cancer cells with little to no measurable (or functional) binding to non-cancerous cells, such as human red blood cells (see Examples below). For one example, the binding avidity of the disclosed immunotoxins to CD47+ cancer cells, such as CD47+CCR4+ T-ALL CCRF-CEM cells, may be about 280 nM. In contrast, the binding avidity of the disclosed immunotoxins to human red blood cells may vary from about 1.0 μM to about 99.0 μM. The immunotoxins may not cause, or cause little, hemagglutination of red blood cells. The immunotoxins may not cause, or cause little, negative side effects such as anemia and thrombocytopenia.

Pharmaceutical Formulations

Immunotoxins of this disclosure may be administered as a pharmaceutical formulation. The formulation may include an immunotoxin and a pharmaceutically acceptable carrier. Immunotoxins of this disclosure may be formulated into a pharmaceutical dosage form adapted for intravenous, intraperitoneal, intra-arterial, or subcutaneous administration to a subject. Injection by such routes may use an injection device, such as an IV drip device, infusion pump, and/or tuberculin syringe.

In embodiments, the immunotoxins may be administered concurrently with one or more excipients. Suitable excipients may vary depending upon the particular dosage form chosen. In addition, suitable pharmaceutically acceptable excipients may be chosen for a particular function that they may serve in the formulation. Alternatively or additionally, certain pharmaceutically acceptable excipients may be chosen for their ability to facilitate the production of stable dosage forms, enhance bioavailability, and/or minimize side effects.

Excipients that may be used include buffering agents, carriers, diluents, fillers, binders, disintegrants, lubricants, glidants, granulating agents, coating agents, wetting agents, solvents, co-solvents, suspending agents, emulsifiers, coloring agents, anticaking agents, humectants, chelating agents, plasticizers, viscosity agents, antioxidants, preservatives, stabilizers, and surfactants. The skilled artisan will appreciate that certain pharmaceutically acceptable excipients may serve more than one function and may serve alternative functions depending on how much of the excipient is present in the formulation and what other ingredients are present in the formulation.

The therapeutically effective concentration or dosage of immunotoxin administered to a subject may vary depending on, for example, the nature of the formulation, mode of administration, particular condition to be treated, and condition and mass of the patient. Dosage levels are typically sufficient to achieve a tissue concentration at the site of action that is at least comparable to a concentration that has been shown to be active in vitro, in vivo, or ex vivo. In an example, an immunotoxin is provided in a liquid formulation for intraperitoneal administration at a concentration of about 15μg/kg once daily for 5 consecutive days. In some embodiments, the once daily administration for 5 consecutive days may be repeated at least once after three weeks. In many embodiments, the dosing may be repeated between 2 and 8 times consecutively. In some embodiments, the dosing may be from about 9 to about 18 μg/kg/day. The composition may be administered by intravenous infusion over about 15 to about 60 for from about 1 to about 5 days consecutively.

Therapeutic Methods

The compositions containing the immunotoxins described herein are suitable for treating at least one symptom of a $CD47^+$ cancer. $CD47^+$ cancers are those in which at least some of the cancer cells express the CD47 transmembrane protein on their surface. $CD47^+$ cancers targeted by the therapeutic methods disclosed herein include T-ALL, cutaneous T-cell lymphoma (CTCL) (including human $CD47^+CD25^+CCR4^+CD30^+$ CTCL), peripheral T-cell lymphoma (PTCL), lung cancer (including human $CD47^+$ lung cancer), triple-negative breast cancer, head and neck cancer, melanoma, and bladder cancer. ALL, CTCL, and PTCL are blood cancers; lung, breast, head and neck, melanoma, and bladder are solid cancers.

Administration of one or more immunotoxins disclosed herein may cause death of $CD47^+$ cancer cells. Without being limited to any mechanism or mode of action, the toxin portion, e.g., the DT390 domain, may be internalized by a $CD47^+$ cancer cell where it inhibits protein synthesis and ultimately causes cell death. Consistent with this theory, immunotoxins may inhibit cell growth, such as the growth of $CD47^+$ cancer cells, for example $CD47^+CCR4^+$ T-ALL CCRF-CEM cells. Immunotoxins may deplete a concentration of cancerous cells in a patient's blood, such as human $CD47^+CCR4^+$ T-ALL CCRF-CEM cells (see Examples, below). Immunotoxins may be useful in reducing or preventing metastases, such as liver infiltration by $CD47^+$ cancer cells (see Examples below). The present immunotoxins may also be useful in reducing or preventing metastases to the spleen, bone marrow, brain, spinal cord, and other tissues and organs. The disclosed immunotoxins may reduce death and/or prolong survival of subjects with various $CD47^+$ cancers.

The immunotoxin formulations of this disclosure can be administered to a subject diagnosed with active cancer or in remission from cancer. The frequency and duration of immunotoxin administration may vary. In many embodiments, the dosing schedule may be similar to other toxin-based drugs, for example Ontak® and Elzonris®, for example administration of the immunotoxin-therapeutic daily for ten consecutive days every 3 weeks. In embodiments, an effective amount of immunotoxin may be administered daily for about ten days, for example seven to 12 consecutive days. In many embodiments the disclosed immunotoxin may be administered daily for more than about 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, or 12 days and less than about 12 days, 11 days, 10 days, 9 days, 8 days, 7 days, or 6 days. In embodiments, an effective amount of immunotoxin may be administered more than once a day, twice daily, or three times daily. In some embodiments, the disclosed pharmaceutical immunotoxin compositions may be administered on a weekly basis, for example one, two, three, four, five, six, or more times per week. Monthly administrations may also be implemented, such that immunotoxin formulations are administered one, two, three, four, or more times per month.

In some embodiments, a treatment regimen may be a set number of consecutive days of administration followed by a period where no immunotoxin is administered, for example more than 5 days and less than about a month, for example more than 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, 14 days, 2.5 weeks, 3 weeks, 3.5 weeks, 4 weeks, 5 weeks, or more and less than about 2 months, 1.5 months, 5 weeks, 4 weeks, 3.5 weeks, 3 weeks, 2.5 weeks, 14 days, 13 days, 12 days, 11 days, 10 days, 9 days, 8 days, or 7 days.

The number of times per day, week, or month that the disclosed formulations are administered to a subject, along with the entire duration of the treatment period, may depend on the severity or type of condition a subject is experiencing or is expected to experience. For example, embodiments in which an immunotoxin is administered to treat existing cancer may involve more frequent administrations than embodiments in which an immunotoxin is administered to prevent or delay the recurrence of cancer. Embodiments in which an immunotoxin is administered to prevent or delay the recurrence of cancer may involve a longer treatment period than embodiments in which an immunotoxin is administered to treat existing cancer. The length of the treatment period may also be patient-specific and re-evaluated periodically by a doctor or other health care provider.

Therapeutic efficacy

The disclosed therapeutic methods and pharmaceutical compositions may be effective at significantly depleting or reducing the concentration of CD47+ cells in a patient's tissue or blood. In some embodiments, after administration of the disclosed immunotoxin with two targeting portions to a patient, the concentration of CD47+ cells in the peripheral blood of a patient with a CD47+ cancer may be reduced by more than about 70%, for example more than about 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. In some embodiments, after treatment, the patient may have no detectable CD47+ cancer cells in a target organ, or tissue or peripheral blood. In contrast, an immunotoxin with only one targeting portion, or an anti-CD47 antibody, or an immunotoxin with a non-CD47 targeting portion may reduce the number of CD47+ cells by less than about 50%, for example about 8-30%. In some embodiments, for example where the CD47+ cancer cells are blast cells, the percentage of blast cells in a sample of tissue, for example the liver, spleen, or peripheral blood, of a treated patient may be less than about 10%, 9%, 8%, 7%, 6%, 5%, 3%, 2%, or 1%, relative to the starting percentage before administration of the disclosed immunotoxin, at a point after cessation of treatment, for example one week after treatment with the disclosed immunotoxin, for example after 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days.

The disclosed therapeutic methods and pharmaceutical compositions may be effective at curing a patient or subject with a CD47+ cancer. For one example, the patient or subject may be free of CD47+ cancer cells after one, two, three, four, or more rounds of treatment, and may continue to be free of CD47+ cancer cells 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 1.5 years, 2 years, 2.5 years, 3 years, 3.5 years, 4 years, 4.5 years, 5 years or more after treatment has stopped.

Definitions

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

As used herein, the term "about" can mean relative to the recited value, e.g., amount, dose, temperature, time, percentage, etc., ±10%, ±9%, ±8%, ±7%, ±6%, ±5%, ±4%, ±3%, ±2%, or ±1%.

The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. The term "comprises" means "includes." Also, "comprising A or B" means including A or B, or A and B, unless the context clearly indicates otherwise. It is to be further understood that all molecular weight or molecular mass values given for compounds are approximate, and are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of this disclosure, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Antibody

Within the definition of "antibody" according to the invention are full-length antibodies, antibody fragments, and antigen binding proteins. Also included are various antibodies and other immunoglobulins generated by biotechnological or protein engineering methods or processes. Full-length antibodies may be for example monoclonal, recombinant, chimeric, deimmunized, humanized, and human antibodies, as well as antibodies from other species such as mouse, hamster, rabbit, rat, goat, or non-human primates.

Antibody fragments include antigen-binding portions of the antibody including, inter alia, Fab, Fab', F(ab')2, Fv, domain antibody (dAb), complementarity determining region (CDR) fragments, CDR-grafted antibodies, single-chain antibodies (scFv), single chain antibody fragments, chimeric antibodies, diabodies, triabodies, tetrabodies, minibody, linear antibody; chelating recombinant antibody, a tribody or bibody, an intrabody, a nanobody, a small modular

immunopharmaceutical (SMIP), an antigen-binding-domain immunoglobulin fusion protein, single domain antibodies, a VHH containing antibody, or a variant or a derivative thereof, and polypeptides that contain at least a portion of an immunoglobulin that is sufficient to confer specific antigen/target binding to the polypeptide, such as a polypeptide comprising one, two, three, four, five or six CDR sequences, as long as the antibody retains the desired biological activity.

Amino acid

"Amino acid identity," "residue identity," "identity," and the like, as used herein refers to the structure of the functional group (R group) on the polypeptide backbone at a given position or residue. Naturally occurring amino acid identities are (name/3-letter code/one-letter code): alanine/ala/A; arginine/arg/R; asparagine/asn/N; aspartic acid/asp/D; cysteine/cys/C; glutamine/gln/Q; glutamic acid/glu/E; glycine/gly/G; histidine/his/H; isoleucine/ile/I; leucine/leu/L; lysine/lys/K; methionine/met/M; phenylalanine/phe/F; proline/pro/P; serine/ser/S; threonine/thr/T; tryptophan/trp/W; tyrosine/tyr/Y; and valine/val/V. Variants and derivatives of the naturally occurring amino acids are also contemplated here.

An amino acid within a molecule may be substituted to create an engineered molecule. The amino acid (aa or a.a.) residue can be replaced by a residue having similar physiochemical characteristics, that is a 'conservative substitution' – e.g., substituting one aliphatic residue for another (such as Ile, Val, Leu, or Ala for one another), or substitution of one polar residue for another (such as between Lys and Arg; Glu and Asp; or Gln and Asn). Other such conservative substitutions, for example based on size, charge, polarity, hydrophobicity, chain rigidity/orientation, etc., are well known in the art of protein engineering. Polypeptides comprising conservative amino acid substitutions can be tested in any one of the assays described herein to confirm that a desired activity, e.g. binding, specificity, and/or function of a native or reference polypeptide is achieved.

While conservative substitutions within the interior of a protein, i.e. buried or non-solvent accessible residues/positions, may in some cases alter the structure of the protein, affect folding of the protein, and/or modify the protein's function, conservative substitutions at or near the protein's surface, i.e. exposed or solvent-accessible residues/positions may cause little or no discernable change to the protein's structure and/or function, unless the altered surface protein is necessary for an interaction with another molecule, peptide, or protein. It is well within the abilities of the skilled artisan to alter the disclosed protein sequences by introducing conservative substitutions at up to 20% of the residues/positions without disrupting or changing the protein's structure and/or function.

Amino acids can be grouped according to similarities in the properties of their side chains (in A. L. Lehninger, in Biochemistry, second ed., pp. 73-75, Worth Publishers, New York (1975)): (1) non-polar: Ala (A), Val (V), Leu (L), Ile (I), Pro (P), Phe (F), Trp (W), Met (M); (2) uncharged polar: Gly (G), Ser (S), Thr (T), Cys (C), Tyr (Y), Asn (N), Gln (Q); (3) acidic: Asp (D), Glu (E); (4) basic: Lys (K), Arg (R), His (H). Alternatively, naturally occurring residues can be divided into groups based on common side-chain properties: (1) hydrophobic: leucine, Met, Ala, Val, Leu, Ile; (2) neutral hydrophilic: Cys, Ser, Thr, Asn, Gln; (3) acidic: Asp, Glu; (4) basic: His, Lys, Arg; (5) residues that influence chain orientation: Gly, Pro; (6) aromatic: Trp, Tyr, Phe. Non-conservative substitutions will entail exchanging a member of one of these classes for another class. Particular conservative substitutions include, for example; Ala into Gly or into Ser; Arg into Lys; Asn into Gln or into His; Asp into Glu; Cys into Ser; Gln into Asn; Glu into Asp; Gly into Ala or into Pro; His into Asn or into Gln; Ile into Leu or into Val; Leu into Ile or into Val; Lys into Arg, into Gln or into Glu; Met into Leu, into Tyr or into Ile; Phe into Met, into Leu or into Tyr; Ser into Thr; Thr into Ser; Trp into Tyr; Tyr into Trp; and/or Phe into Val, into Ile or into Leu.

Treating

The terms "treat", "treating" and "treatment" may further refer to eliminating, reducing, suppressing, or ameliorating, either temporarily or permanently, either partially or completely, a clinical symptom, manifestation or progression of an event, disease or condition associated with the oncological disorders and diseases described herein. As is recognized in the pertinent field, methods and drugs employed as therapies may reduce the severity of a given disease state, but need not abolish every manifestation of the disease to be regarded as useful. Similarly, a prophylactically administered treatment need not be completely effective in preventing the onset of a condition to constitute a viable prophylactic method or agent. Simply reducing the impact of a disease (for example, as disclosed herein, decreasing cancer cell growth rate, reducing tumor size, reducing tumor weight, reducing or preventing metastasis, etc. and/or reducing the number or severity of associated symptoms, or by increasing the effectiveness of another treatment, or by producing another beneficial effect), or reducing the likelihood that the disease will occur or worsen in a subject, is sufficient. One embodiment of the invention is directed to a method for determining the efficacy of treatment comprising administering to a patient therapeutic treatment in an amount, duration, and repetition sufficient to induce a sustained improvement over pre-existing conditions, or a baseline indicator that reflects the severity of the particular disorder.

Treating cancer, as contemplated herein, encompasses treating, delaying, or reversing at least one symptom of cancer. Accordingly, "treating" or "treatment" or "amelioration" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to

prevent, postpone, or slow down (lessen) the targeted pathological condition, disorder, and/or symptom. A subject is successfully "treated" for cancer if, after receiving a therapeutic amount of an immunotoxin according to methods of this disclosure, the subject shows observable and/or measurable reduction in, or absence of, the total number of tumor cells, the size of a tumor, and/or the location of tumor cell growth or infiltration (e.g., metastases). The terms "treat" or "treating" are used consistently herein for ease of illustration only and thus should not be construed as limiting.

Prevent

The term "prevention" as used herein means the avoidance of the occurrence or of the re-occurrence of a disease as specified herein, by the administration of an active compound, for example the disclosed peptide molecules, according to the invention to a subject in need thereof.

Polypeptide

As used herein, the terms "protein" and "polypeptide" may be used interchangeably to designate a series of amino acid residues connected to each other by peptide bonds between the alpha-amino and carboxy groups of adjacent residues. The terms "protein" and "polypeptide" refer to a polymer of amino acids, including modified amino acids (e.g., phosphorylated, glycated, glycosylated, etc.) and amino acid analogs, regardless of its size or function. "Protein" and "polypeptide" are often used in reference to relatively large polypeptides, whereas the term "peptide" is often used in reference to small polypeptides, but usage of these terms in the art overlaps. The terms "protein" and "polypeptide" may be used interchangeably herein when referring to a gene product and fragments thereof. Thus, exemplary polypeptides or proteins include gene products, naturally occurring proteins, homologs, orthologs, paralogs, fragments and other equivalents, variants, fragments, and analogs of the foregoing.

Dose

The terms "dosage" or "dose" as used herein denote any form of the active ingredient formulation that contains an amount sufficient to produce a therapeutic effect with a single administration.

Effective Amount

An "effective amount" of an immunotoxin is an amount sufficient to carry out a specifically stated purpose. An "effective amount" may be determined empirically and in a routine manner, in relation to the stated purpose. The term "therapeutically effective amount" refers to an amount of an immunotoxin to "treat" cancer in a subject. The term "effective amount" refers to an amount of a compound of the invention or other active ingredient sufficient to provide a therapeutic or prophylactic benefit in the treatment or prevention of a disease or to delay or minimize symptoms associated with a disease. Further, a therapeutically effective

amount with respect to a compound of the invention means that amount of therapeutic agent alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or prevention of a disease. Used in connection with a compound of the present disclosure, the term can encompass an amount that improves overall therapy, reduces or avoids symptoms or causes of disease, or enhances the therapeutic efficacy or synergies with another therapeutic agent.

The phrase "therapeutically effective amount" means an amount of a compound of the present invention that (i) treats the particular disease, condition, or disorder, (ii) attenuates, ameliorates, or eliminates one or more symptoms of the particular disease, condition, or disorder, or (iii) prevents or delays the onset of one or more symptoms of the particular disease, condition, or disorder described herein. In the case of cancer, the therapeutically effective amount of the drug may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy can be measured, for example, by assessing the time to disease progression (TTP) and/or determining the response rate (RR).

Subject

As used herein, "subject" or "patient" means a human or other mammal. Non-human subjects may include, but are not limited to, various mammals including domestic pets and/or livestock. The animal can be a mammal such as a non-primate and a primate (e.g., monkey and human). In one embodiment, a patient is a human, such as a human infant, child, adolescent or adult.

The term "mammal" includes, but is not limited to, humans, mice, rats, guinea pigs, monkeys, dogs, cats, horses, cows, pigs, and sheep.

"Subject in need", "patient" or those "in need of treatment" include those already with existing disease (in one example, T-ALL), as well as those at risk of the disease. The terms also include human and other mammalian subjects that receive either prophylactic or therapeutic treatments as disclosed herein.

Amelioration

The term "amelioration" as used herein refers to any improvement of a disease state (for example cancer) of a patient, by the administration of one or more treatments and/or compositions, according to the present disclosure, to such patient or subject in need thereof. Such an improvement may be seen as a slowing down the progression or stopping the progression of

the disease of the patient, and/or as a decrease in severity of disease symptoms, an increase in frequency or duration of disease symptom-free periods or a prevention of impairment or disability due to the disease.

Reducing

"Reducing," "reduce," or "reduction" means decreasing the severity, scope, or degree of cancer or a symptom or cause thereof.

Administration

"Administration of" and "administering a" compound, composition, or agent should be understood to mean providing a compound, composition, or agent; a prodrug of a compound, composition, or agent; or a pharmaceutical composition as described herein. The compound, composition, or agent can be provided or administered by another person to the subject (e.g., intravenously) or it can be self-administered by the subject (e.g., orally). The compound, composition, or agent may be an immunotoxin and may be administered intravenously, intraperitoneally, via infusion, or intravenous bag.

"Intravenous" administration refers to administering a drug (e.g., the disclosed immunotoxin and/or pharmaceutically acceptable forms thereof) into a vein of a patient, e.g., by infusion (slow therapeutic introduction into the vein).

Intraperitoneal administration or injection refers to administering a drug (e.g., the disclosed peptide and/or pharmaceutically acceptable forms thereof) into the peritoneum of a patient.

"Infusion" or "infusing" refers to the introduction of a drug into the body through a vein for therapeutic purposes. Generally, this is achieved via an intravenous (IV) bag.

"Sub-cutaneous" administration may refer to introduction of a drug below the surface of the skin. Generally, this may be accomplished with a syringe and needle, for one example a tuberculin syringe and needle.

An "intravenous bag" or "IV bag" is a bag that can hold a solution which can be administered via the vein of a patient, which may be referred to as an IV drip. In one embodiment, the solution is a saline solution (e.g. about 0.9% or about 0.45% NaCl). Optionally, the IV bag is formed from polyolefin or polyvinyl chloride.

Pharmaceutical composition

"Pharmaceutical compositions" or "pharmaceutical formulations" are compositions that include an amount (for example, a unit dosage) of one or more of the disclosed immunotoxins together with one or more non-toxic pharmaceutically acceptable additives, including carriers, diluents, and/or adjuvants, and optionally other biologically active ingredients. Such pharmaceutical compositions can be prepared by standard pharmaceutical formulation

techniques such as those disclosed in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa. (19th Edition).

As used herein, a "pharmaceutically acceptable excipient" or a "pharmaceutically acceptable carrier" means a pharmaceutically acceptable material, composition, or vehicle involved in giving form or consistency to the pharmaceutical composition. Each excipient or carrier should be compatible with other ingredients of the pharmaceutical composition when comingled such that interactions that would substantially reduce the efficacy of the immunotoxin formulations of this disclosure when administered to a subject and interactions that would result in pharmaceutical compositions that are not pharmaceutically acceptable are avoided. In addition, each excipient or carrier should be of sufficiently high purity to render it pharmaceutically acceptable.

In this description, a "pharmaceutically acceptable salt" is a pharmaceutically acceptable, organic or inorganic acid or base salt of a compound of the invention. Representative pharmaceutically acceptable salts include, e.g., alkali metal salts, alkali earth salts, ammonium salts, water-soluble and water-insoluble salts, such as the acetate, amsonate (4,4-diaminostilbene-2,2-disulfonate), benzenesulfonate, benzonate, bicarbonate, bisulfate, bitartrate, borate, bromide, butyrate, calcium, calcium edetate, camsylate, carbonate, chloride, citrate, clavulariate, dihydrochloride, edetate, edisylate, estolate, esylate, fiunarate, gluceptate, gluconate, glutamate, glycollylarsanilate, hexafluorophosphate, hexylresorcinate, hydrabamine, hydrobromide, hydrochloride, hydroxynaphthoate, iodide, lactate, lactobionate, laurate, malate, maleate, mandelate, mesylate, methylbromide, methylnitrate, methylsulfate, mucate, napsylate, nitrate, N-methylglucamine ammonium salt, 3-hydroxy-2-naphthoate, oleate, oxalate, palmitate, pamoate (1,1-methene-bis-2-hydroxy-3-naphthoate, einbonate), pantothenate, phosphate/diphosphate, picrate, polygalacturonate, propionate, p-toluenesulfonate, salicylate, stearate, subacetate, succinate, sulfate, sulfosaliculate, suramate, tannate, tartrate, teoclate, tosylate, triethiodide, and valerate salts. A pharmaceutically acceptable salt can have more than one charged atom in its structure. In this instance the pharmaceutically acceptable salt can have multiple counterions. Thus, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counterions.

EXAMPLES

The following examples illustrate various aspects of the disclosure, and should not be considered limiting.

The following materials and methods were employed in the subsequent Examples.

In vivo efficacy studies of bi-CD47-IT

NSG (NOD scid gamma) mice (6–8 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) for the in vivo efficacy studies of bi-CD47-IT against T-ALL. The in vivo experiments were approved by the University of Colorado Anschutz Medical Campus Animal Care and Use Committee. All experimental mice were intravenously injected on day 0 with 1.0×10^7 human CD47+ T-ALL tumor cells (T-ALL cell lines or PDX cells). The immunotoxin was intraperitoneally injected at 8.43×10^{-10} moles/kg, once daily for 10 consecutive days. The dosing schedule of the immunotoxins was based on our previous publication. In this study, we assessed the in vivo efficacy of bi-CD47- IT in two T-ALL tumor cell lines (CCRF-CEM and Molt-4), and two T-ALL PDX samples (PDX sample #1 and #2)-based T-ALL CDX and PDX mouse models. 1) CCRF-CEM cell line-based early T-ALL CDX mouse model. The immunotoxin treatment started on day 4, when ~0.3% T- ALL blast cells were detected in the peripheral blood. This CDX mouse model was also used for serial study of the in vivo efficacy of bi-CD47-IT. 2) CCRF-CEM cell line-based overt T-ALL CDX mouse model. The immunotoxin treatment started on day 7, when 6% T-ALL blast cells were detected in the peripheral blood. The overt T-ALL mouse model was defined as that when >1% T-ALL blast cells were detected in the peripheral blood. The different overt T-ALL mouse models were used to mimic the different late-stage T-All patients. 3) Molt-4 cell line-based overt T-ALL CDX mouse model. The immunotoxin treatment started on day 4, when 1.3-2.2 % T-ALL blast cells were detected in the peripheral blood. 4) PDX sample #1-based overt T-ALL PDX mouse model. The immunotoxin treatment started on day 7, when ~30% human T-ALL blast cells were detected in the peripheral blood. 5) PDX sample #2-based overt T-ALL PDX mouse model. The immunotoxin treatment started on day 7, when 5% human T-ALL blast cells were detected in the peripheral blood. The tumor-bearing mice were monitored daily for signs and symptoms of illness and scored at least twice weekly based on the parameters as previously reported. The animals were humanely euthanized when the body condition score exceeded the limit, or the animal lost more than 15% of its pre-injection body weight.

Human T-ALL cell lines, PDX cells, and antibodies

Human T-ALL CCRF-CEM and Molt-4 cell lines were obtained from ATCC (Cat# CCL-119 and CRL-1582, Manassas, VA). Human T-ALL PDX sample #1 and #2 tumor cells were generously provided by Dr. Eduard Davila's laboratory (University of Colorado Anschutz Medical Campus). The T-ALL PDX tumor cells were in vivo amplified using immunodeficient NSG mice. The original T-ALL PDX tumor cells were washed with 10 mL of modified PBS (1% FBS and 1× penicillin/streptomycin) and intravenously injected into NSG mice via the tail vein.

When the T-ALL PDX tumor cells reach 1,000 cells/mL in the peripheral blood, the tumor-bearing mice were euthanized and the T-ALL PDX cells were harvested from the spleens and purified using CD3+ magnetic beads (Miltenyi Biotec, Cat# 130-097-043). The purified T-ALL PDX tumor cells were then directly intravenously injected into the experimental NSG mice at 5×10^6 cells per mouse. The antibodies used in this study are listed below:

Antibody name	Clone	Source	Cat#
Anti-DT mAb		Meridian Bioscience	C86036M
Goat anti-mouse IgG-HRP		Santa Cruz	SC-2354
Anti-6xHis mAb		Biolegend	652508
FITC-Anti-human CD47 mAb	B6H12	BD Biosciences	556045
PE-Anti-human CD47 mAb	B6H12	BD Biosciences	556046
Anti-human CD47 mAb	B6H12	Thermo Fisher	14-0479-82
PerCP/Cy5.5 Anti-mouse CD45 mAb	C363-16A	Biolegend	103314
Magrolimab (Humanized anti-human CD47 IgG4 mAb)	Hu5F9-G4	MedChemExpress	HY-P99029
PE-Anti-human HLA/ABC Ab	W6/32	Biolegend	311406
APC-Anti-Human CD5 Ab	UCHT2	Biolegend	300612
PE/Cyanine7-Anti-Human CD7 Ab	4H9/CD7	Biolegend	311406
FITC-Anti-Human CD8a Ab	RPA-T8	Biolegend	301050.

DNA construction

As shown in Figure 1A, mono-CD47-IT and bi-CD47-IT contain two domains, DT390 1 and antihuman CD47 scFv (B6H12). A linker consisting of four glycines and a serine residue (G_4S; SEQ ID NO: 26) connected DT390 domain to anti-human CD47 scFv (B6H12) domain(s). The anti-human CD47 scFv (B6H12) domains of the bi-CD47-IT were joined by three tandem G_4S (SEQ ID NO: 26) linkers, $(G_4S)3$ (SEQ ID NO: 27). Six histidines (6× His tag; SEQ ID NO: 28) were added to the C-terminus of each construct to facilitate the immunotoxin purification.

Mono-CD47-IT DNA construct.

Codon-optimized anti-human CD47 scFv (B6H12) DNA was synthesized by GenScript (Piscataway, NJ) and cloned into the pwPICZαDT390 vector between the NcoI and EcoRI sites, yielding the mono-CD47-IT DNA construct.

Bi-CD47-IT DNA construct.

The first anti-human CD47 scFv (B6H12) DNA was amplified using PCR primers CD47-Nco carrying XhoI and NcoI sites, and CD47-Bam1 carrying BamHI and EcoRI sites. The codon-optimized, synthesized anti-human CD47 scFv (B6H12) DNA was used as the PCR template. The amplified PCR product was separated using DNA agarose gel electrophoresis. The

expected PCR product band was cut out and extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). The extracted DNA was digested using XhoI and EcoRI, cleaned using the QIAquick PCR Purification Kit (Qiagen), and cloned into pwPICZα plasmid vector for sequencing confirmation. The first anti-human CD47 scFv (B6H12) DNA was cut out using NcoI and BamHI and extracted using the QIAquick Gel Extraction Kit as Insert #1. A similar approach was used to obtain the second anti-human CD47 scFv (B6H12) DNA. The primers used to amplify the second anti-human CD47 scFv (B6H12) DNA were CD47-Bam2 carrying XhoI and BamHI sites, and CD47-Eco carrying an EcoRI site. The gel-purified PCR product was digested using XhoI and EcoRI, cleaned using the QIAquick PCR Purification Kit, and cloned into pwPICZα plasmid vector for sequencing confirmation. The second anti-human CD47 scFv (B6H12) DNA was cut out using BamHI and EcoRI and extracted using the QIAquick Gel Extraction Kit as Insert #2. Insert #1 and insert #2 [NcoI-scFv(B6H12)-BamHIscFv(B6H12)-EcoRI] were cloned together into pwPICZα-DT390 vector between the NcoI and EcoRI to generate the bi-CD47-IT DNA construct. BiscFv(B6H12) DNA (cloned into pwPICZα) was also constructed using a similar approach. The PCR primers used for the DNA construction are listed below at Table 1.

TABLE 1

Name and Sequence (with restriction site(s) underlined) 5'->3'	SEQ ID NO:
CD47-Nco CCGCTCGAGCCATGGGGTGGTGGTGGTTCTGAGATTGTTTTGACTCAATCTCCA XhoI NcoI	9
CD47-Bam1 CCGGAATTCCGCCGCGGATCCACCACCACCAGAACCACCACCACCGGAAGAAAC EcoRI BamHI AGTAACCAAAGTACC	10
CD47-Bam2 CCGCTCGAGGGATCCGGTGGTGGTGGTTCTGAGATTGTTTTGACTCAATCTCCA XhoI BamHI	11
CD47-Eco CCGGAATTCTTAGTGGTGGTGGTGGTGGTGGGAAGAAACAGTAACCAAAGTACC EcoRI	12
CD47-XhoI CCGCTCGAGAAGAGAGAGGCTGAAGCTGAGATTGTTTTGACTCAATCTCCA XhoI	13

Protein expression

Mono-CD47-IT and bi-CD47-IT DNA constructs were linearized by SacI digestion and transformed into the diphtheria toxin-resistant yeast Pichia pastoris cells using the Gene Pulser Xcell Electroporation System (Bio-Rad, Hercules, CA). The transformed cells were spread on YPD agar plates (1% yeast extract, 2% peptone, 1.5% agar, 2% dextrose) containing 100 μg/mL

zeocin and incubated at 30°C for 3–4 days. Six colonies were randomly picked and cultured in 5 mL YPD at 30°C for 24 h at 250 rpm and then in YPG (1% yeast extract, 2% peptone, 1% glycerol) for another 24 hours. The immunotoxin induction was carried out with 2 mL BMMYC (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 7.0, 1.34% yeast nitrogen base without amino acids, 4×10^{-5} % biotin, 0.5% methanol and 1% casamino acids) for 48 hours at 25°C and 225 rpm. Methanol (0.5%) was added twice daily to maintain the methanol level. Antifoam (Emerald Performance Materials LLC, Vancouver, WA) was added to all growth and induction media at a concentration of 0.02%. Phenylmethanesulfonyl fluoride (PMSF, 1 mM, Sigma, St. Louis, MO) was added to inhibit immunotoxin degradation during the induction phase. Penicillin (100 U/mL) and streptomycin (100 µg/mL) were added to all growth and induction media to inhibit bacterial contamination. The culture supernatants were analyzed using 4–12% SDS gels. One clone of mono-CD47-IT or bi-CD47-IT was selected for large-scale expression. The Excella E24 incubator shaker (Eppendorf, Framingham, MA) was used for large-scale expression. The seed culture was prepared by inoculating a single colony into YPD medium and then incubating at 25°C and 225 rpm overnight. 5% of the seed culture was transferred to 1 L PYREX shaker flasks containing 250 mL YPD medium and cultured at 30°C and 250 rpm for 24 hours. The cells were centrifuged at 491 g for 5 minutes, and the cell pellet was resuspended in 250 mL YPG medium and cultured at 30°C and 250 rpm for 24 hours. For the induction phase, cells were centrifuged at 491 g for 5 minutes, and the cell pellet was resuspended in 125 mL BMMYC induction medium and induced at 25°C and 225 rpm for 48 hours. Methanol (0.5%) was added twice daily to maintain the methanol level. After the induction, the yeast cells were pelleted by centrifugation at 1,692 g, 4°C for 10 minutes. The supernatant was collected for the first-step purification. Antifoam, PMSF, and penicillin/streptomycin were also added to the expression medium, as described for the small-scale preparation.

Protein purification

Ni-Sepharose™ 6 fast flow resin (Cytiva, Marlborough, MA) was used for the first-step purification of the mono-CD47-IT and bi-CD47-IT. The resin was packed in an XK50 column (Cytiva), equilibrated with 20 mM Tris-HCl pH 7.4, 0.5 M NaCl, and 5 mM imidazole. The sample was loaded onto the equilibrated column in 0.5 M NaCl, 20 mM Tris-HCl pH 7.4, 5 mM imidazole. The column was washed with 20 mM Tris-HCl pH 7.4, 0.5 M NaCl, and 5 mM imidazole, and the bound immunotoxins were eluted with 20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, and 500 mM imidazole. The purification fractions were analyzed using 4-12% SDS gels. The fractions containing the immunotoxin of interest were pooled and dialyzed using 3.5 kDa cut-off

Spectra/Por membrane tubing (Repligen, Waltham, MA) against 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5% glycerol at 4°C with stirring. The dialysis buffer was replaced once. Based on the calculated theoretical PI (PI=5.79 for mono-CD47-IT and 6.03 for bi-CD47-IT), strong anion exchange resin Poros 50 HQ (Thermo Fisher Scientific, Waltham, MA) was packed in an XK16/20 column (Cytiva) for the second-step purification. The column was equilibrated with 20 mM TrisHCl, pH 8.0, 1 mM EDTA, and 5% glycerol. The dialyzed sample was loaded onto the column and washed with 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5% glycerol. The bound immunotoxin was eluted with 100 mM and 200 mM sodium borate, and then 200 mM sodium borate plus 50 mM NaCl (250 mM salt in total) in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 5% glycerol. The purified fractions were analyzed using 4–12% SDS gels. The fractions containing the immunotoxin of interest were pooled and dialyzed using the 3.5 kDa cut-off Spectra/Por membrane tubing against PBS, PH 7.4 plus 5% glycerol at 4°C with stirring. The dialysis buffer was replaced once. Protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific). C21 immunotoxin (C21-IT, a non-related DT390-based immunotoxin as negative control), anti-porcine CD3 immunotoxin (pCD3-IT) and single-chain, fold-back, diabody anti-human CCR4 immunotoxin (CCR4-IT, positive control for in vivo study) were also expressed and purified using the same DT-resistant yeast Pichia pastoris expression system in our laboratory. BiscFv(B6H12) protein was also expressed and purified using the yeast Pichia pastoris expression system in our laboratory.

Western Blot analysis

Western blot analysis was performed as described previously. Briefly, protein samples were separated and transferred onto nitrocellulose membranes. The membranes were blocked and washed at room temperature with shaking. The proteins were detected using mouse anti-His Tag or anti-DT primary antibodies and goat anti-mouse IgG-HRP secondary antibody. The proteins were detected using the TMB membrane peroxidase substrate (KPL Cat# 50-77-02, Milford, MA).

Alexa Flour 488-labeling of bi-CD47-IT

Bi-CD47-IT was labeled using Alexa Flour 488 microscale protein labeling kit (Thermo Fisher Scientific) following the manufacturer's instruction. In brief, bi-CD47-IT was concentrated down to a concentration of ~1 mg/mL, The 1/10 volume of sodium bicarbonate solution was added to the protein solution and mixed well. An appropriate volume of Alexa Flour 488 reactive dye solution was added according to the equation of the manufacturer's instruction and incubated for 15 minutes at room temperature. The conjugate reaction mixture was uploaded onto the resin bed surface and centrifuged at 16,000× g for 1 minute. The purified

dye-labeled bi-CD47-IT was collected into an Eppendorf tube. The labeled-immunotoxin concentration was measured using nanodrop and stored at 4°C for use.

Flow cytometry binding avidity analysis of bi-CD47-IT

Human CD47+ T-ALL tumor cells (CCRF-CEM, Molt-4, PDX sample #1, PDX sample #2) were stained with Alexa Flour 488-labeled bi-CD47-IT at a range of concentrations (0.2 to 600 nM). The FITC-labeled anti-human CD47 mAb (BD Bioscience, Cat# 556045, San Jose, CA) was used as a positive control. Alexa Flour 488-labeled isotype mouse IgG1 served as a negative control at a final concentration of 200 nM. 1×10^6 tumor cells were aliquoted into a 12×75 mm tube and incubated with serial diluted Alexa Flour 488-labeled bi-CD47-IT for 30 minutes at 4°C. The tumor cells were washed with 2 mL of cold flow cytometry buffer twice and spun down at 300× g for 5 minutes. The supernatant was discarded and resuspended in 300 µL of flow cytometry buffer. 10 µL of 7-AAD was added and stained at room temperature for 10 minutes. Flow cytometry was carried out using a CytoFLEX Flow cytometer (Beckman Coulter, Brea, CA), and the data was analyzed using FlowJo software (FLOWJO, LLC, Ashland, OR).

K_D determination

Binding of the bi-CD47-IT to human CD47+ T-ALL tumor cells was performed using a wide concentration range (0.1-2000 nM) of Alexa Flour 488-labeled bi-CD47-IT. K_D determination was performed based on the flow cytometry data using nonlinear regression with the saturation binding equation (GraphPad Prism 9.4.1, San Diego, CA). The median fluorescence intensity (MFI) was plotted versus the Alexa Flour 488-labeled bi-CD47-IT concentrations. Nonlinear regression was based on the equation $Y = Bmax \times X/(K_D + X)$, where Y = MFI at the given Alexa Flour 488 labeled bi-CD47-IT after subtracting the background, X = Alexa Flour 488-labeled bi-CD47-IT concentration, and Bmax = the maximum specific binding in the same units as Y. The same procedure was applied for the flow cytometry binding avidity analysis and K_D determination of human red blood cells, human lymphocytes, and human monocytes.

In vitro efficacy analysis of bi-CD47-IT

The in vitro efficacy of bi-CD47-IT was determined in human CD47+ T-ALL tumor cells using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) as described previously. This assay measures the luminescence produced by ATP production from metabolically active cells. Increasing concentrations of bi-CD47-IT cause cell death and a corresponding reduction in ATP-related fluorescence. The luminescence signals were recorded using a BioTek Synergy LX Multi-Mode Reader (Agilent, Santa Clara, CA). The pCD3-IT was

included as negative immunotoxin control. In brief, 1×10^4 tumor cells in 100 µL were added to each well of the 96-well plate. The serial diluted immunotoxins starting at 1×10^{-7} M to 1×10^{-14} M were added and mixed well to a triple-well set for each diluted immunotoxin. The reaction plate was incubated for 48 hours at 37°C with 5% CO_2. The plate was equilibrated at room temperature for 30 minutes. An equal volume of the pre-prepared lyophilized enzyme/substrate CellTiter-Glo mixture was added to each well. The plate was mixed for 2 minutes at 1000 rpm on a shaker to induce cell lysis. The plate was incubated at room temperature for 10 minutes to stabilize luminescent signal in the dark. The luminescence signal was measured using a microplate reader.

Flow cytometry analysis to monitor the in vivo depletion of the human T-ALL blast cells

The peripheral blood samples were collected via the tail vein from the tumor-bearing mice. The PBMCs were isolated from the collected blood samples using micro-processing procedure as described below. The heparinized mouse blood was diluted to 650 µL using Hanks Balanced Salt Solution containing calcium and magnesium (HBSS). The 800 µL of the Histopaque-1077 was overlayed above the diluted blood solution and centrifuged at 14,220× g for 4 minutes. The puffy coat was transferred to a new tube and the cells were spun down at 14,220× g for 1 minute. The supernatant was discarded and 500 µL of the red cell lysis buffer was added into the cell pellets to break the contaminated red blood cells. The reaction tube was mixed well and incubated at room temperature for 5 minutes. The isolated PBMC were spun down at 5,223× g for 4 minutes and resuspended with 900 µL of the FACS buffer. The cells were counted and ready for flow cytometry analysis. The murine splenocyte and bone marrow single-cell suspension was prepared by mincing spleen and bone marrow tissue with back plunger from a sterile 3 cc syringe and crushing through 70 µM cell strainer. Red blood cells were lysed by incubation with red blood cell lysis buffer (Sigma) for 2 minutes at room temperature. The cells were then washed twice with HBSS by centrifugation at 300× g for 5 minutes, suspended in flow cytometry buffer, and counted. 100 µL of 1×10^6 cells were aliquoted and stained with Fc blocker (anti-mouse CD16/32 mAb from Biolegend, Cat# 156604), LIVE/DEAD™ Fixable Near-IR, and fluorescence-labelled mAbs (PE anti-human CD47, PerCP/Cy5.5 anti-mouse CD45, APC-anti-human CD5, PE/Cyanine7-antihuman CD7, FITC-anti-human CD8, PE-anti-human HLA/ABC) at 4°C for 30 minutes. The cells were washed with 2 mL of the cold flow cytometry buffer twice again and spun down at 300× g for 5 minutes. The supernatant was discarded, and the cells were resuspended in 300 µL of the flow cytometry buffer and subjected to the flow cytometry analysis using a CytoFLEX Flow cytometer (Beckman Coulter). The flow cytometry data were analyzed using FlowJo software (FLOWJO, LLC).

Histology analysis

The murine tissues were harvested surgically from the tumor-bearing mice. The collected murine tissues were fixed in 10% formalin, embedded in paraffin, and subsequently sectioned. The tissues were stained with hematoxylin-eosin by the University of Colorado Histology Shared Resource Core. The slides were photographed using Echo Revolve Microscope (San Diego, CA).

Hemagglutination assay

Peripheral blood samples were obtained from two healthy human donors. The collected human peripheral blood samples were washed with 1× PBS twice to make a 0.8% working solution. 50 µL of the working solution was distributed to each well of a U-bottom 96-well plate. Serially diluted bi-CD47-IT, pCD3-IT (negative immunotoxin control), Magrolimab (anti-human CD47 mAb, clone Hu5F9-G4, as positive control) were prepared in 1× PBS. 100 µL were distributed in the 96-well plate containing red blood cells and gently mixed with a multichannel pipet. The plate was placed into a tissue culture incubator (5% CO_2, 37°C) and photographed after ~20-hour incubation.

Toxicity study of bi-CD47-IT in humanized mice

NSG-SGM3 mice (strain# 013062, 6–8 weeks old) were obtained from Jackson Laboratories as breeding pairs. To generate the humanized mice, baby NSG-SGM3 mice (1-2 days old) were subjected to hepatic injection with 0.5×10^5 human CD34+ stem cells from cord blood (VitalantCord Blood Services, Pittsburgh, PA) per mouse following 100 cGy irradiation. Humanized mice were characterized when ≥25% human CD45+ cells were detected in the peripheral blood by flow cytometry analysis. The humanized mice were used for bi-CD47-IT toxicity studies. Bi-CD47-IT was intraperitoneally (IP) injected at a dosage of 8.43×10^{-10} moles/kg on day 0, once daily for 10 consecutive days. The peripheral blood samples were collected weekly via tail vein to monitor the depletion of human lymphocytes (human CD45+ cells). All experiments were approved by the University of Colorado Anschutz Medical Campus Animal Care and Use Committee.

Sudan black B staining of the human T-ALL blast cells

Fresh peripheral blood samples were collected from the tumor-bearing mice and the blood smear slides were prepared. The slides were stained using Sudan Black B Staining Kit (IHC WORLD, Woodstock, MD) according to the manufacturer's instruction.

In vitro macrophage-mediated phagocytosis assay

In vitro macrophage-mediated phagocytosis was measured by flow cytometry analysis. Monocytes from healthy human donors were enriched with EasySep™ Human Monocyte

Isolation Kit (Stemcell Technologies, Vancouver, Canada), and differentiated into macrophages in the presence of 100 ng/mL M-CSF7 (GenScript, NJ, USA). Monocytes were cultured at 37°C in a humidified incubator with 5% CO_2 for 6 days. Differentiated macrophages were labeled with CellTracker™.

Deep Red Dye (ThermoScitific) according to manufacturer's instructions and plated in 96 well round bottom plate at 1.0×10^5 cells/well in 50 µL of serum free culture medium. Target cells (TALL CCRF-CEM) were labeled with CellTracker™ Green CMFDA Dye according to manufacturer's instructions and added to the macrophages-containing wells at 2.0×10^5 cells/well in 50 µL of serum free culture medium. Magrolimab, human CD47 mAb(B6H12), Bi-CD47-IT and BiscFv(B6H12) were added to a final concentration at 20 µg/mL, with or without daratumumab at 20 µg/mL. The plates were incubated for 4.5 hours at 37°C, 5% CO_2. After incubation, the cells were harvested, washed, and analyzed by CytoFlex. The phagocytosis rate was defined as the percentage of Green CMFDA+ cells within Deep Red+ macrophages.

T-ALL minimal residual disease analysis by real-time quantitative PCR

Total RNA was extracted from the peripheral blood and bone marrow of the clinically cured mice using TRIzol™ (Invitrogen, Cat# 15596026) following the manufacturer's instructions. One microgram of RNA from each sample was reverse transcribed using the QuantiTect Reverse.

Transcription Kit (QIAGEN, Cat# 205311) according to the manufacturer's instructions. Realtime quantitative polymerase chain reaction (qPCR) was performed in duplicate with 2× PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Cat# 25742) on a QuantStudio 3 Real-time system (Thermo Fisher Scientific). In brief, 1 µL of cDNA and 10 µM of forward and reverse targeting primers were added to each reaction. The expression levels of six T-ALL MRD markers (LMO1, LMO2, LYL, TAL1, TLX1, TLX3) were determined using the 2-ΔΔCT method, with GAPDH serving as the internal control. T-ALL MRD markers (LMO1, LMO2, LYL, TAL1, TLX1, TLX3) and the corresponding qPCR primers (Table 2) were reported by Muller et al., 2022.

TABLE 2

LMO1-Forward, 5' GGC AAG CGC AAT GAG GCA GC 3'	SEQ ID NO: 14
LMO1-Reverse, 5' GGC GCG TCT AGC CAC AGG TC 3'	SEQ ID NO: 15
LMO2-Forward, 5' GCC GAT GCT CTC CGT CCA GC 3'	SEQ ID NO: 16
LMO2-Reverse, 5' CGG CAG TCA CAG CAG GCA C 3'	SEQ ID NO: 17
LYL-Forward, 5' AGC AGC CGG TTG AAG CGG AG 3'	SEQ ID NO: 18

LYL-Reverse, 5' CGC TCC CGG CTG TTG GTG AA 3'	SEQ ID NO: 19
TAL1-Forward, 5' GCC GAG CTC CGC AAG CTG AT 3'	SEQ ID NO: 20
TAL1-Reverse, 5' CAG TCT TGG CCC GCT GGG TG 3'	SEQ ID NO: 21
TLX1-Forward, 5' CAC CGT GCC CTC TGT GCC TG 3'	SEQ ID NO: 22
TLX1-Reverse, 5' GGA CGT GCG CGG CTT CTT CT 3'	SEQ ID NO: 23
TLX3-Forward, 5' GCG CTT CCA CCG CCA GAA GT 3'	SEQ ID NO: 24
TLX3-Reverse, 5' ATG CGG TTC GCT TGC TGC CT 3'	SEQ ID NO: 25

Statistical analysis

The IC_{50}s were determined using Two-way ANOVA (GraphPad Prism 9.4.1, GraphPad Software, San Diego, CA). The p-values for the survival curves were calculated using the Mantel-Cox logrank test (GraphPad Prism 9.4.1). The p-values for other comparisons were calculated using the two-tailed Student t-test (GraphPad Prism 9.4.1). $p < 0.05$ was considered statistically significant.

Example 1: Expression of mono-CD47-IT and bi-CD47-IT

Codon-optimized anti-human CD47 scFv DNA was synthesized and cloned into the truncated DT390-containing expression vector pwPICZα-DT390 (**FIG. 1**) as previously described (Wang et al, 2011) Development of a diphtheria toxin based antiporcine CD3 recombinant immunotoxin. Bioconjug Chem 22, 2014-2020). Both mono-CD47-IT and bi-CD47-IT were expressed and purified using a DT-resistant *Pichia pastoris* yeast expression system. The final two-step purification yields were ~10 mg per liter of the harvested supernatant for both mono-CD47-IT and bi-CD47-IT. The purified mono-CD47-IT and bi-CD47-IT were analyzed using SDS-PAGE and western blot. The expected molecular weights of ~69 kDa and ~95 kDa were detected for mono-CD47-IT and bi-CD47-IT, respectively (**FIG. 2A–2C**). The weak lower molecular weight bands in both the SDS gel and western blot analyses are degradation products of mono-CD47-IT or bi-CD47-IT.

Example 2: Bi-CD47-IT produced using a unique diphtheria toxin resistant yeast Pichia pastoris expression system

Codon-optimized anti-human CD47 scFv DNA was synthesized and cloned into the truncated DT390-containing yeast expression vector pwPICZα-DT390 (Figure 1A) as previously described. Both mono-CD47-IT and bi-CD47-IT were expressed and purified using a unique DT-resistant *Pichia pastoris* yeast expression system. The final two-step purification yields were

~10 mg per liter of the harvested supernatant for both mono-CD47-IT and bi-CD47-IT. The purified mono-CD47-IT and bi-CD47-IT were analyzed using SDS-PAGE and western blot, and the expected molecular weights of ~69 kDa and ~95 kDa were detected for mono-CD47-IT and bi-CD47-IT, respectively (Figure 1B-D). The weak lower molecular weight bands in both the SDS gel and western blot analysis are broken-down products of mono-CD47-IT or bi-CD47-IT.

The in vitro binding avidities of mono-CD47-IT and bi-CD47-IT were evaluated in human CD47+CCR4+ T-ALL CCRF-CEM cells by flow cytometry analysis. As shown in Figure 1E, Alexa Flour 488-labeled mono-CD47-IT and bi-CD47-IT bound to CD47+CCR4+ T-ALL CCRF- CEM cells in a dose-dependent fashion. The K_D value of bi-CD47-IT was determined to be 281 nM and the K_D value of mono-CD47-IT was not calculable due to the low binding avidity (Figure 1F). The in vitro efficacy of mono-CD47-IT and bi-CD47-IT for CD47+CCR4+ T-ALL CCRF- CEM cells was assessed using the CellTiter-Glo® luminescent cell viability assay. Both mono- CD47-IT and bi-CD47-IT effectively inhibited CD47+CCR4+ T-ALL CCRF-CEM cell growth, with IC50 values of 1.4×10^{-9} M and 2.14×10^{-11} M for mono-CD47-IT and bi-CD47-IT, respectively (Figure 1G). Thus, bi-CD47-IT was ~65-fold more effective than mono-CD47-IT in vitro.

Example 3: Bi-CD47-IT is highly effective against T-ALL in a T-ALL CCRF-CEM CDX mouse model

A T-ALL CCRF-CEM CDX mouse model was first used to assess the in vivo efficacy of bi-CD47- IT. Human CD47+CCR4+ T-ALL CCRF-CEM cells were intravenously injected into NSG mice. Beginning 4 days after the tumor cell injection, the mice were treated with 8.43×10^{-10} mol/kg of bi-CD47-IT or the control immunotoxins (C21-IT, mono-CD47-IT, CCR4-IT) by daily intraperitoneally injection for 10 consecutive days. As shown in Figure 2A, compared with the negative control C21-IT, mono-CD47-IT, bi-CD47-IT, and CCR4-IT (positive control) significantly prolonged the median survival (24 days for the negative control C21-IT group versus 28, 30, and 43 days for the mono-CD47-IT, CCR4-IT, and bi-CD47-IT groups, respectively). CCR4-IT was included as a positive immunotoxin control because we have previously demonstrated that CCR4-IT was significantly effective in the same T-ALL CCRF-CEM CDX mouse model. Strikingly, although with low in vitro binding avidity (K_D=281 nM, Figure 1F) to CD47+CCR4+ T-ALL CCRF-CEM cells, bi-CD47-IT was markedly more effective in prolonging the survival of the tumor-bearing mice than the positive control CCR4-IT, which has a very high in vitro binding avidity to CD47+CCR4+ T-ALL CCRF-CEM cells (K_D=0.74 nM) (median survival, 43 days versus 30 days).

On day 23 after the tumor cell injection, Applicants euthanized two tumor-bearing mice from each experimental group and collected the peripheral blood, spleen, and liver. As shown in Figure 2B, flow cytometry analysis demonstrated that most of the CD47+CCR4+ T-ALL CCRF-CEM cells (95–98%) were effectively depleted in the peripheral blood of the tumor-bearing mice treated with bi-CD47-IT. In contrast, only 8–30% of the CD47+CCR4+ T-ALL CCRF-CEM cells were depleted in the peripheral blood of the tumor-bearing mice treated with the positive control CCR4-IT. Only 0–15% of the CD47+CCR4+ T-ALL CCRF-CEM cells were depleted in the peripheral blood of the tumor-bearing mice treated with mono-CD47-IT. As shown in Figure 2C, gross necropsy examination demonstrated that the harvested spleens were enlarged to different degrees in the tumor-bearing mice treated with C21-IT (negative control), mono-CD47-IT, and CCR4-IT (positive control). In contrast, the spleen size was normal in the tumor-bearing mice treated with bi-CD47-IT, indicating that bi-CD47-IT effectively depleted the CD47+CCR4+ T-ALL CCRF- CEM cells in vivo. Immunohistochemistry analysis demonstrated that bi-CD47-IT very effectively depleted the CD47+CCR4+ T-ALL CCRF-CEM cells in the spleen. In contrast, CD47+ tumor cells were heavily observed in the spleen of the tumor-bearing mice treated with C21-IT, mono-CD47- IT, and the positive control CCR4-IT (Figure 2D). Liver pathology analysis found no tumor cell infiltration in the tumor-bearing mice treated with bi-CD47-IT, but tumor cell infiltration was observed in the tumor-bearing mice treated with C21-IT, mono-CD47-IT, and the positive control CCR4-IT (Figure 2E). Taken together, all the above in vivo data consistently demonstrated that bi-CD47-IT highly effectively depleted the CD47+CCR4+ T-ALL CCRF-CEM cells in vivo.

To study the in vivo efficacy of bi-CD47-IT further, Applicants performed a serial study in the same T- ALL CCRF-CEM CDX mouse model. Applicants euthanized two tumor-bearing mice from each treatment group on day 4, 14, and 21 post the tumor cell injection to monitor the human T-ALL blast cells in the peripheral blood, spleen, liver, bone marrow, brain, and spinal cord. The data demonstrated that the 10-day treatment of bi-CD47-IT significantly prolonged the median survival of tumor-bearing mice from 24 days (C21-IT control group) to 43 days (Figure 3A), and very effectively depleted the T-ALL blast cells in the peripheral blood (Figure 3B and Figures 8A-B), spleen (Figure 3C-D and Figure 8C-D), bone marrow (Figure 3E and Figure 8E-G), brain and spinal cord (Figure 3F and Figure 8H-I), and liver (Figure 3G). The deep depletion lasted for ~3 weeks (Figure 3B-G), indicating that the 10- day treatment within 3 weeks as one cycle might be an appropriate possible dosing schedule as reported for other FDA-approved diphtheria toxin-based immunotoxin drugs including Ontak® and Elzonris®. On day 28, the T-ALL blast cells started to rebound back, which indicates that the second round of treatment or another treatment is needed before day 28.

Example 4: Bi-CD47-IT was still highly effective against T-ALL in an overt T-ALL CCRF-CEM CDX mouse model

To try to imitate the conditions of most T-ALL clinical cases, Applicants further assessed the in vivo efficacy of bi-CD47-IT in an overt human T-ALL CCRF-CEM CDX mouse model. CD47+ T-ALL CCRF-CEM tumor cells were intravenously injected into the NSG mice on day 0. The immunotoxin treatment started on day 7, when 6% T-ALL blast cells were detected in the peripheral blood (Figure 9A). The tumor-bearing mice were treated with 8.43×10^{-10} mol/kg of bi-CD47-IT or C21-IT (negative immunotoxin control) by daily intraperitoneally injection for 10 consecutive days. On day 21, two tumor-bearing mice from each group were euthanized and T-ALL blast cells were monitored in the collected blood, spleen, liver, bone marrow, brain and spinal cord. Compared with the negative control C21-IT, bi-CD47-IT still significantly prolonged the median survival of the tumor-bearing mice from 23 days to 35 days (Figure 4A) in this overt CDX mouse model and depleted the T-ALL blast cells very effectively in the collected peripheral blood (Figure 4B, Figure 9B), spleen (Figure 4C, Figure 9C-D), bone marrow (Figure 4D, Figure 9E), brain and spinal cord (Figure 4E, Figure 9F-G), and liver (Figure 4F).

Example 5: Bi-CD47-IT was even more effective against T-ALL in an overt T-ALL Molt-4 CDX mouse model

Applicants assessed the in vivo efficacy of bi-CD47-IT in the second human T-ALL cell line Molt- 4-based overt T-ALL CDX mouse model. Human T-ALL Molt-4 cells were intravenously injected into the NSG mice on day 0. The immunotoxin treatment started on day 4, when 1.3-2.2 % T-ALL blast cells were detected in the peripheral blood (Figure 10A). The tumor-bearing mice were treated with 8.43×10^{-10} mol/kg of bi-CD47-IT or C21-IT by daily intraperitoneally injection for 10 consecutive days. On day 14 and 23, two tumor-bearing mice from each group were euthanized and human T-ALL blast cells were monitored in the collected blood, spleen, liver, bone marrow, brain and spinal cord. As shown in Figure 5A, surprisingly, 60% (3 of 5) of the tumor-bearing mice treated with bi-CD47-IT were cured – i.e. the mice survived very well in the end of the study. Bi-CD47-IT very effectively depleted T-ALL blast cells in the peripheral blood (Figure 5B, Figure 10B-C), spleen (Figure 5C, Figure 10D), bone marrow (Figure 5D, Figure 10E), and liver (Figure 5E). However, no blast cells were detected by flow cytometry analysis in the brain and spinal cord from both negative control C21-IT treatment group and bi-CD47-IT treatment group (Figure 10F-G), which might be associated with the 60% cure in this overt T-ALL Molt-4 CDX mouse model. On day 131 post the tumor cell injection, blast cells were still not detected by flow cytometry analysis in the peripheral blood, spleen and bone marrow collected from the cured mice (Figure 10H). To further confirm the cured mice, T-

ALL minimal residue disease (MRD) analysis was performed using real-time quantitative PCR to the blood and bone marrow of the cured mice on day 131. The human T-ALL MRD analyses were negative except for LMO2 in the bone marrow (Figure 10I). The T-ALL MRD markers include LMO1, LMO2, LYL, TAL1, TLX1, TLX3 (See Table 2). It was reported that LMO2 expression was associated with longer overall survival and LMO2 is a promising new and good prognostic marker. In addition, we also performed a pilot study in the same T-ALL Molt-4 CDX mouse model. 2 of 6 tumor-bearing mice were also cured by the same 10-day treatment (data not shown). Therefore, the partial cure efficacy result is reliable in this mouse model.

Example 6: Bi-CD47-IT was highly effective against T-ALL in two overt T-ALL PDX mouse models

Applicants assessed the in vivo efficacy of bi-CD47-IT in two overt T-ALL PDX mouse models. Flow cytometry analysis confirmed that the two T-ALL PDX samples were CD47+ (data not shown). In the first T-ALL PDX mouse model, T-ALL PDX sample #1 tumor cells were amplified using NSG mice, harvested, purified, and intravenously injected into the NSG mice on day 0. The immunotoxin treatment started on day 7, when ~30% human T-ALL blast cells were detected in the peripheral blood (Figure 11A). The mice were treated with 8.43×10^{-10} mol/kg of bi-CD47-IT or C21-IT control by daily intraperitoneally injection for 10 consecutive days. On day 28, two tumor-bearing mice from each group were euthanized and the T-ALL blast cells were monitored in the collected blood, spleen, liver, bone marrow, brain, and spinal cord. Compared with the negative control C21-IT, bi-CD47-IT still significantly prolonged the median survival of the tumor-bearing mice from 33 days to 54 days (Figure 6A) in this late overt T-ALL PDX mouse model and still effectively depleted the T-ALL blast cells in the peripheral blood (Figure 6B-C, Figure 11A-C), spleen (Figure 6D, Figure 11D-E), bone marrow (Figure 6E, Figure 11F), brain, spinal cord (Figure 6F, Figure 11G-H), and liver (Figure 6G).

In the second overt T-ALL PDX mouse model, T-ALL PDX sample #2 tumor cells were amplified using NSG mice, harvested, purified, and intravenously injected into the NSG mice on day 0. The immunotoxin treatment started on day 7, when 5% T-ALL blast cells were detected in the peripheral blood (Figure 11I). On day 33, two tumor-bearing mice from each group were euthanized and the T-ALL blast cells were monitored in the collected blood, spleen, liver, bone marrow, brain, and spinal cord. Compared with the negative control C21-IT, bi-CD47-IT also significantly prolonged the median survival of the tumor-bearing mice from 53 days to 85 days (Figure 6H) and effectively depleted the T-ALL blast cells in the peripheral blood (Figure 6I- J, Figure 11I-K), spleen (Figure 6K, Figure 11L-M), bone marrow (Figure 6L, Figure 11N), brain, spinal cord (Figure 6M, Figure 11O-P), and liver (Figure 6N).

Example 7: Targeted therapy is the mechanism of bi-CD47-IT

Bi-CD47-IT has potential as a checkpoint inhibitor to block the CD47-SIRPα pathway to facilitate the phagocytosis of the macrophage and dendritic cells to the tumor cells for immunotherapy. To study this potential, Applicants constructed and expressed the bivalent anti-human CD47 scFv, biscFv(B6H12), without DT390, using yeast Pichia pastoris expression system. In vitro macrophage-based phagocytosis analysis demonstrated that biscFv(B6H12) (binding domain only of bi-CD47-IT) and bi-CD47-IT did not induce phagocytosis. In contrast, Magrolimab (a humanized anti-human CD47 IgG4 mAb, clone Hu5F9-G4) and anti-human CD47 mAb (clone B6H12) as two positive controls induced significant macrophage-mediated phagocytosis (Figure 12). BiscFv(B6H12) control was added to the in vivo efficacy study of bi-CD47-IT in the second overt T-ALL PDX mouse model. The results demonstrated that biscFv(B6H12) did not prolong the median survival of the tumor-bearing mice (Figure 6H) and did not induce the depletion of the T-ALL blast cells in the peripheral blood (Figure 6I-J, Figure 11I-K), spleen (Figure 6K, Figure 11L-M), bone marrow (Figure 6L, Figure 11N), brain, spinal cord (Figure 6M, Figure 11O-P), and liver (Figure 6N). The data indicates that the in vivo mechanism of bi-CD47-IT is via targeted therapy, not via immunotherapy.

Example 8: Bi-CD47-IT showed no toxicity to normal human tissues

CD47 is also expressed on normal tissues with low binding avidities, including human red blood cells and lymphocytes. Specificity/on-target toxicity is a major concern in the development of CD47-based therapies. Therefore, we first analyzed the in vitro binding avidity and hemagglutination of bi-CD47-IT in human red blood cells. As shown in Figure 7A, with up to 1 μM of bi-CD47-IT, no binding was observed on human red blood cells from two different healthy donors. K_D values were not determinable. In contrast, Magrolimab bound to human red blood cells very strongly with K_D value of 9.96 nM (Figure 7B, Figure 13A). Applicants also demonstrated that bi-CD47-IT bound to T-ALL CCRF-CEM cells significantly stronger than to human monocytes, lymphocytes, and red blood cells (Figure 7C, Figure 13B). The data demonstrated that the engineered bi-CD47-IT possessed the "optimal" binding avidity with no binding up to 1 μM to human red blood cells and with weak binding to human monocytes and lymphocytes. As shown in Figure 7D, with up to 1500 μg/mL of bi-CD47-IT, no hemagglutination was observed in human red blood sample, unlike the positive control Magrolimab which induced strong hemagglutination activity (≥1.2 μg/mL). Anti-human CD47 mAb (clone B6H12, the parent mAb of anti-human CD47 scFv used for construction of the bi-CD47-IT) also induced strong hemagglutination activity, similar to Magrolimab (data not shown). Applicants performed a toxicity study of bi-CD47-IT in humanized mice. In these

studies, Bi-CD47-IT treatment started on day 0 at 8.43×10^{-10} mol/kg by daily intraperitoneally injection for 10 consecutive days. The data demonstrated that bi-CD47-IT transiently depleted human lymphocytes for ~4 weeks (Figure 7E, Figure 13C). The human lymphocytes rebounded back to normal range in ~4 weeks. No clinical adverse events were observed.

All references disclosed herein, whether patent or non-patent, are hereby incorporated by reference as if each was included at its citation, in its entirety. In case of conflict between reference and specification, the present specification, including definitions, will control.

Although various embodiments have been described above with a certain degree of particularity, or with reference to one or more individual embodiments, those skilled in the art could make numerous alterations to the disclosed embodiments without departing from the spirit or scope of this disclosure. Other embodiments are therefore contemplated. All matter contained in the above description and the accompanying drawings is illustrative only of particular embodiments and not limiting. Changes in detail, structure, or order of operation of steps of a method may be made without departing from the basic elements described herein.

CLAIMS

1. An anti-human CD47 immunotoxin comprising:

a toxin portion comprising a diphtheria toxin or a fragment thereof; and

a targeting portion comprising at least a first anti-human CD47 antibody or fragment thereof,

wherein the toxin portion is covalently linked to the targeting portion.

2. The anti-human CD47 immunotoxin of claim 1, wherein the diphtheria toxin or fragment thereof includes about 390 amino acids from the diphtheria toxin, having an amino acid sequence at least 80% identical to SEQ ID NO: 2.

3. The anti-human CD47 immunotoxin of claim 1 or claim 2, wherein the toxin portion is located at or near an N terminus of the anti-human CD47 immunotoxin.

4. The anti-human CD47 immunotoxin of any one of claims 1-3, wherein the targeting portion further comprises a second anti-human CD47 antibody or fragment thereof.

5. The anti-human CD47 immunotoxin of any one of claims 1-4, wherein the anti-human CD47 antibody or fragment thereof comprises an antigen-binding portion of the anti-human CD47 antibody or fragment thereof.

6. The anti-human CD47 immunotoxin of claim 5, wherein the antigen-binding portion comprises VH and VL regions from an anti-human CD47 antibody.

7. The anti-human CD47 immunotoxin of any one of claims 1-6, wherein the targeting portion is located at or near a C terminus of the toxin portion.

8. The anti-human CD47 immunotoxin of any one of claims 1-7, wherein the toxin portion is linked to the targeting portion by at least one linker, and the linker comprises four glycine residues and one serine residue.

9. A nucleic acid molecule encoding the anti-human CD47 immunotoxin of any one of claims 1-8.

10. The nucleic acid molecule of claim 9, wherein the nucleic acid molecule is codon-optimized for expression in a methylotropic yeast.

11. A vector comprising the nucleic acid molecule of any one of claims 8-10.

12. A cell comprising the nucleic acid molecule of any one of claims 8-10 or the vector of claim 11.

13. The cell of claim 12, wherein the cell is derived from *Pichia pastoris*.

14. A pharmaceutical composition comprising the anti-human CD47 immunotoxin of any one of claims 1-8 and a pharmaceutically acceptable carrier.

15. A method of treating a subject having a $CD47^-$ cancer, the method comprising:

administering a therapeutically effective amount of an anti-human CD47 immunotoxin, the immunotoxin comprising:

a toxin portion comprising a diphtheria toxin or a fragment thereof; and

a targeting portion comprising at least a first anti-human CD47 antibody or fragment thereof,

wherein the toxin portion is linked to the targeting portion.

16. The method of claim 15, wherein the subject is human.

17. The method of claim 15 or 16, wherein the CD47+ cancer is selected from T-cell acute lymphoblastic lymphoma (T-ALL), T-cell lymphoma (CTCL) (including human CD47+CD25+CCR4+CD30+ CTCL), peripheral T-cell lymphoma (PTCL), lung cancer (including human CD47+ lung cancer), triple-negative breast cancer, head and neck cancer, melanoma, and bladder cancer

18. The method of any one of claims 15-17, wherein the method reduces the concentration of CD47+ cancer cells, relative to the concentration prior to initiating the method, in one or more of the peripheral blood, the liver, spleen, bone marrow, brain, and spinal cord.

19. A method of reducing a population of $CD47^+$ cells, the method comprising:

adding an anti-human CD47 immunotoxin to the population, the immunotoxin comprising:

a toxin portion comprising a diphtheria toxin or a fragment thereof; and

a targeting portion comprising at least a first anti-human CD47 antibody or fragment thereof,

wherein the toxin portion is linked to the targeting portion, and

wherein addition of the anti-human CD47 immunotoxin reduces the number of $CD47^+$ cells compared to no addition of the anti-human CD47 immunotoxin.

20. The method of claim 19, wherein the population of CD47+ cells is found in one or more of peripheral blood, liver, spleen, bone marrow, brain, and spinal cord.

FIG. 1A

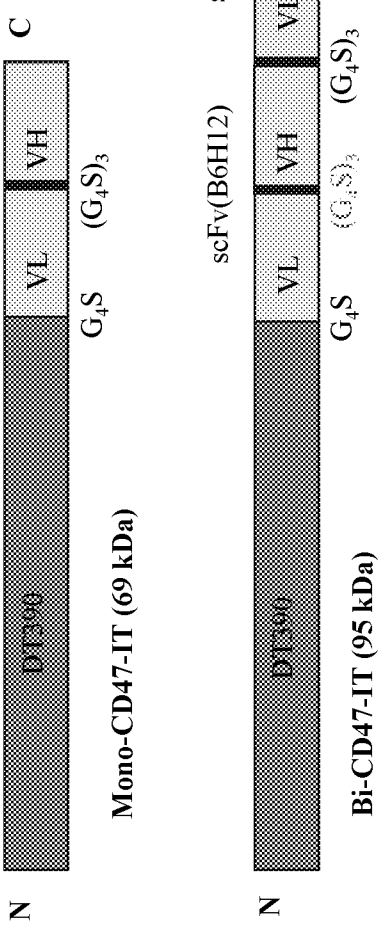


FIG. 1B

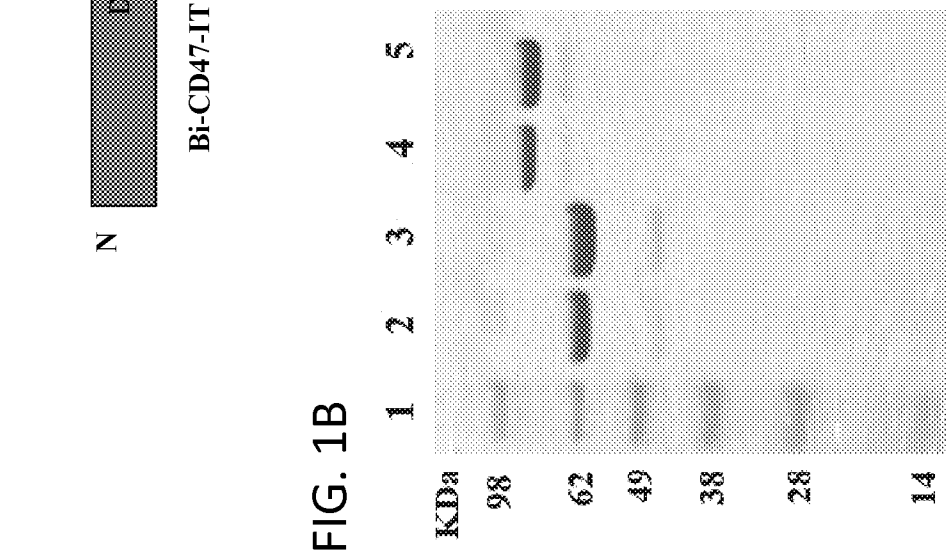


FIG. 1C

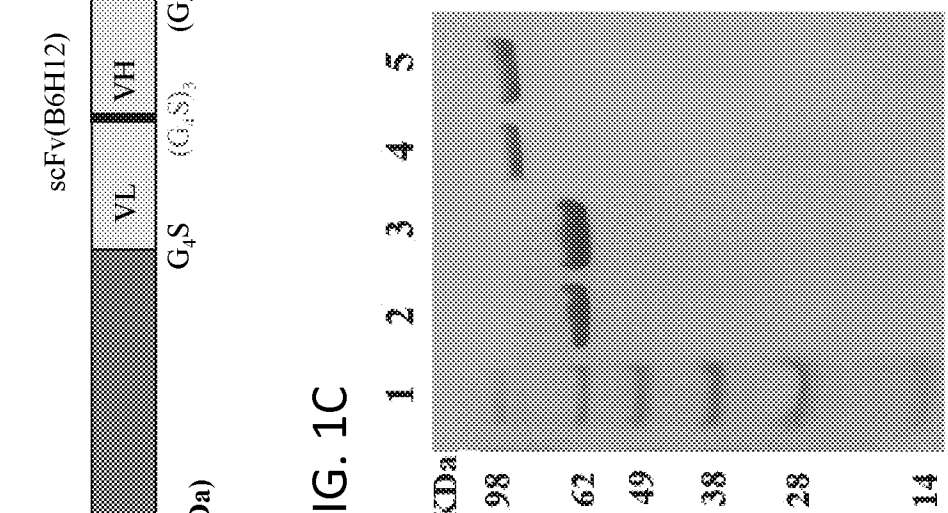
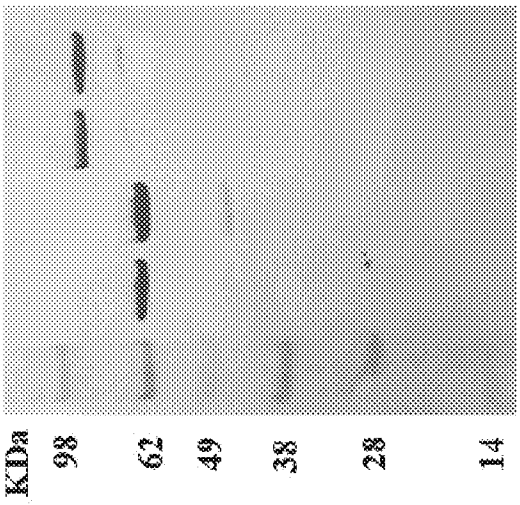


FIG. 1D



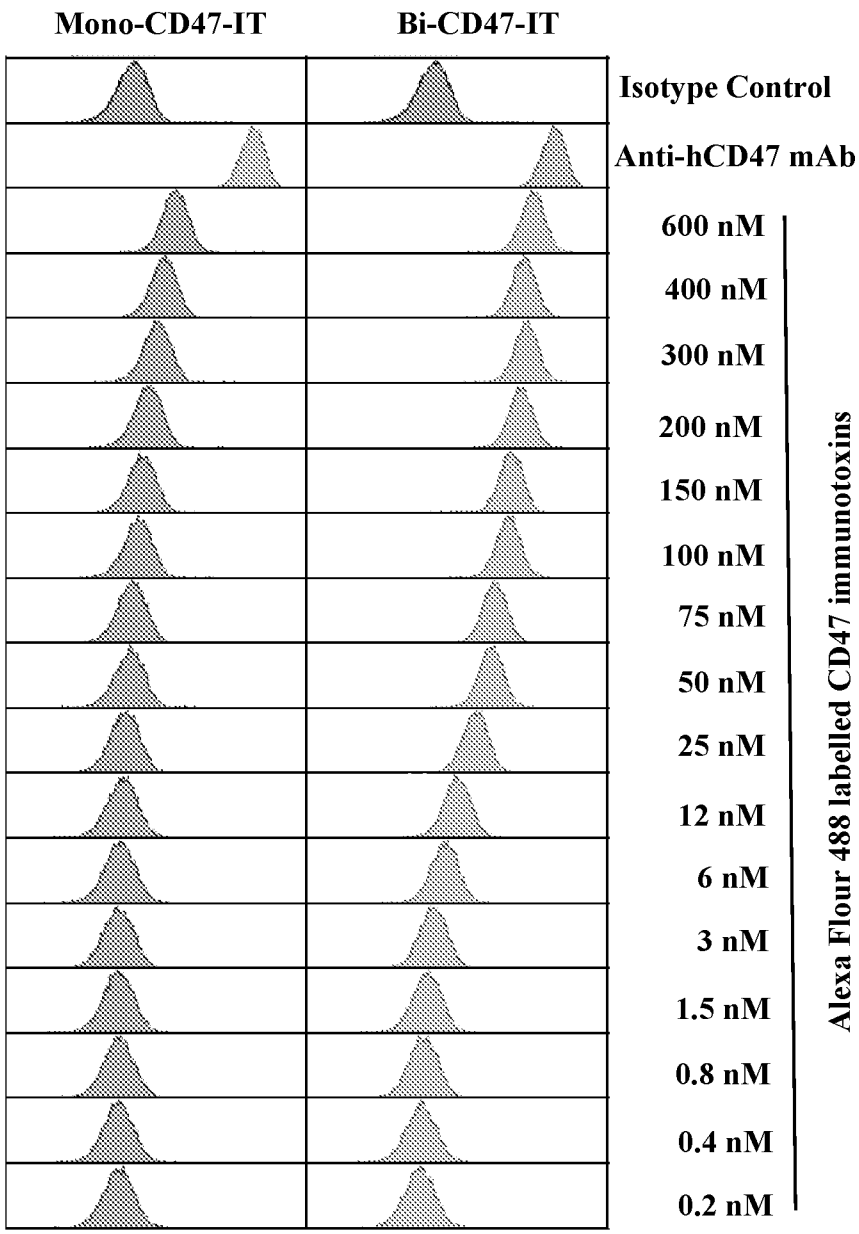

Mono-CD47-IT
Bi-CD47-IT
Isotype Control
Anti-hCD47 mAb
600 nM
400 nM
300 nM
200 nM
150 nM
100 nM
75 nM
50 nM
25 nM
12 nM
6 nM
3 nM
1.5 nM
0.8 nM
0.4 nM
0.2 nM
Alexa Flour 488 labelled CD47 immunotoxins

FIG. 1E

FIG. 1F

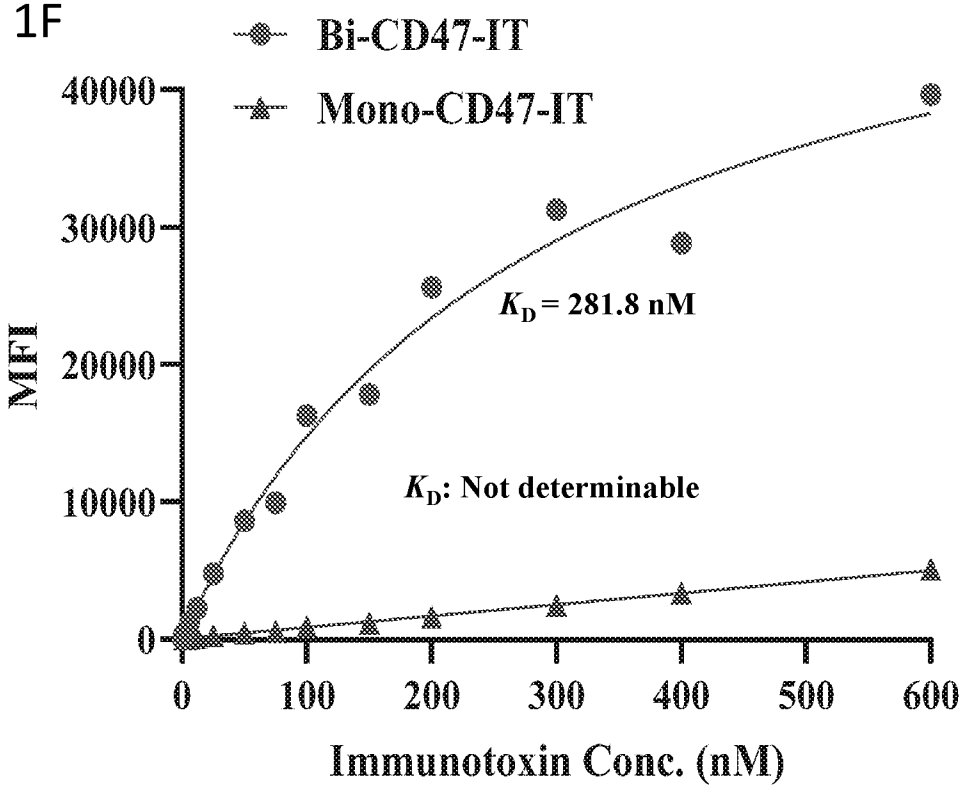

Bi-CD47-IT
Mono-CD47-IT
40000
30000
20000
10000
0
MFI
K_D = 281.8 nM
K_D: Not determinable
0
100
200
300
400
500
600
Immunotoxin Conc. (nM)

FIG. 1G

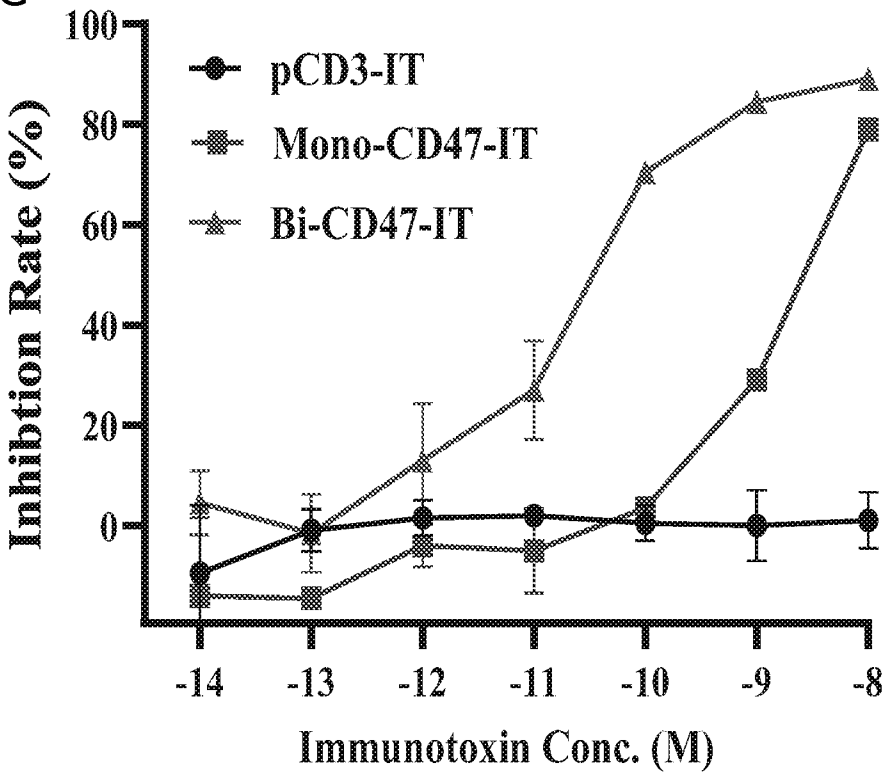

100
80
60
40
20
0
Inhibtion Rate (%)
pCD3-IT
Mono-CD47-IT
Bi-CD47-IT
-14
-13
-12
-11
-10
-9
-8
Immunotoxin Conc. (M)

FIG. 2A

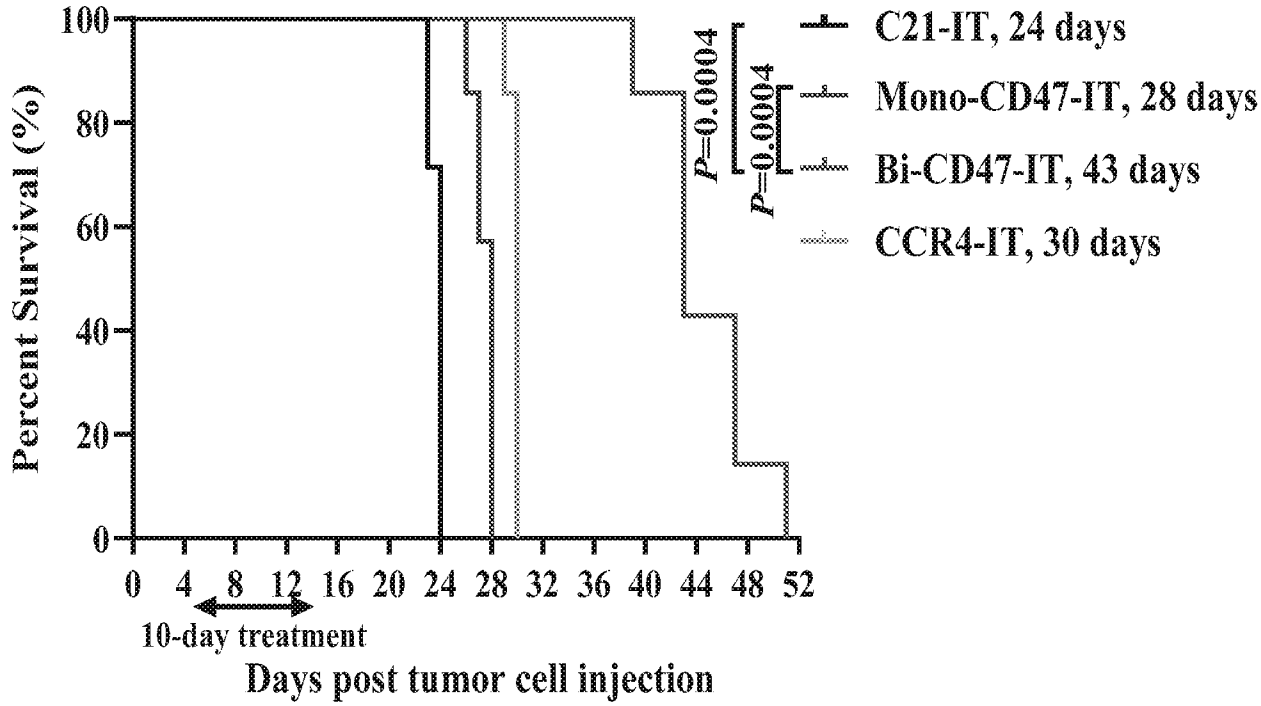
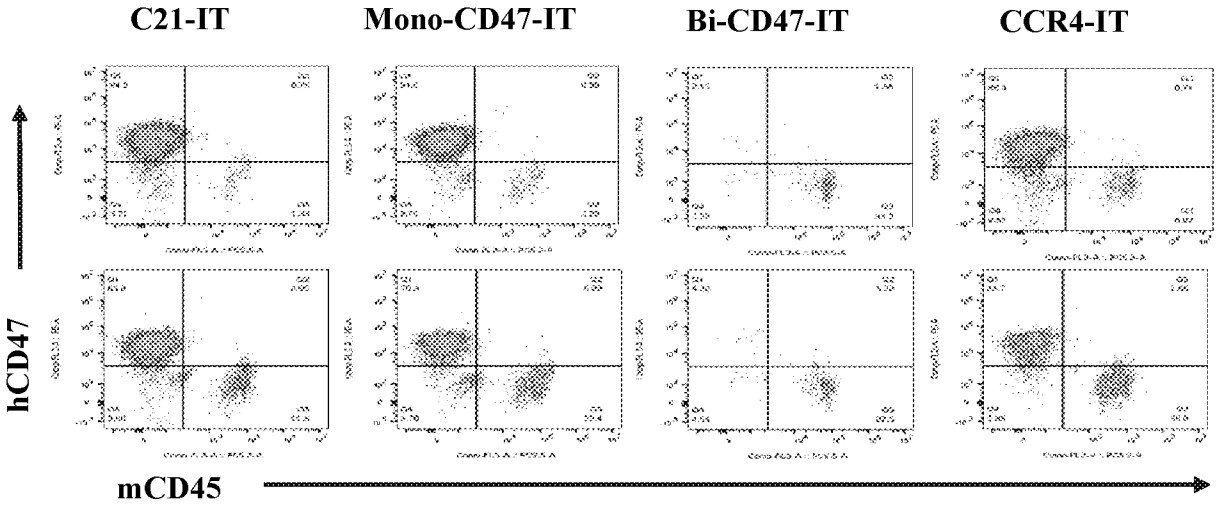

Percent Survival (%)
100
80
60
40
20
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52
10-day treatment
Days post tumor cell injection
P=0.0004
P=0.0004
C21-IT, 24 days
Mono-CD47-IT, 28 days
Bi-CD47-IT, 43 days
CCR4-IT, 30 days

FIG. 2B


C21-IT
Mono-CD47-IT
Bi-CD47-IT
CCR4-IT
hCD47
mCD45

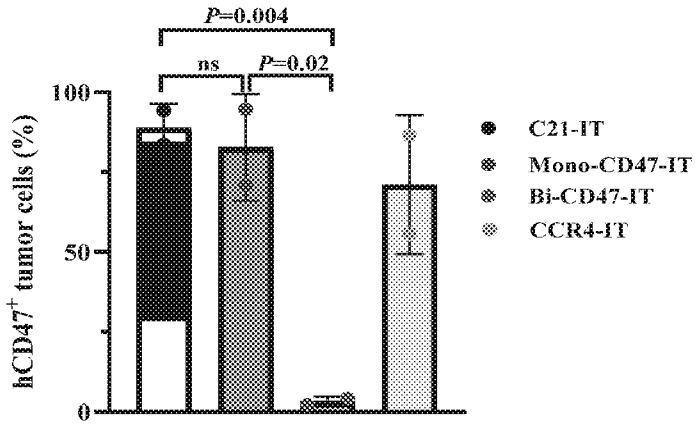
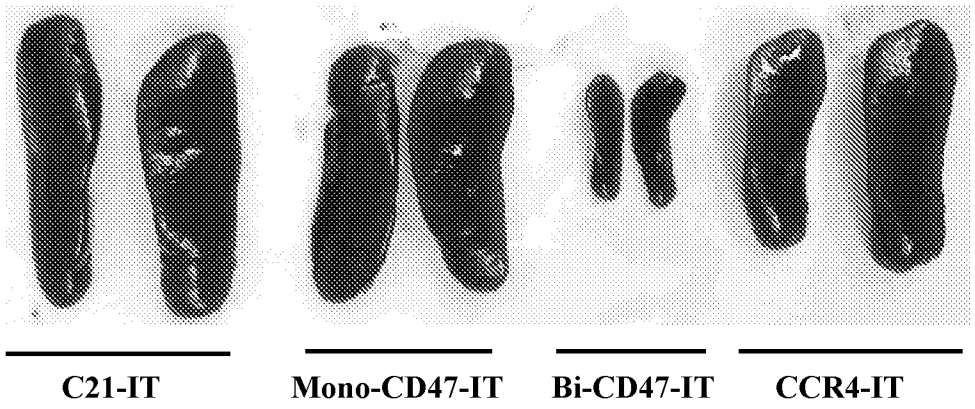

P=0.004
ns
P=0.02
hCD47+ tumor cells (%)
100
50
0
C21-IT
Mono-CD47-IT
Bi-CD47-IT
CCR4-IT

FIG. 2C


C21-IT
Mono-CD47-IT
Bi-CD47-IT
CCR4-IT

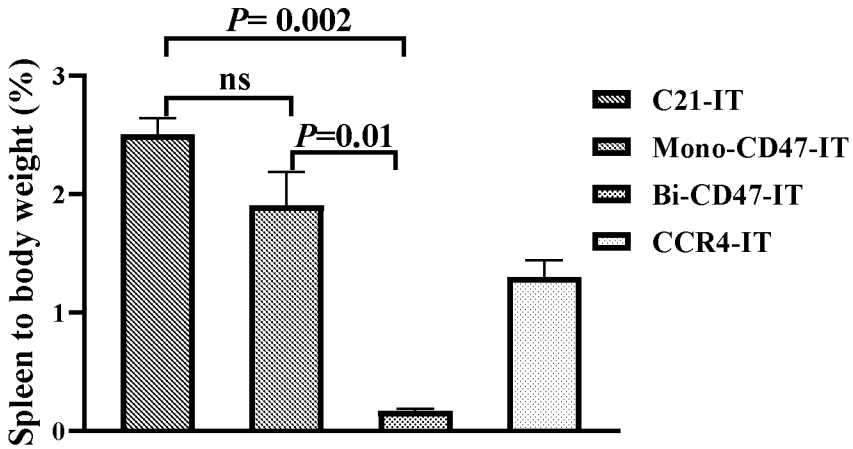
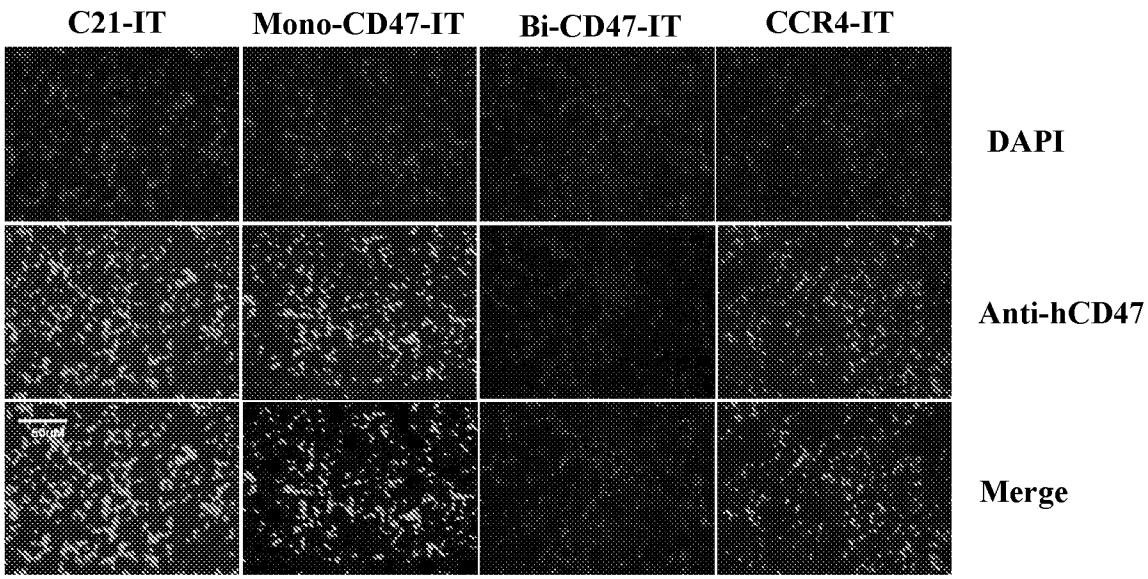

P= 0.002
ns
P=0.01
Spleen to body weight (%)
3
2
1
0
C21-IT
Mono-CD47-IT
Bi-CD47-IT
CCR4-IT

FIG. 2D


C21-IT
Mono-CD47-IT
Bi-CD47-IT
CCR4-IT
DAPI
Anti-hCD47
Merge

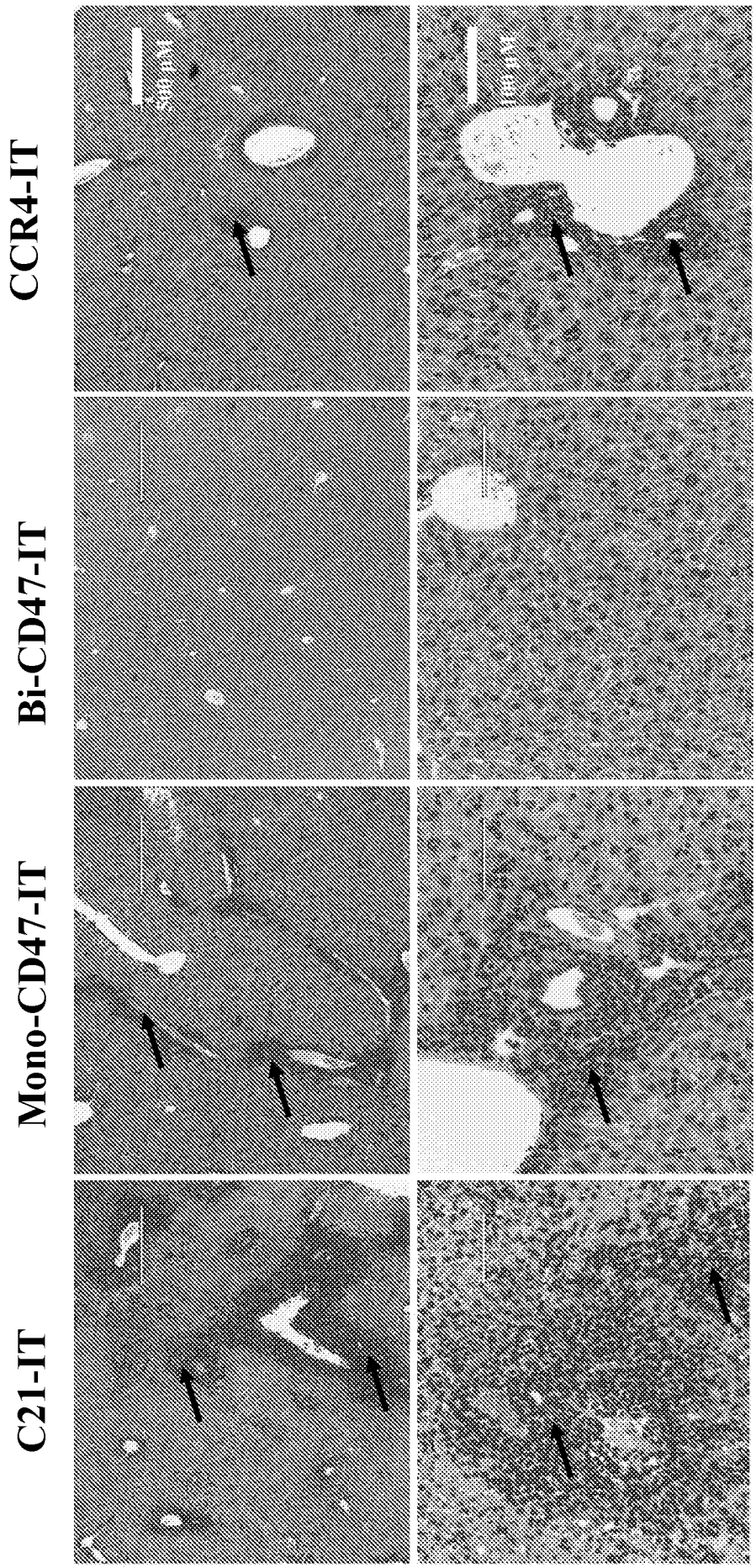


FIG. 2E

FIG. 3A

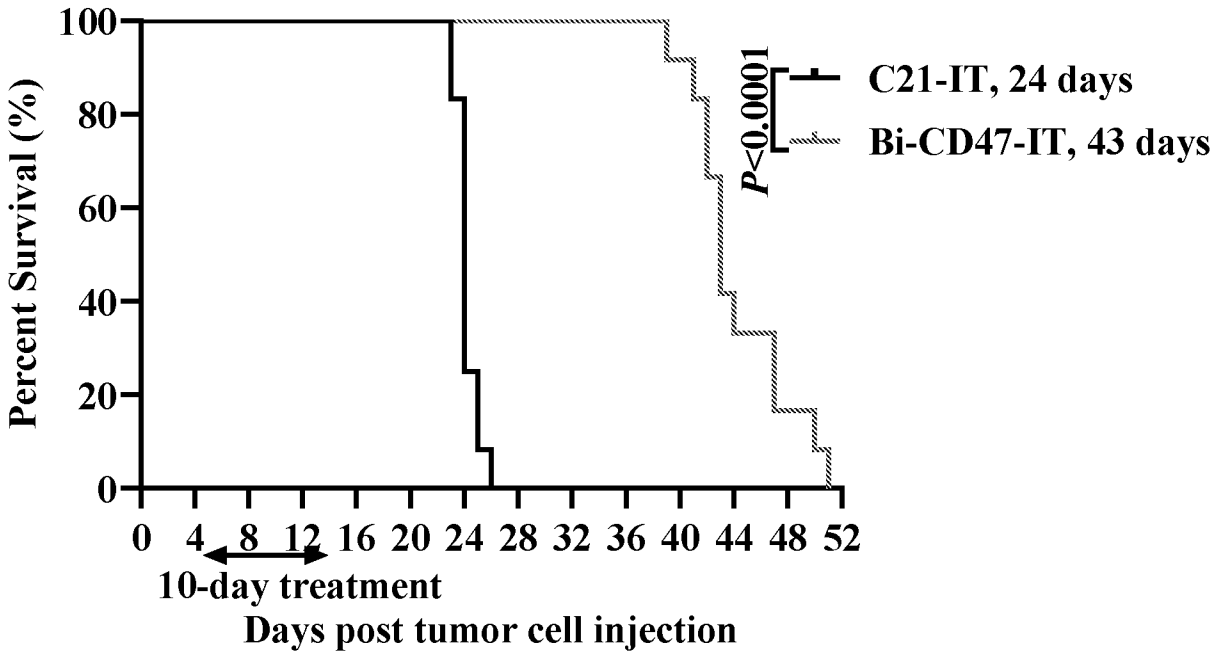

Percent Survival (%)
100
80
60
40
20
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52
10-day treatment
Days post tumor cell injection
P<0.0001
C21-IT, 24 days
Bi-CD47-IT, 43 days

FIG. 3B

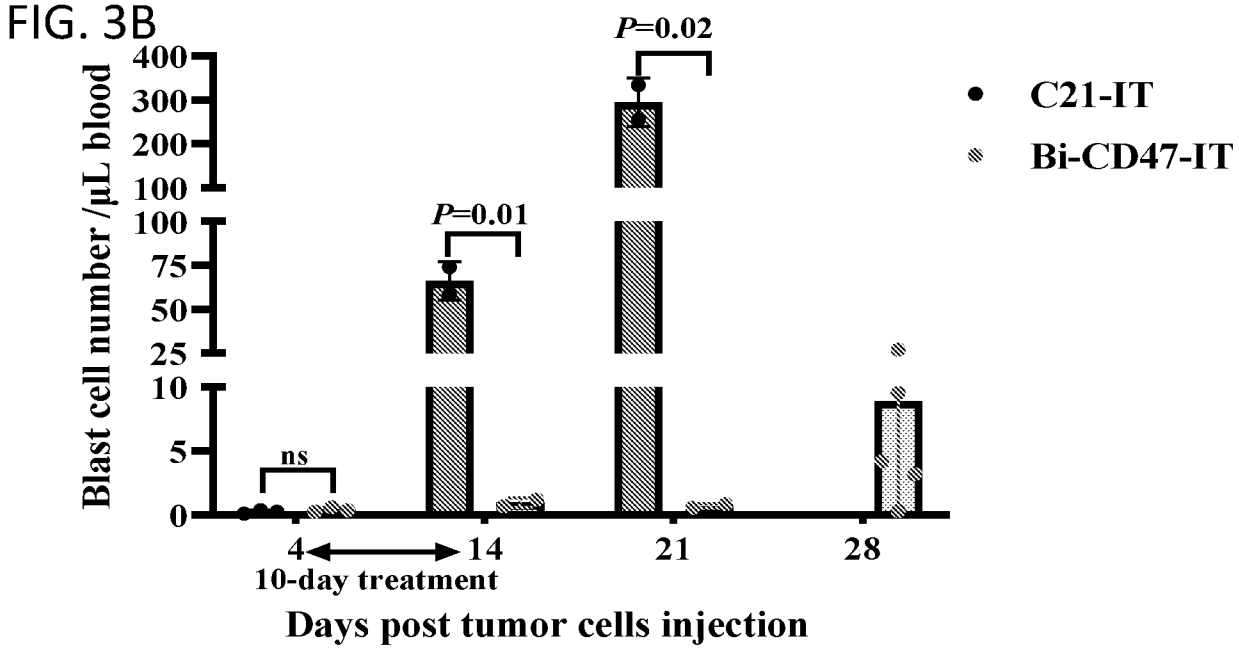
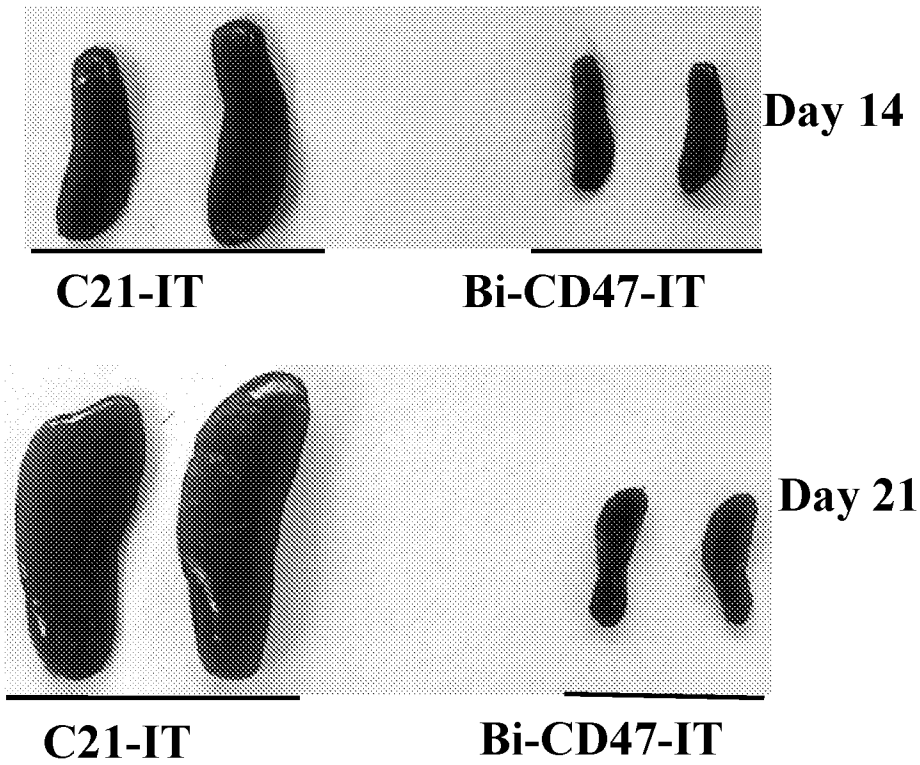

Blast cell number /μL blood
400
300
200
100
100
75
50
25
10
5
0
ns
P=0.01
P=0.02
4
14
21
28
10-day treatment
Days post tumor cells injection
C21-IT
Bi-CD47-IT

FIG. 3C


Day 14
C21-IT
Bi-CD47-IT
Day 21
C21-IT
Bi-CD47-IT

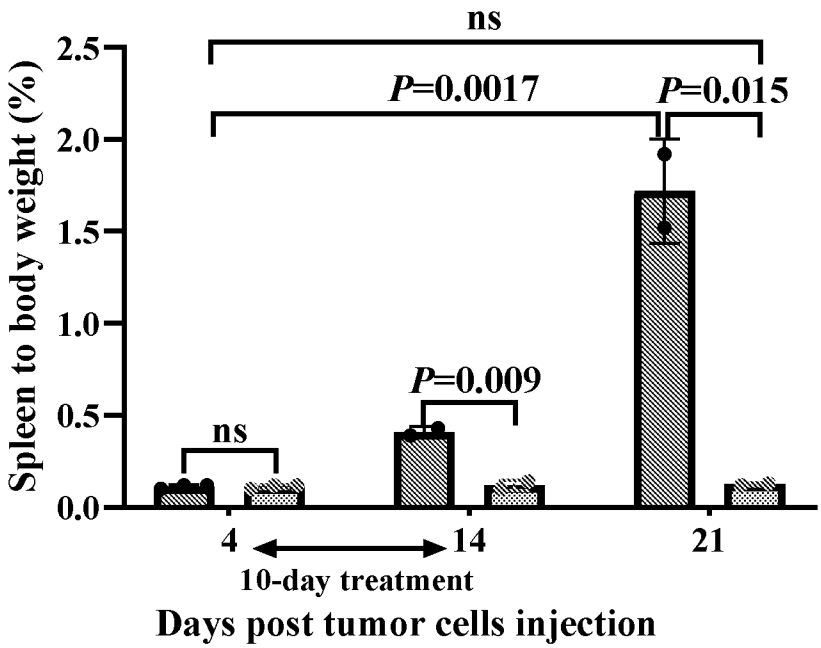

ns
P=0.0017
P=0.015
P=0.009
ns
Spleen to body weight (%)
2.5
2.0
1.5
1.0
0.5
0.0
4
14
21
10-day treatment
Days post tumor cells injection

FIG. 3D

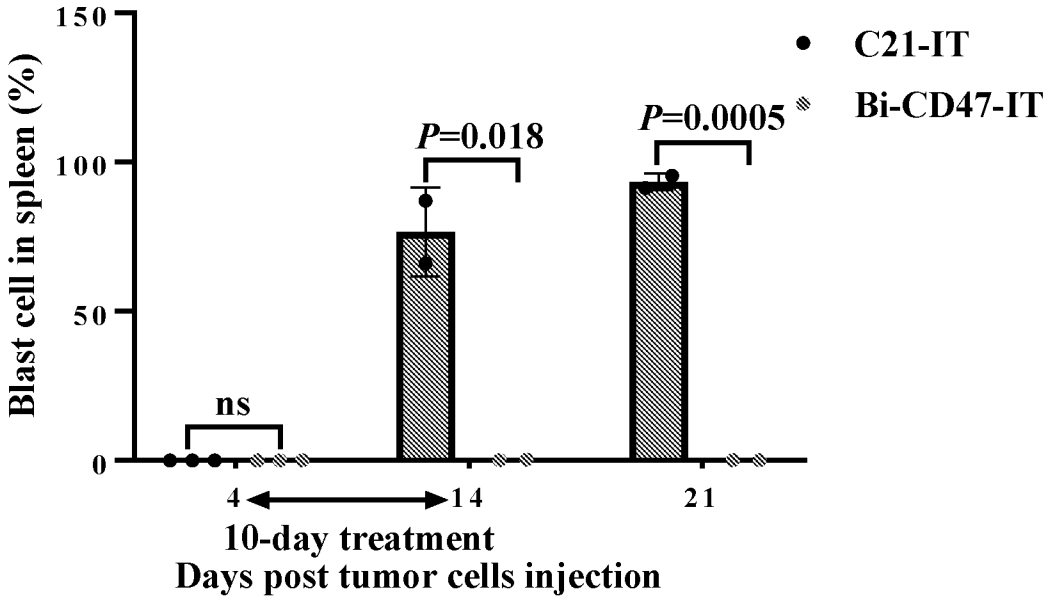

150
100
50
0
Blast cell in spleen (%)
ns
P=0.018
P=0.0005
C21-IT
Bi-CD47-IT
4
14
21
10-day treatment
Days post tumor cells injection

FIG. 3E

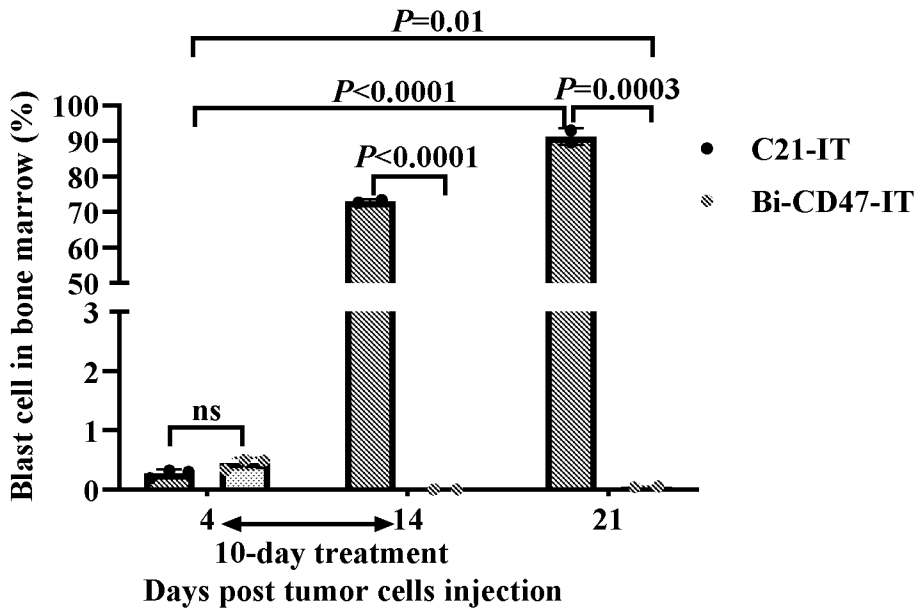

P=0.01
P<0.0001
P=0.0003
P<0.0001
100
90
80
70
60
50
3
2
1
0
Blast cell in bone marrow (%)
ns
C21-IT
Bi-CD47-IT
4
14
21
10-day treatment
Days post tumor cells injection

FIG. 3F

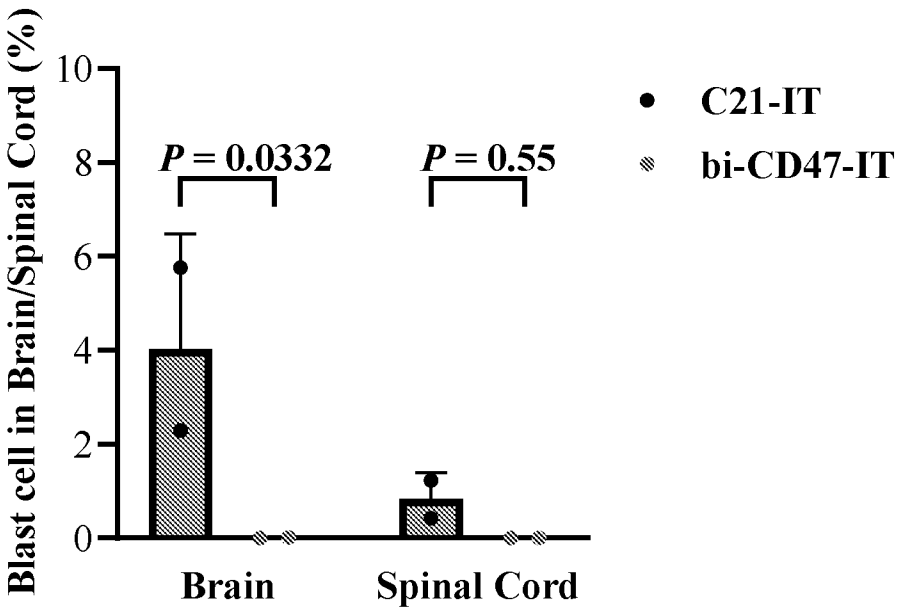

Blast cell in Brain/Spinal Cord (%)
10
8
6
4
2
0
P = 0.0332
P = 0.55
C21-IT
bi-CD47-IT
Brain
Spinal Cord

FIG. 3G

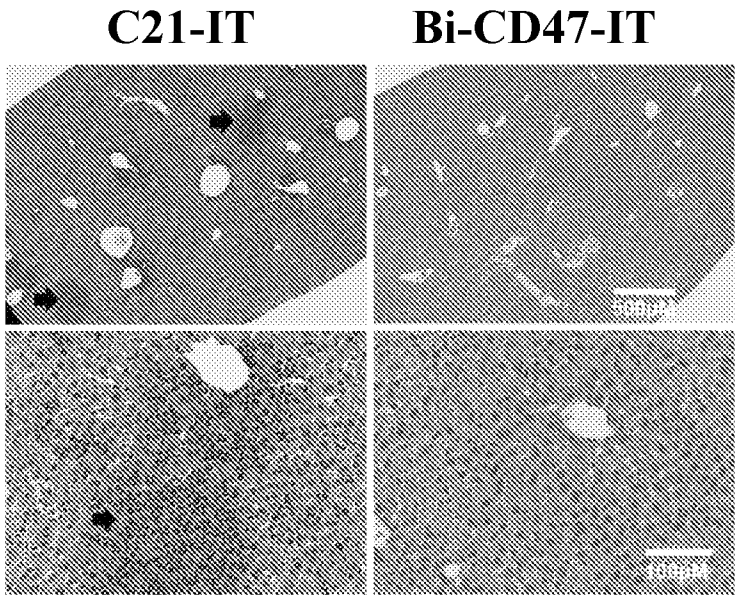

C21-IT
Bi-CD47-IT

FIG. 4A

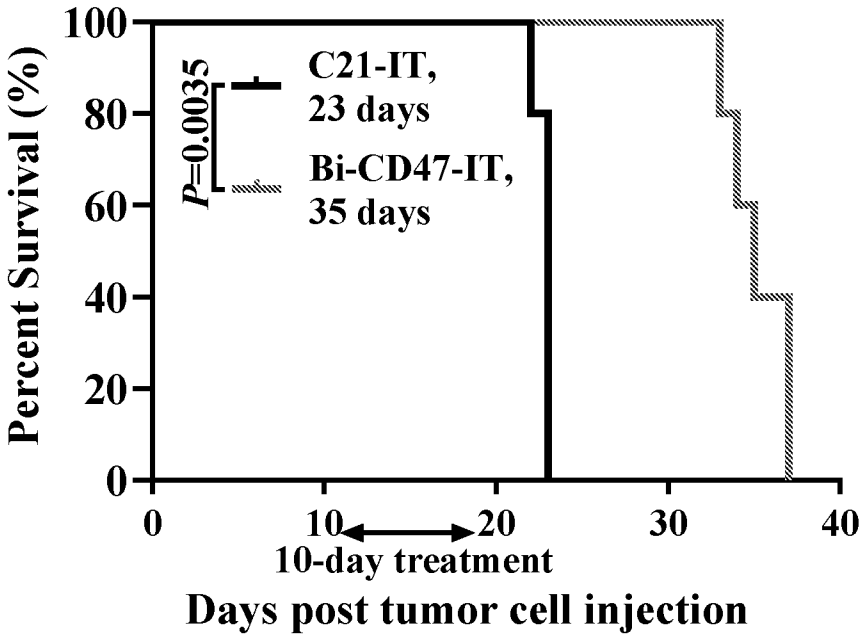

Percent Survival (%)
100
80
60
40
20
0
P=0.0035
C21-IT,
23 days
Bi-CD47-IT,
35 days
0
10
20
30
40
10-day treatment
Days post tumor cell injection

FIG. 4B

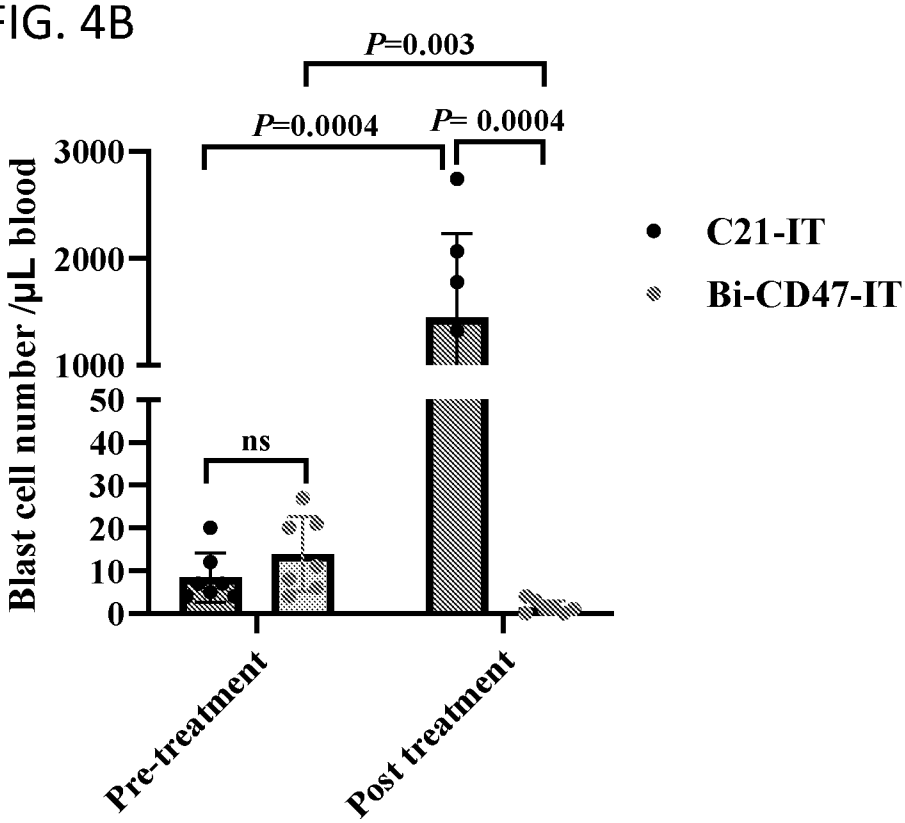

P=0.003
P=0.0004
P= 0.0004
Blast cell number /µL blood
3000
2000
1000
50
40
30
20
10
0
ns
C21-IT
Bi-CD47-IT
Pre-treatment
Post treatment

FIG. 4C

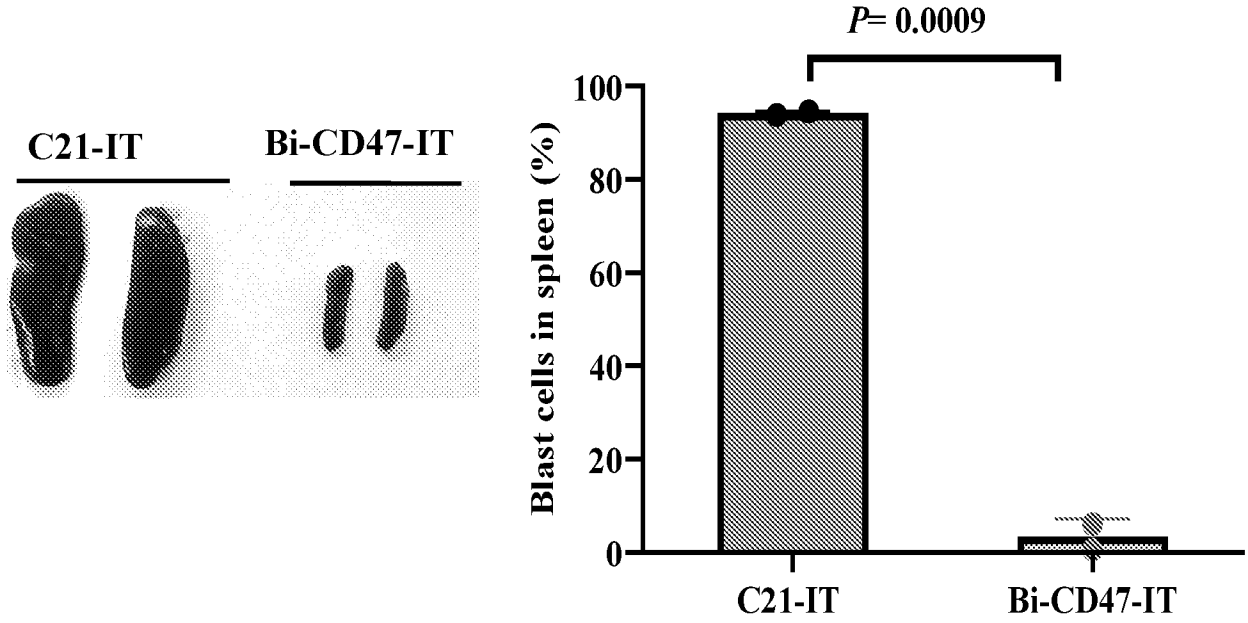

C21-IT
Bi-CD47-IT
P= 0.0009
Blast cells in spleen (%)
100
80
60
40
20
0
C21-IT
Bi-CD47-IT

FIG. 4D

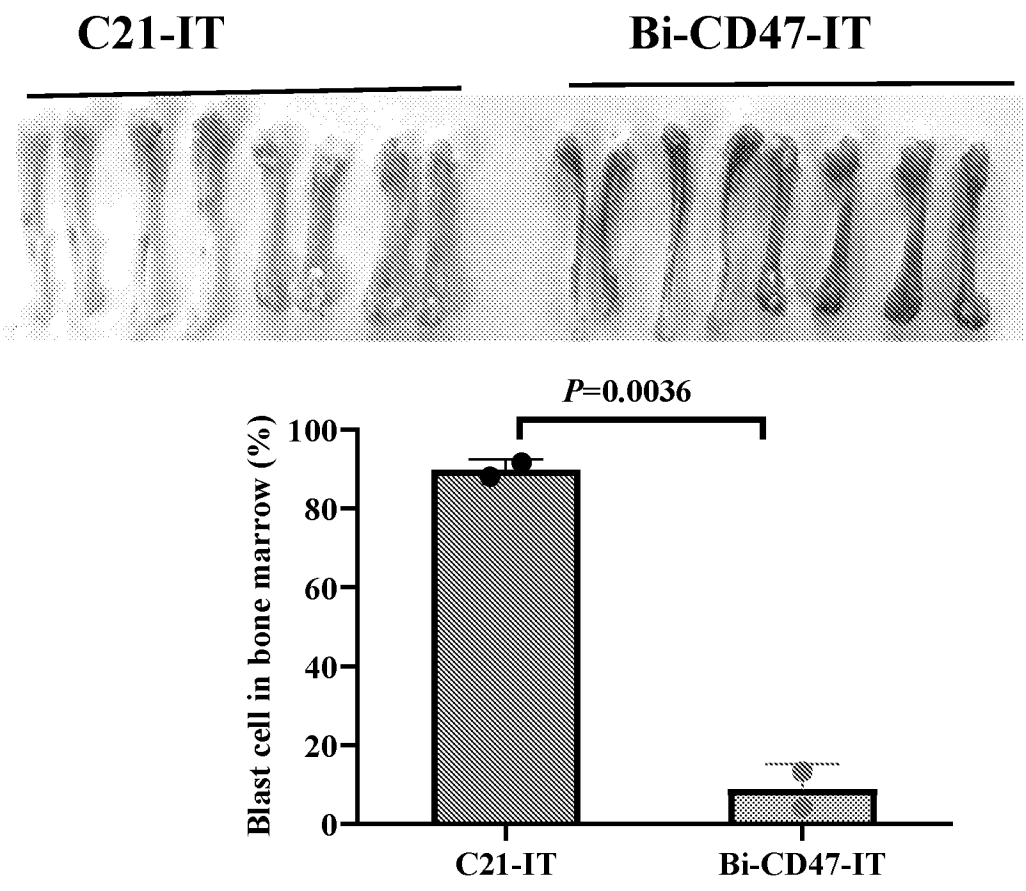

C21-IT
Bi-CD47-IT
P=0.0036
Blast cell in bone marrow (%)
100
80
60
40
20
0
C21-IT
Bi-CD47-IT

FIG. 4E

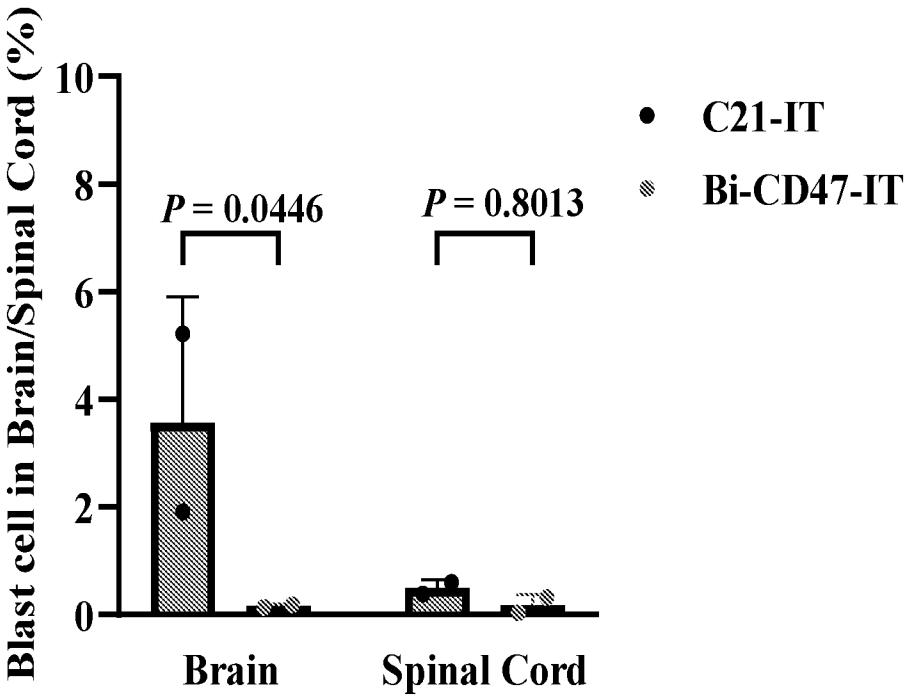

Blast cell in Brain/Spinal Cord (%)
10
8
6
4
2
0
P = 0.0446
P = 0.8013
C21-IT
Bi-CD47-IT
Brain
Spinal Cord

FIG. 4F

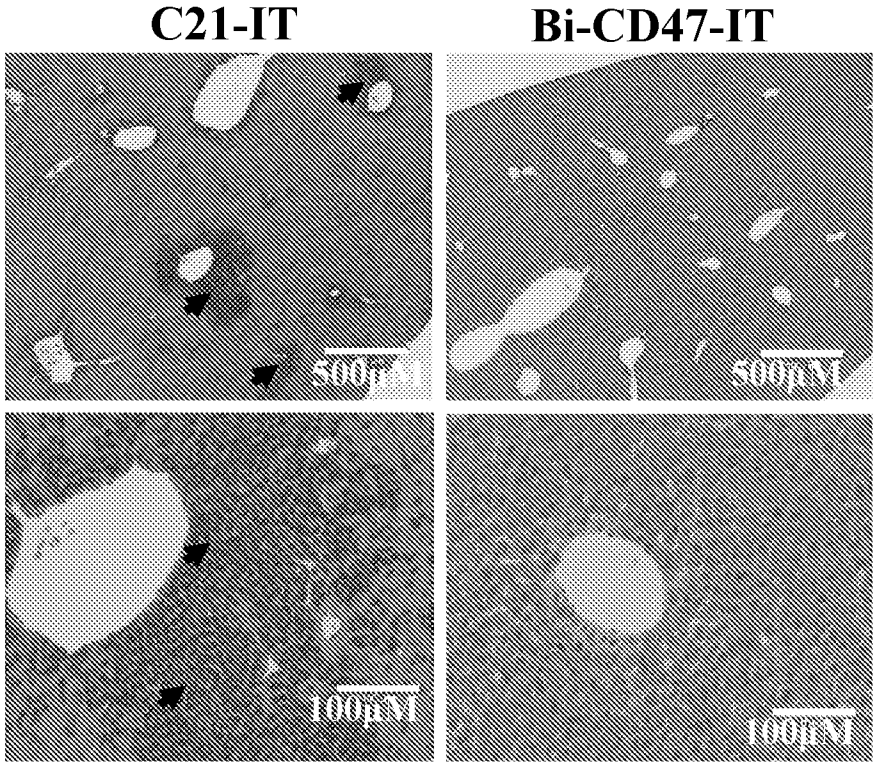

C21-IT
Bi-CD47-IT
500μM
500μM
100μM
100μM

FIG. 5A

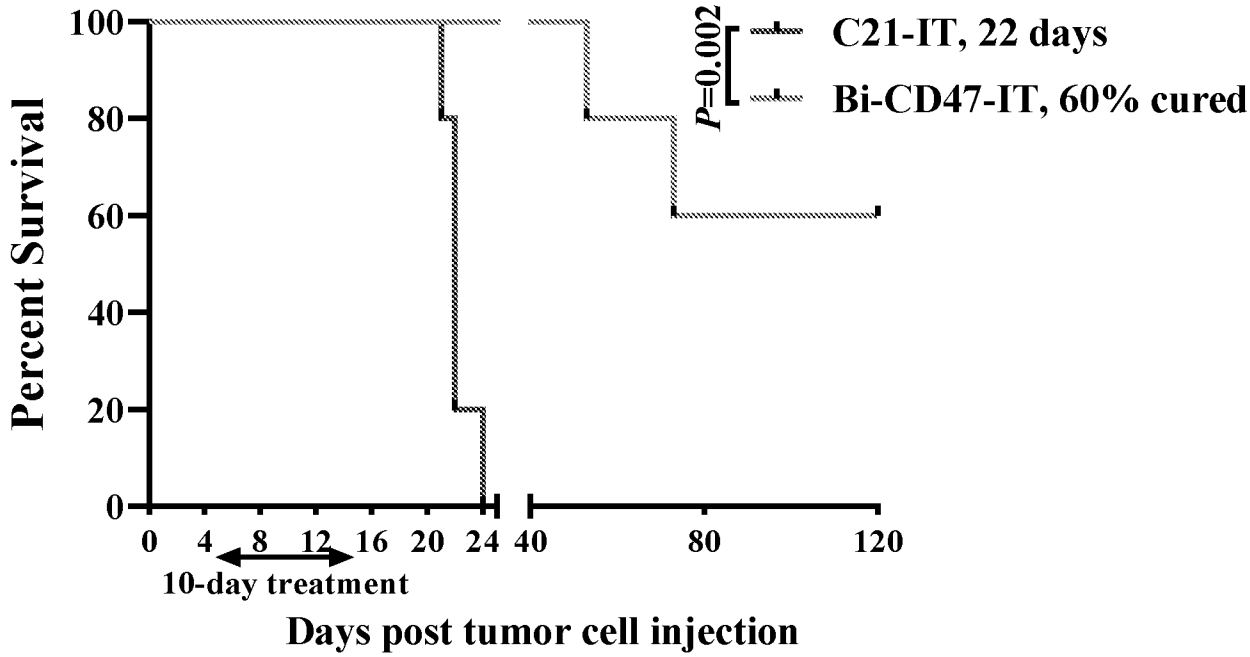

Percent Survival
100
80
60
40
20
0
0 4 8 12 16 20 24 40 80 120
10-day treatment
Days post tumor cell injection
P=0.002
C21-IT, 22 days
Bi-CD47-IT, 60% cured

FIG. 5B

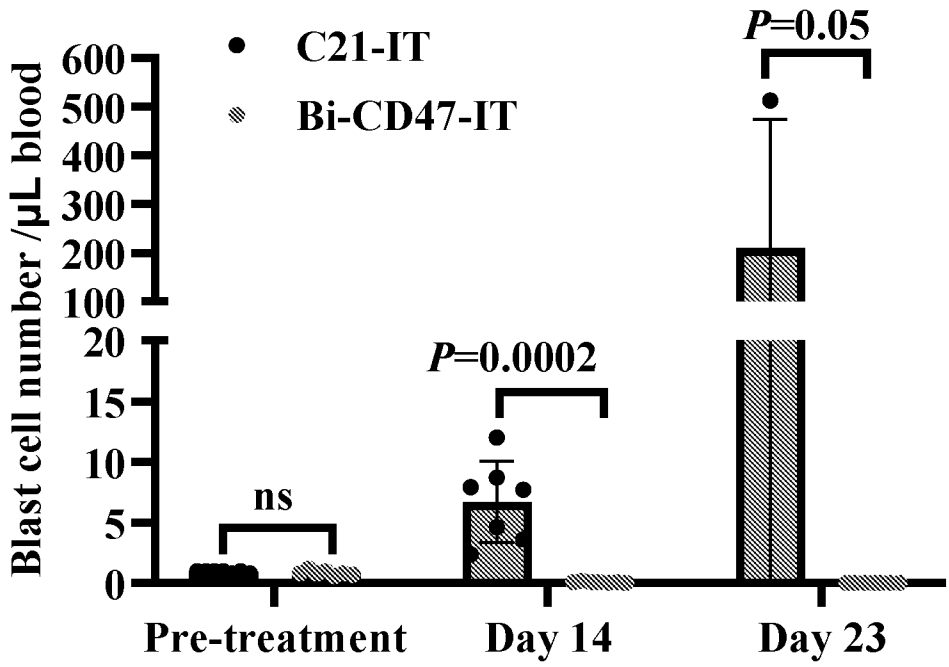
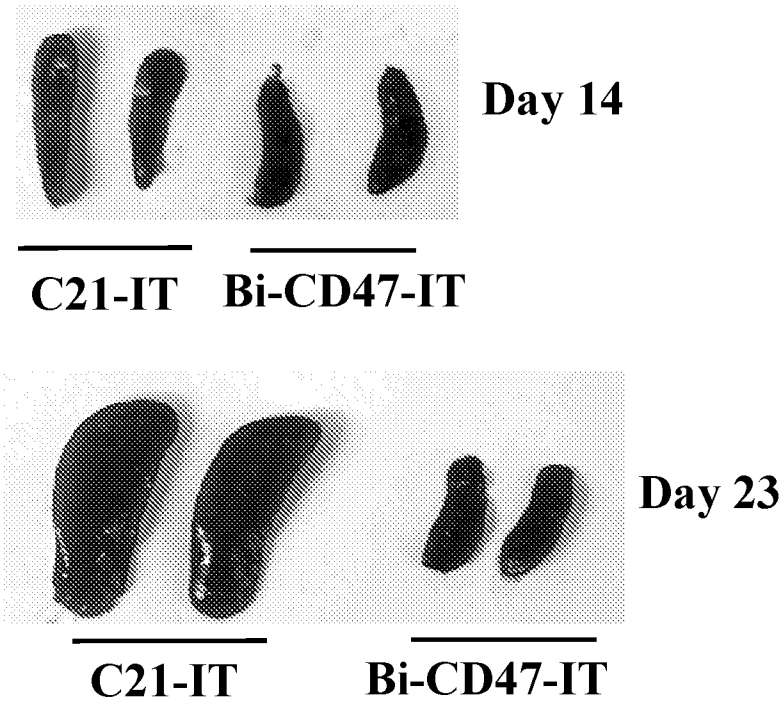

Blast cell number /μL blood
600
500
400
300
200
100
20
15
10
5
0
C21-IT
Bi-CD47-IT
P=0.05
P=0.0002
ns
Pre-treatment
Day 14
Day 23

FIG. 5C


Day 14
C21-IT
Bi-CD47-IT
Day 23
C21-IT
Bi-CD47-IT

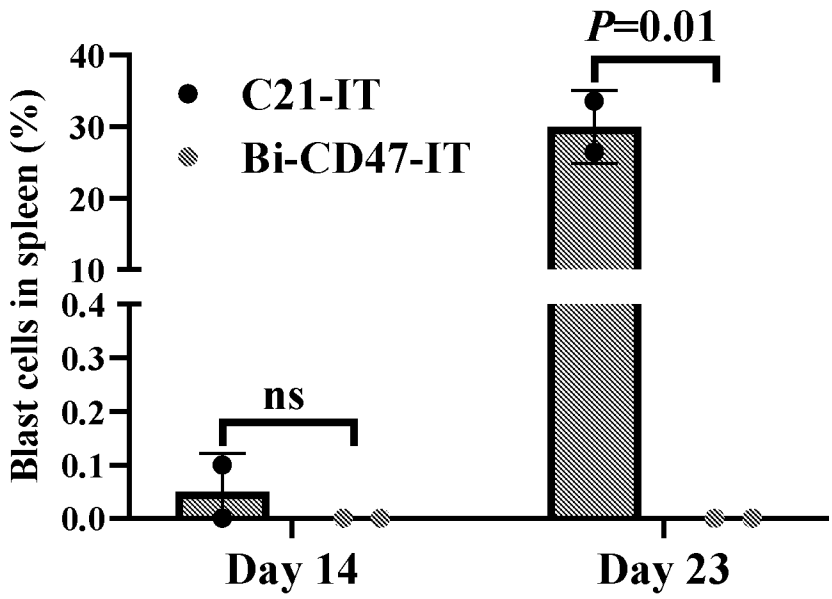
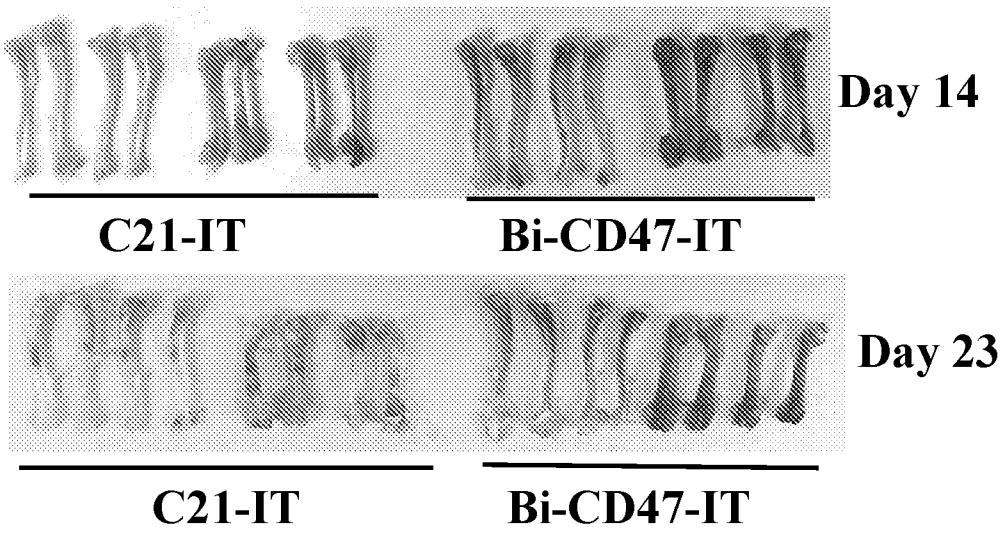

P=0.01
C21-IT
Bi-CD47-IT
Blast cells in spleen (%)
40
30
20
10
0.4
0.3
0.2
0.1
0.0
ns
Day 14
Day 23

FIG. 5D


Day 14
C21-IT
Bi-CD47-IT
Day 23
C21-IT
Bi-CD47-IT

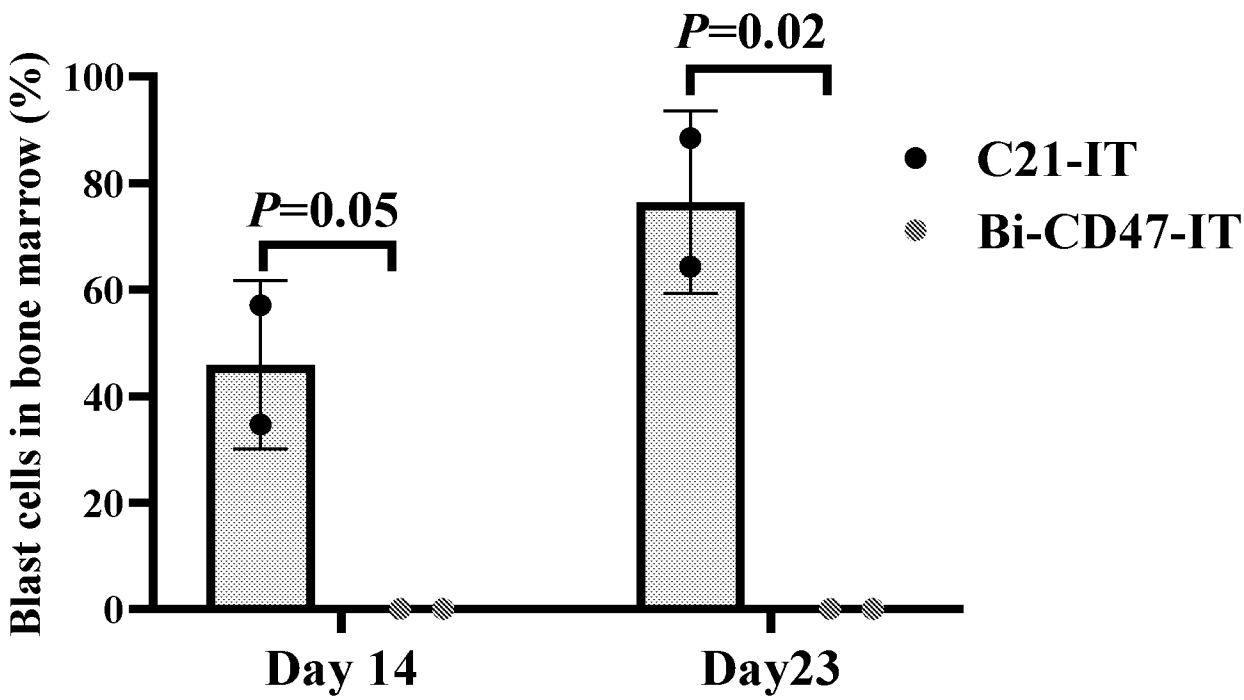

P=0.02
P=0.05
Blast cells in bone marrow (%)
100
80
60
40
20
0
Day 14
Day23
C21-IT
Bi-CD47-IT

FIG. 5E

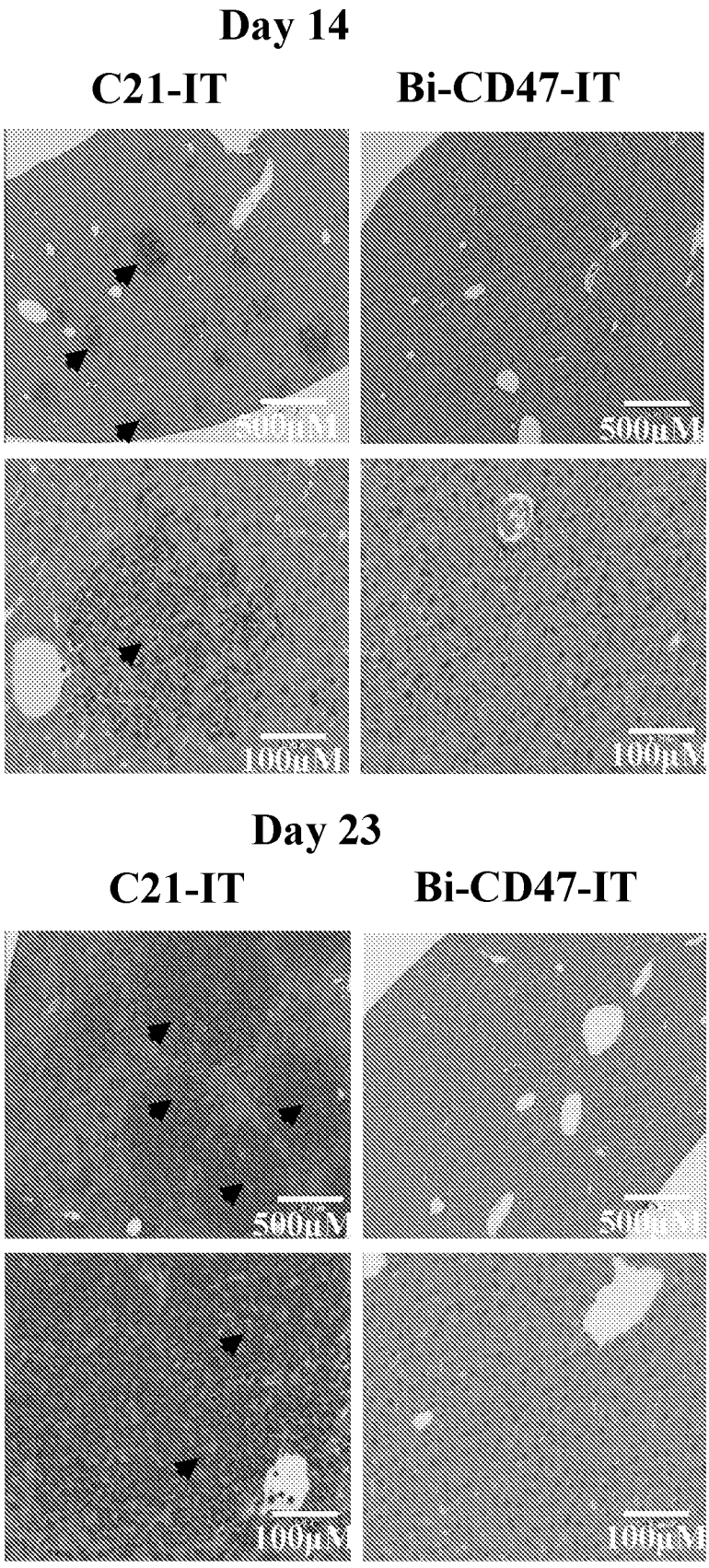

Day 14
C21-IT
Bi-CD47-IT
500μM
500μM
100μM
100μM
Day 23
C21-IT
Bi-CD47-IT
500μM
100μM
100μM

FIG. 6A

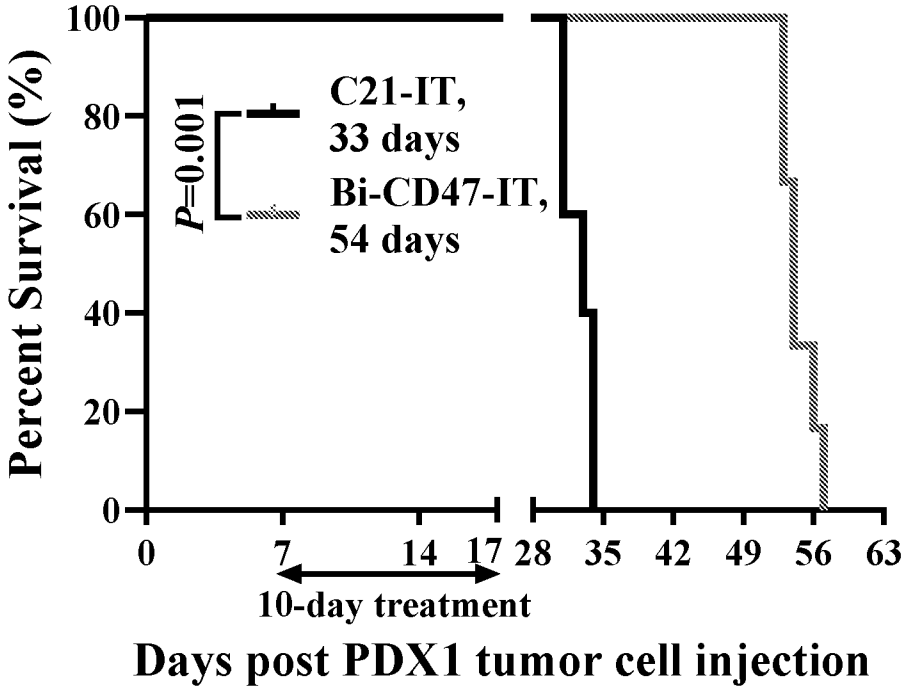

Percent Survival (%)
100
80
60
40
20
0
P=0.001
C21-IT,
33 days
Bi-CD47-IT,
54 days
0
7
14
17
28
35
42
49
56
63
10-day treatment
Days post PDX1 tumor cell injection

FIG. 6B

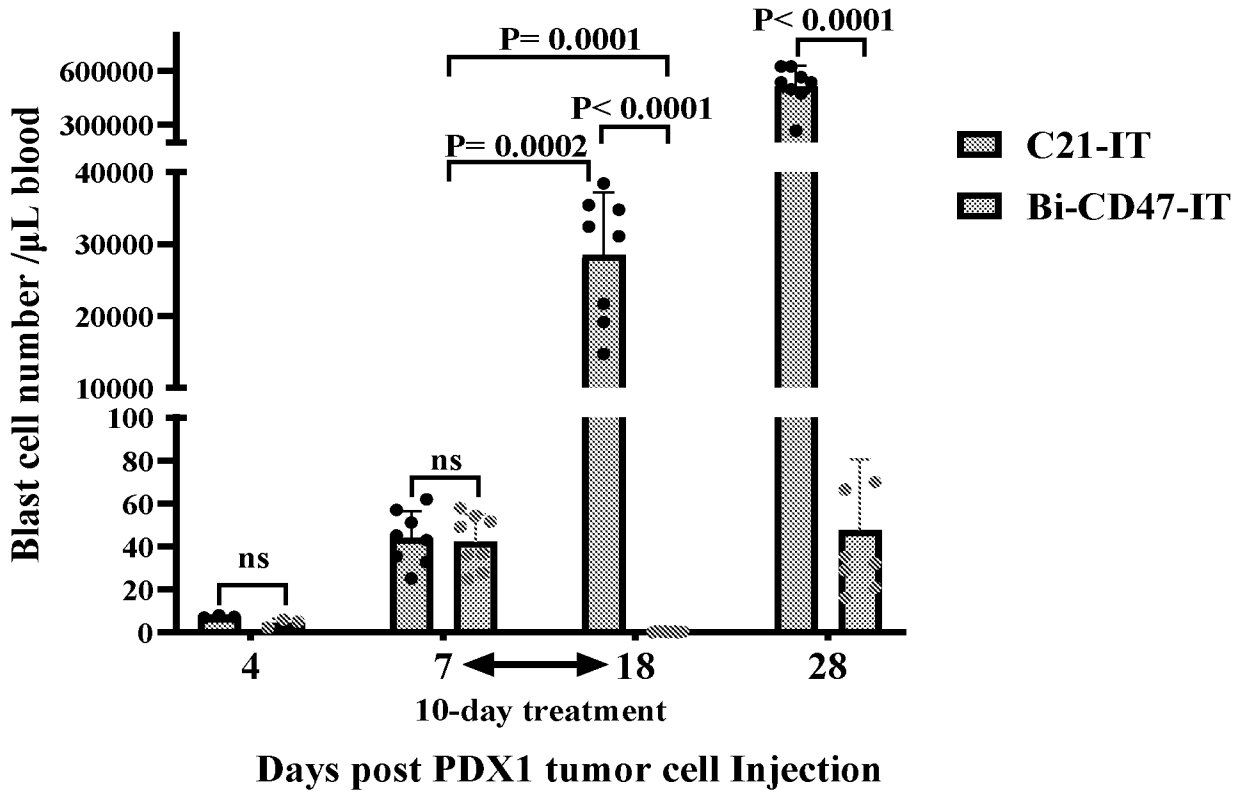

Blast cell number /µL blood
600000
300000
40000
30000
20000
10000
100
80
60
40
20
0
P= 0.0001
P< 0.0001
P= 0.0002
P< 0.0001
ns
ns
4
7
18
28
10-day treatment
C21-IT
Bi-CD47-IT
Days post PDX1 tumor cell Injection

FIG. 6C

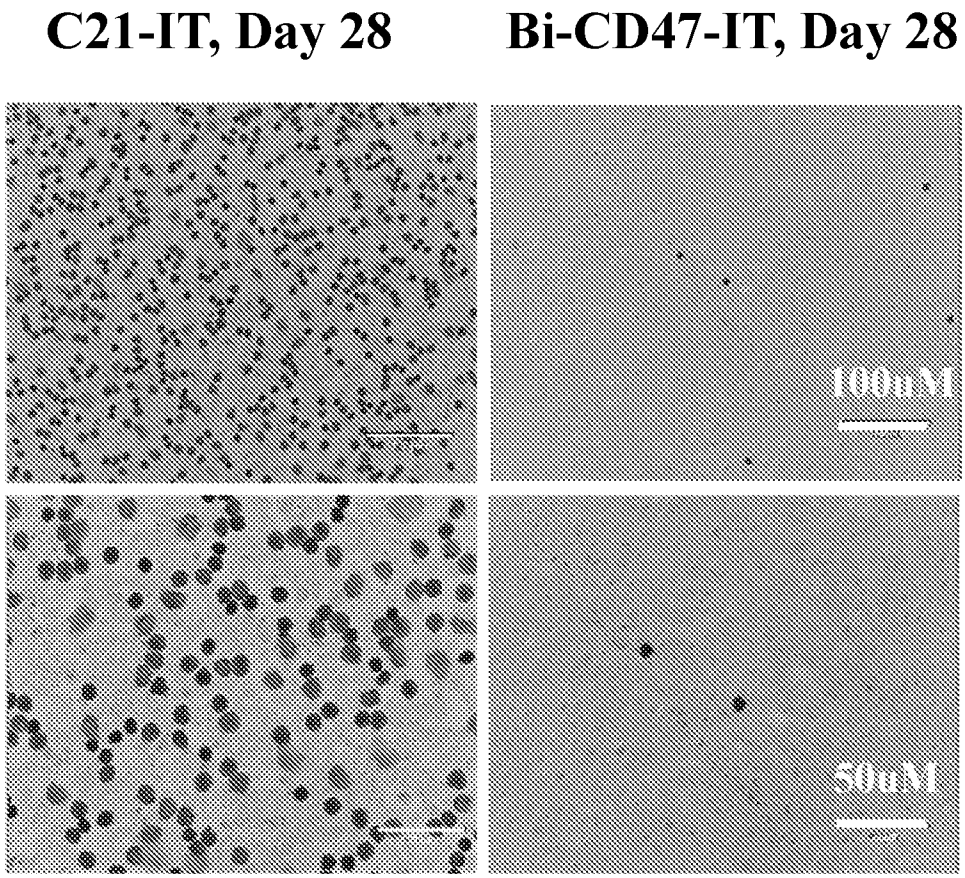

C21-IT, Day 28
Bi-CD47-IT, Day 28
100uM
50uM

FIG. 6D

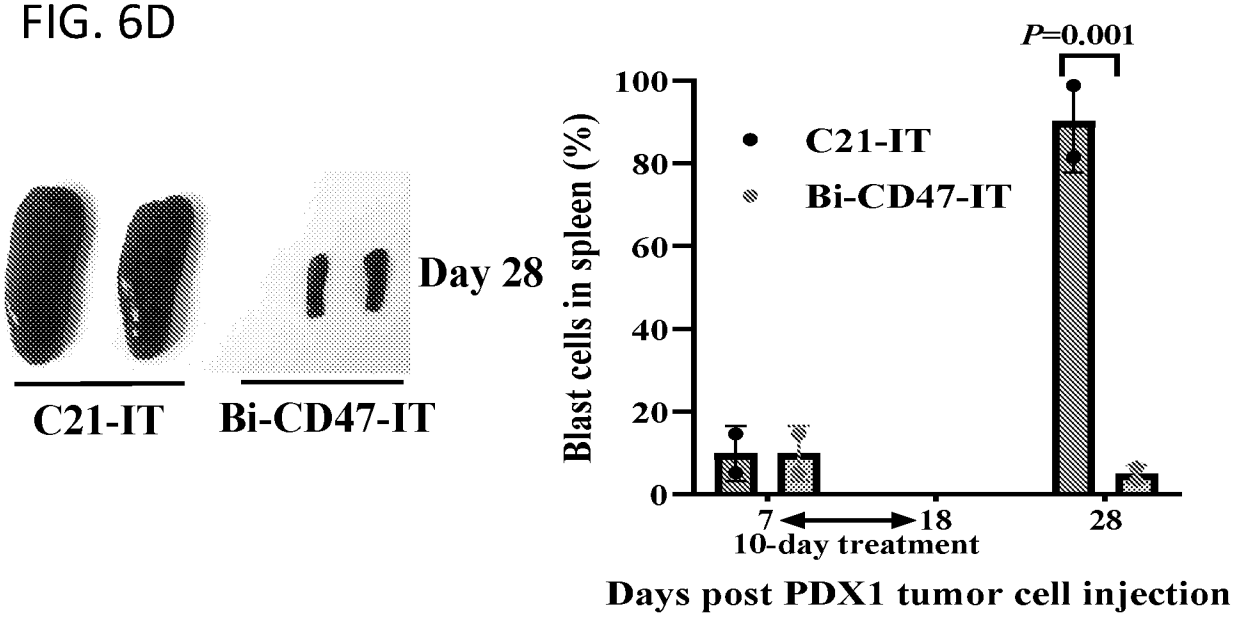

Day 28
C21-IT
Bi-CD47-IT
P=0.001
100
80
60
40
20
0
Blast cells in spleen (%)
C21-IT
Bi-CD47-IT
7
18
28
10-day treatment
Days post PDX1 tumor cell injection

FIG. 6E

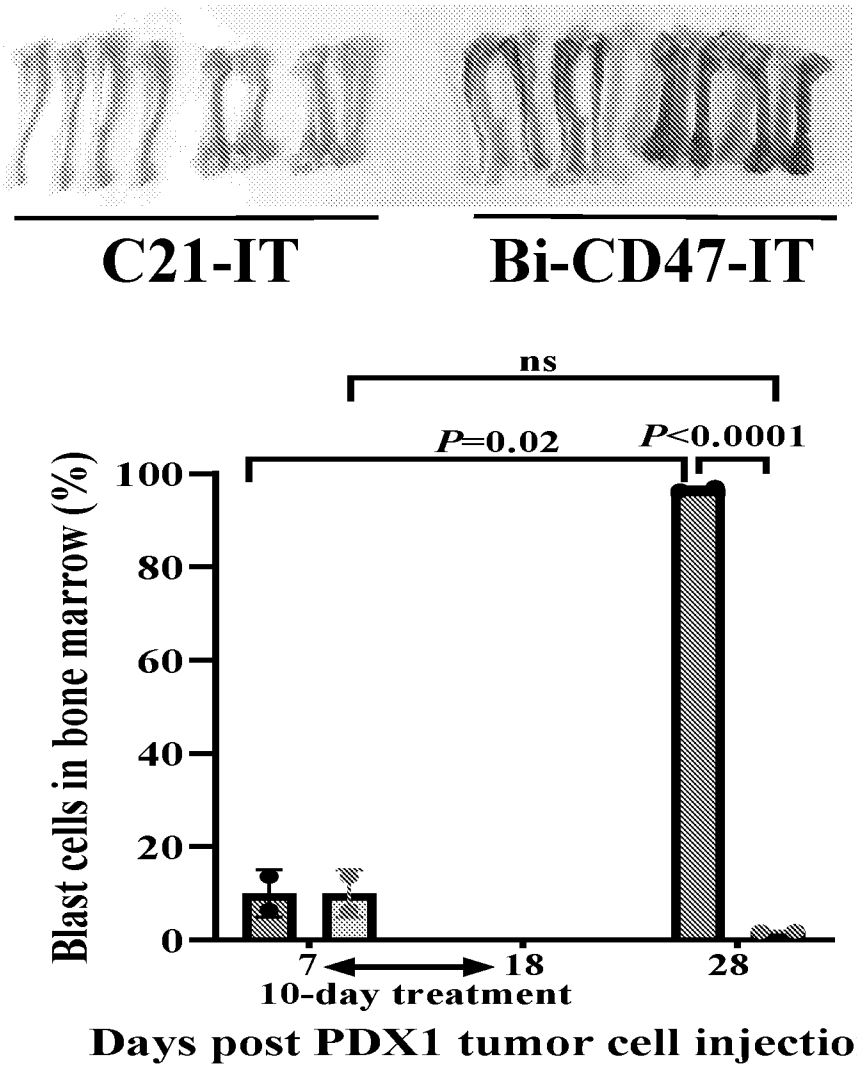

Day 28
C21-IT
Bi-CD47-IT
ns
P=0.02
P<0.0001
Blast cells in bone marrow (%)
100
80
60
40
20
0
7
18
28
10-day treatment
Days post PDX1 tumor cell injection

FIG. 6F

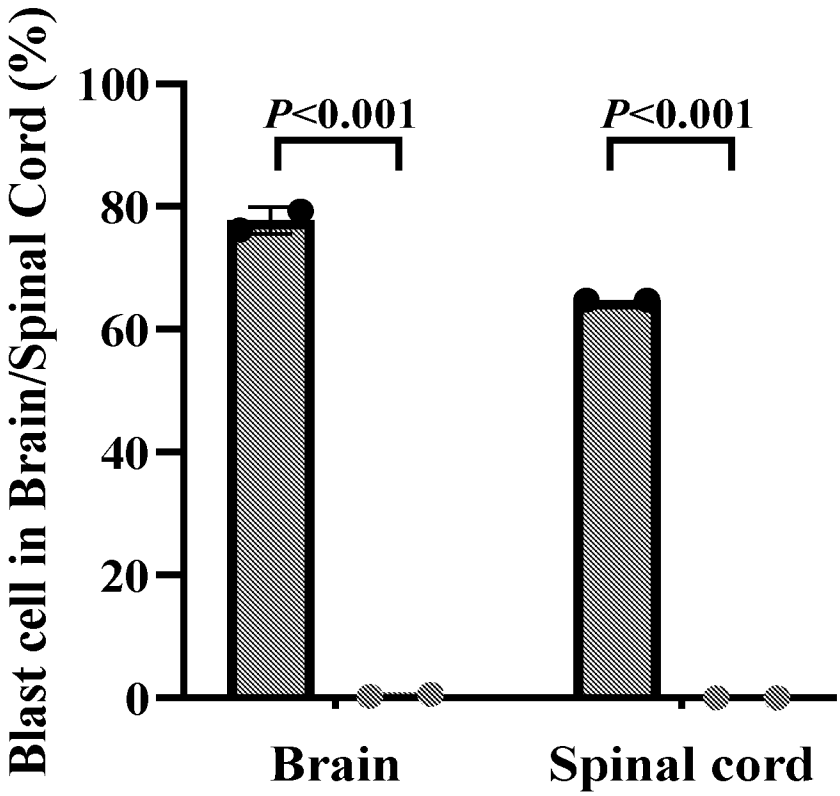

Blast cell in Brain/Spinal Cord (%)
100
80
60
40
20
0
P<0.001
P<0.001
Brain
Spinal cord

FIG. 6G

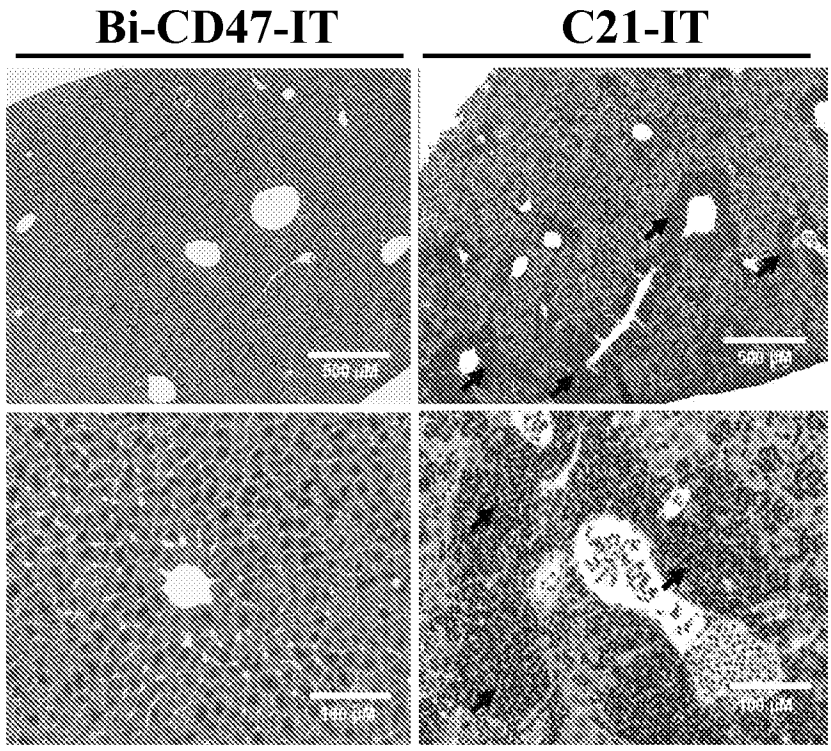

Bi-CD47-IT
C21-IT

FIG. 6H

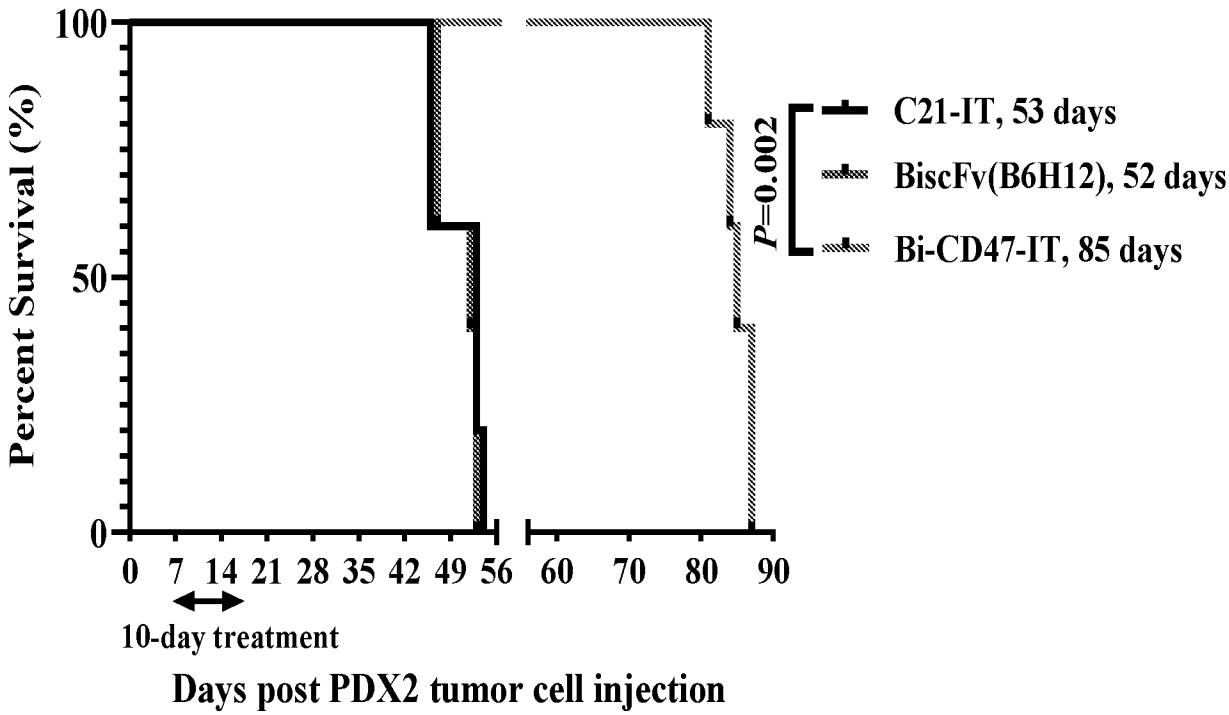

100
50
0
Percent Survival (%)
0 7 14 21 28 35 42 49 56 60 70 80 90
10-day treatment
Days post PDX2 tumor cell injection
P=0.002
C21-IT, 53 days
BiscFv(B6H12), 52 days
Bi-CD47-IT, 85 days

FIG. 6I

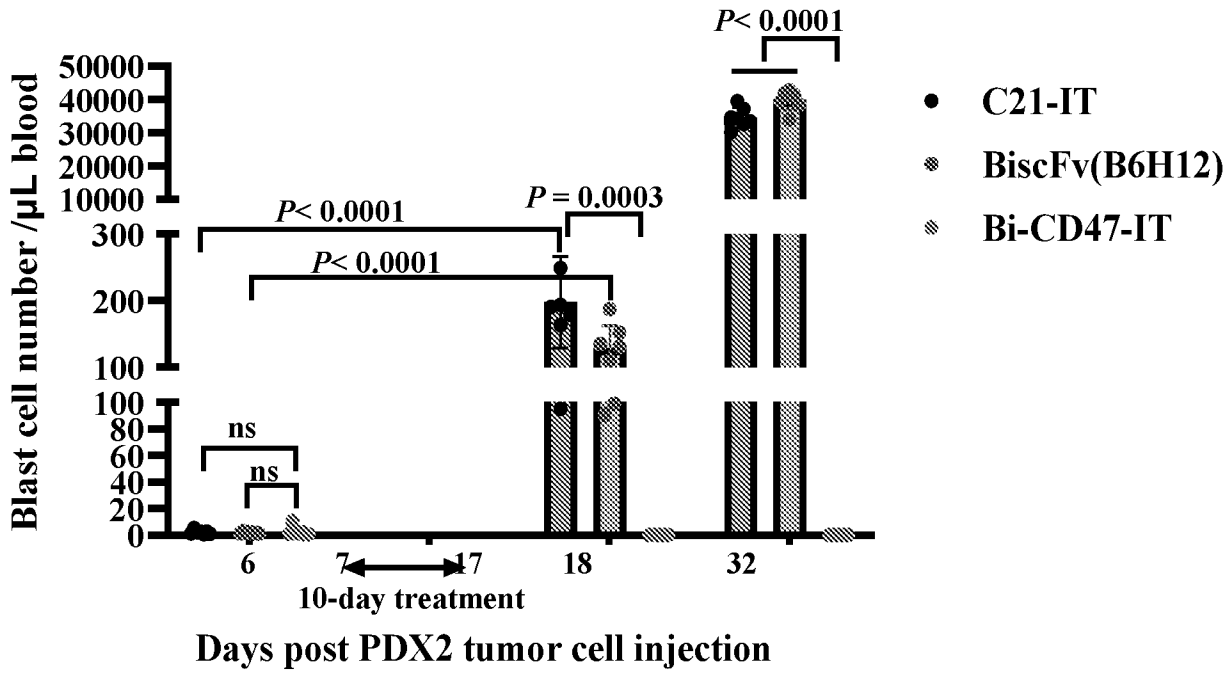

Blast cell number /μL blood
50000
40000
30000
20000
10000
300
200
100
100
80
60
40
20
0
P< 0.0001
P = 0.0003
P< 0.0001
P< 0.0001
ns
ns
6 7 17 18 32
10-day treatment
Days post PDX2 tumor cell injection
C21-IT
BiscFv(B6H12)
Bi-CD47-IT

FIG. 6J

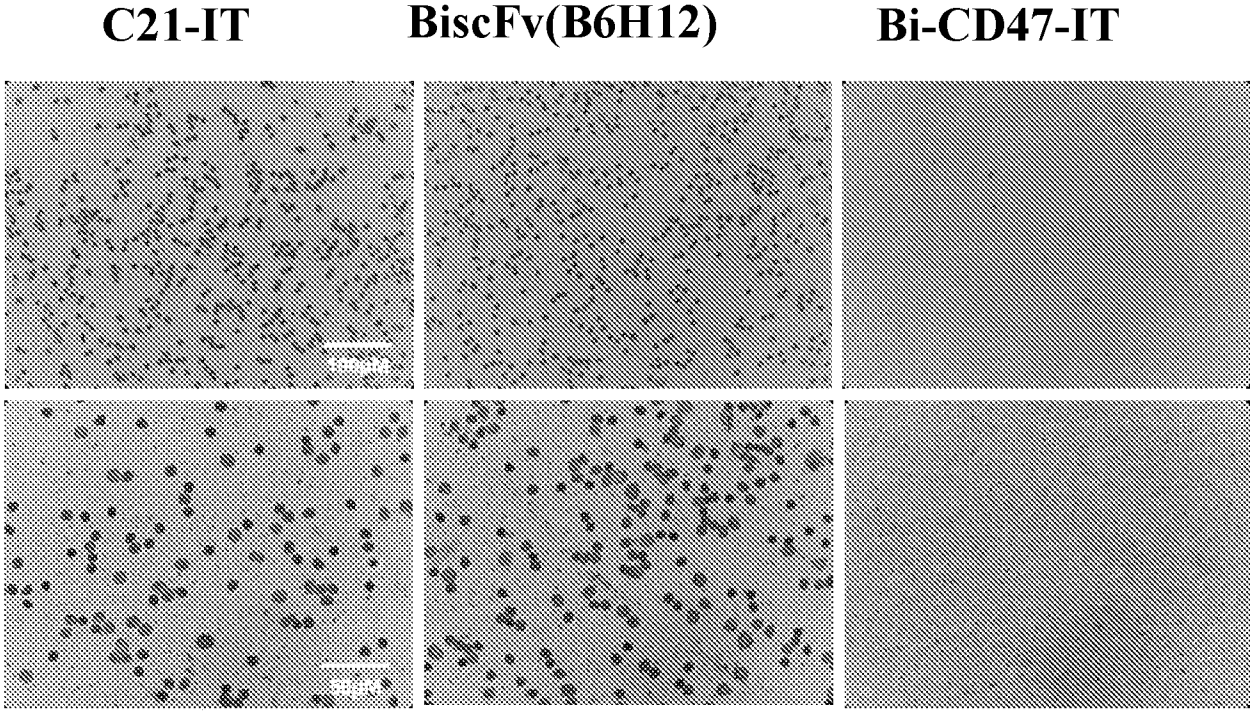

C21-IT
BiscFv(B6H12)
Bi-CD47-IT

FIG. 6K

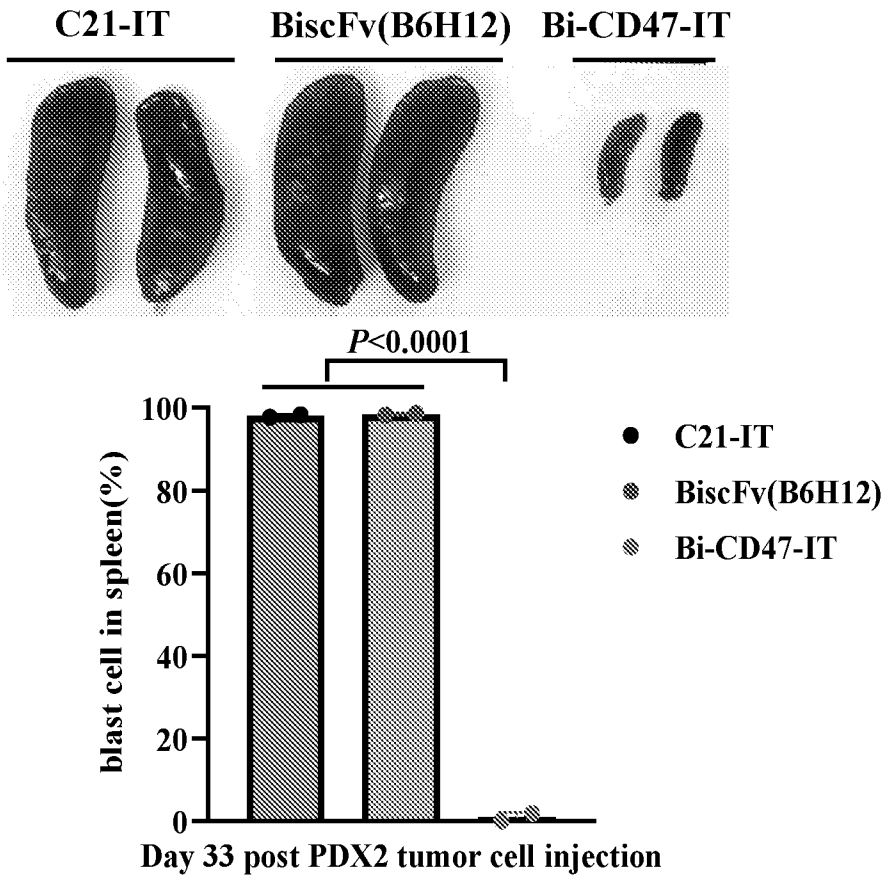

C21-IT
BiscFv(B6H12)
Bi-CD47-IT
P<0.0001
blast cell in spleen(%)
100
80
60
40
20
0
C21-IT
BiscFv(B6H12)
Bi-CD47-IT
Day 33 post PDX2 tumor cell injection

FIG. 6L

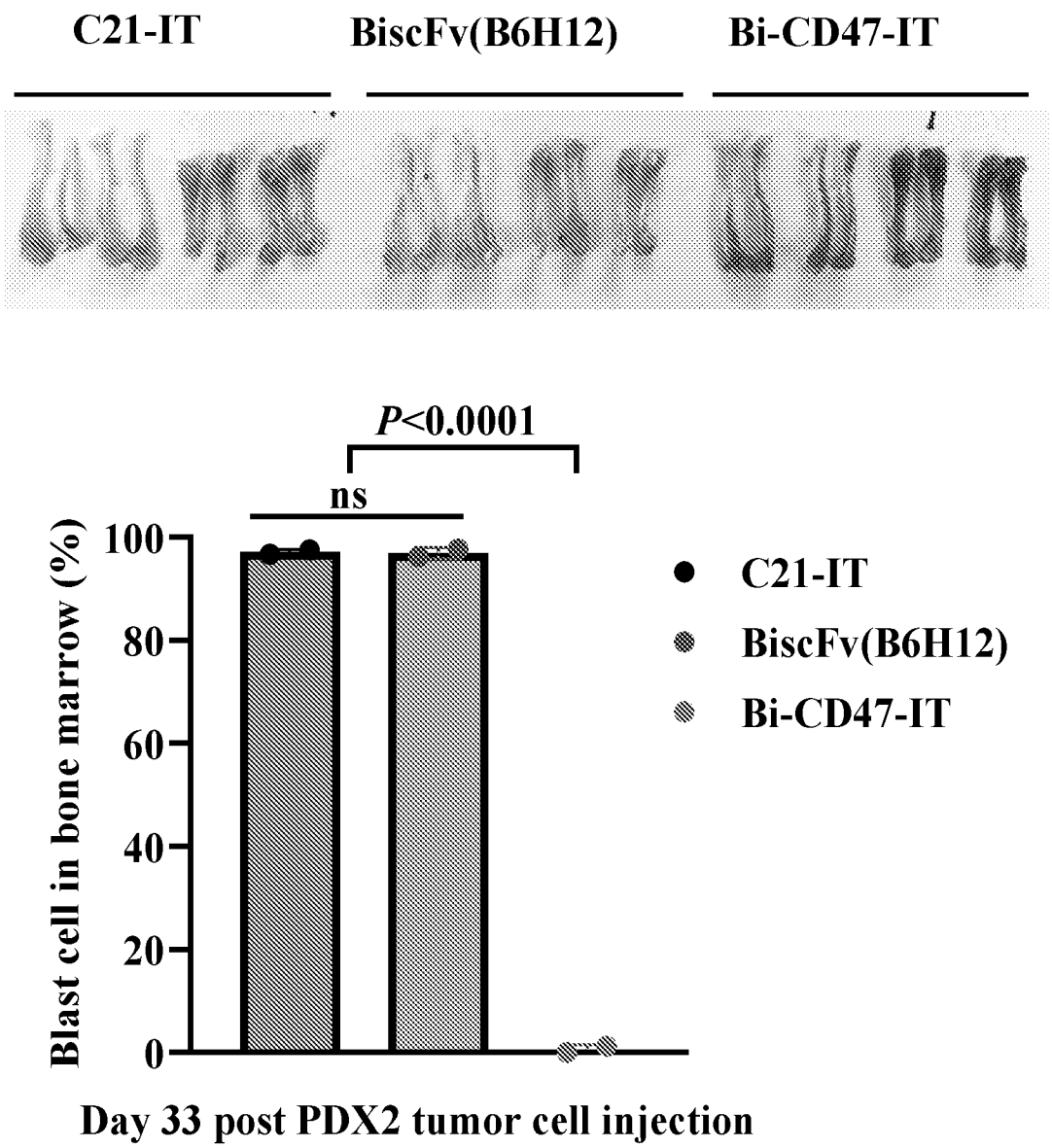

C21-IT
BiscFv(B6H12)
Bi-CD47-IT
P<0.0001
ns
Blast cell in bone marrow (%)
100
80
60
40
20
0
C21-IT
BiscFv(B6H12)
Bi-CD47-IT
Day 33 post PDX2 tumor cell injection

FIG. 6M

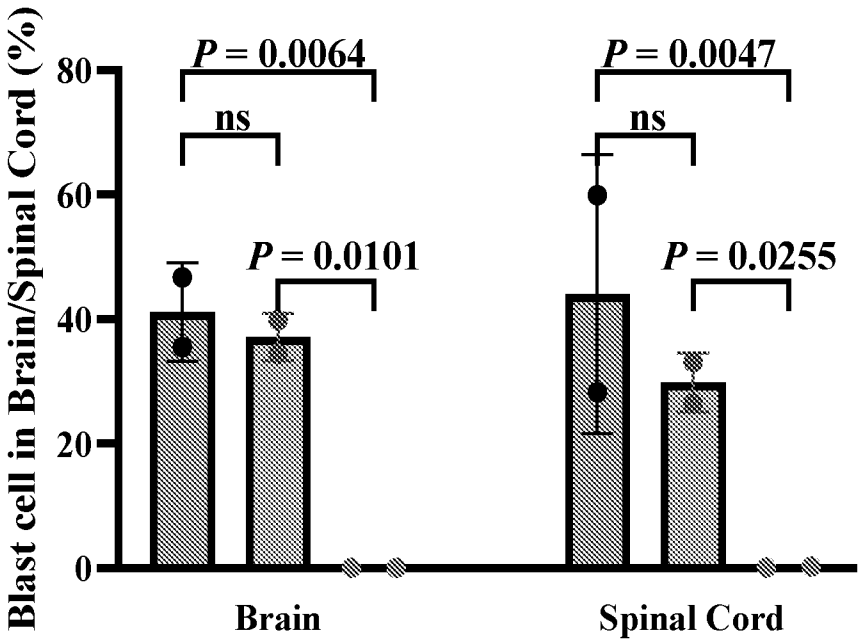
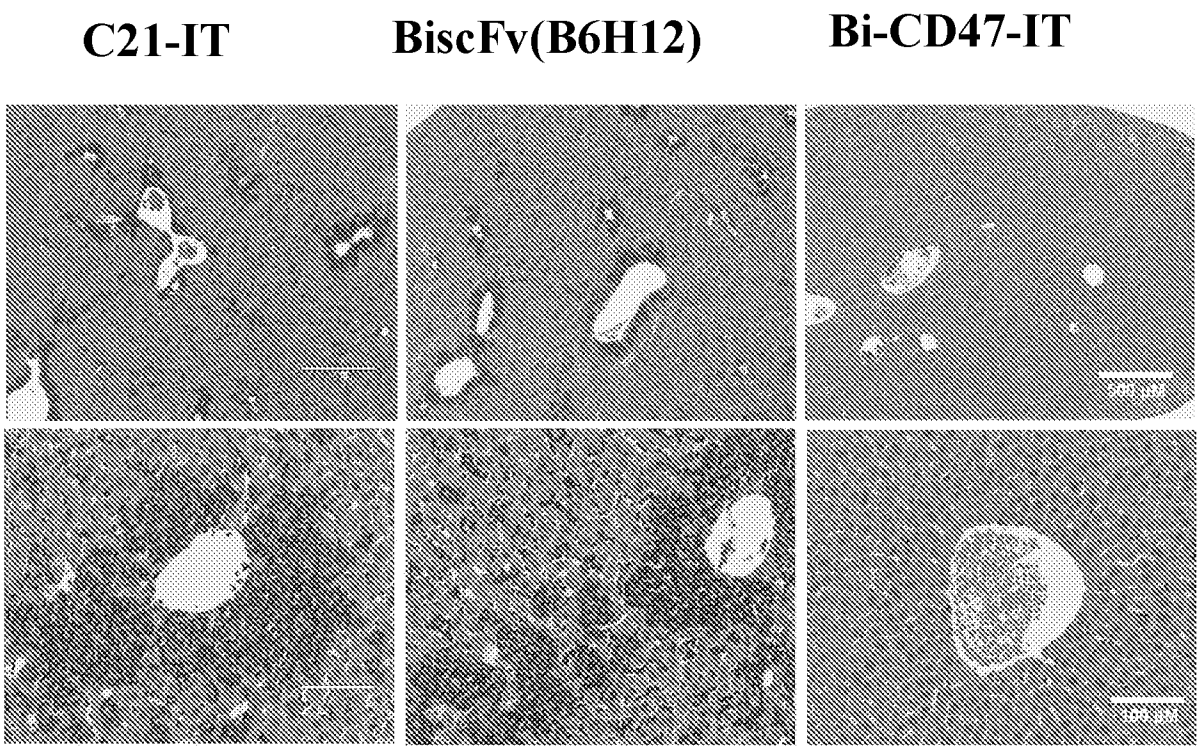
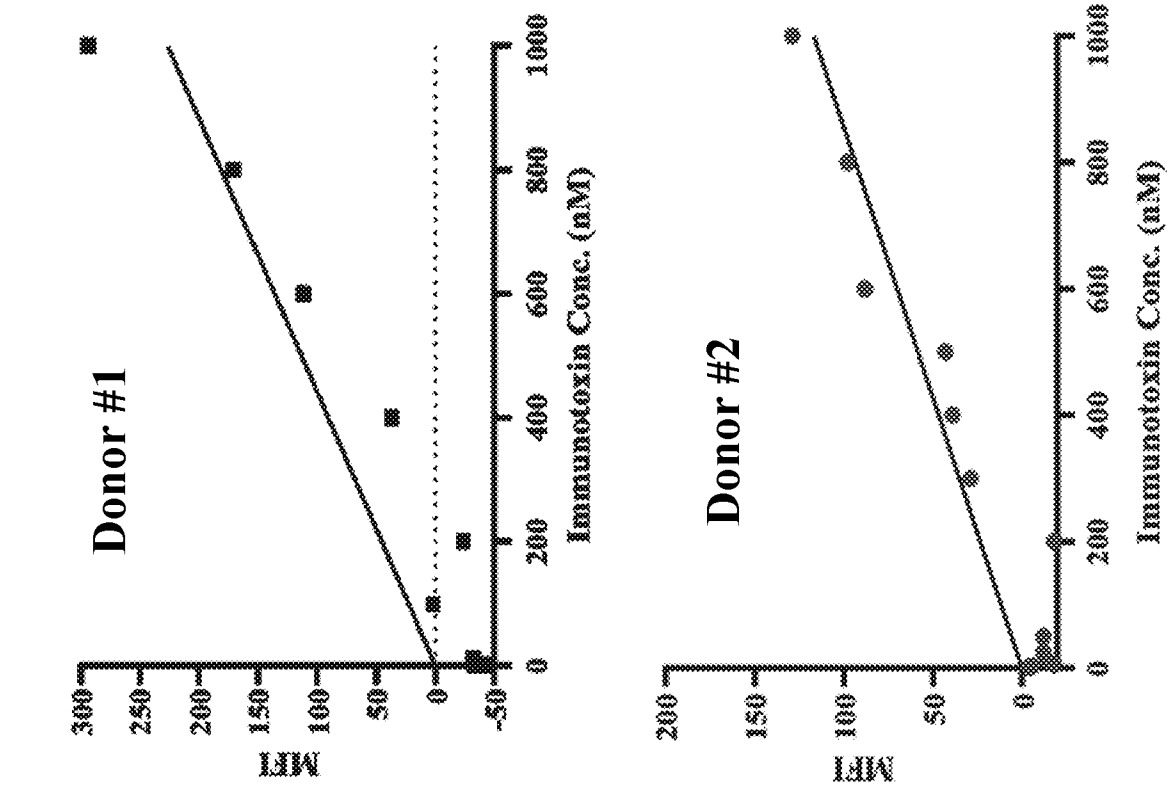

Blast cell in Brain/Spinal Cord (%)
80
60
40
20
0
P = 0.0064
ns
P = 0.0101
P = 0.0047
ns
P = 0.0255
Brain
Spinal Cord

FIG. 6N


C21-IT
BiscFv(B6H12)
Bi-CD47-IT


Donor #1
MFI
Immunotoxin Conc. (nM)
Donor #2
MFI
Immunotoxin Conc. (nM)

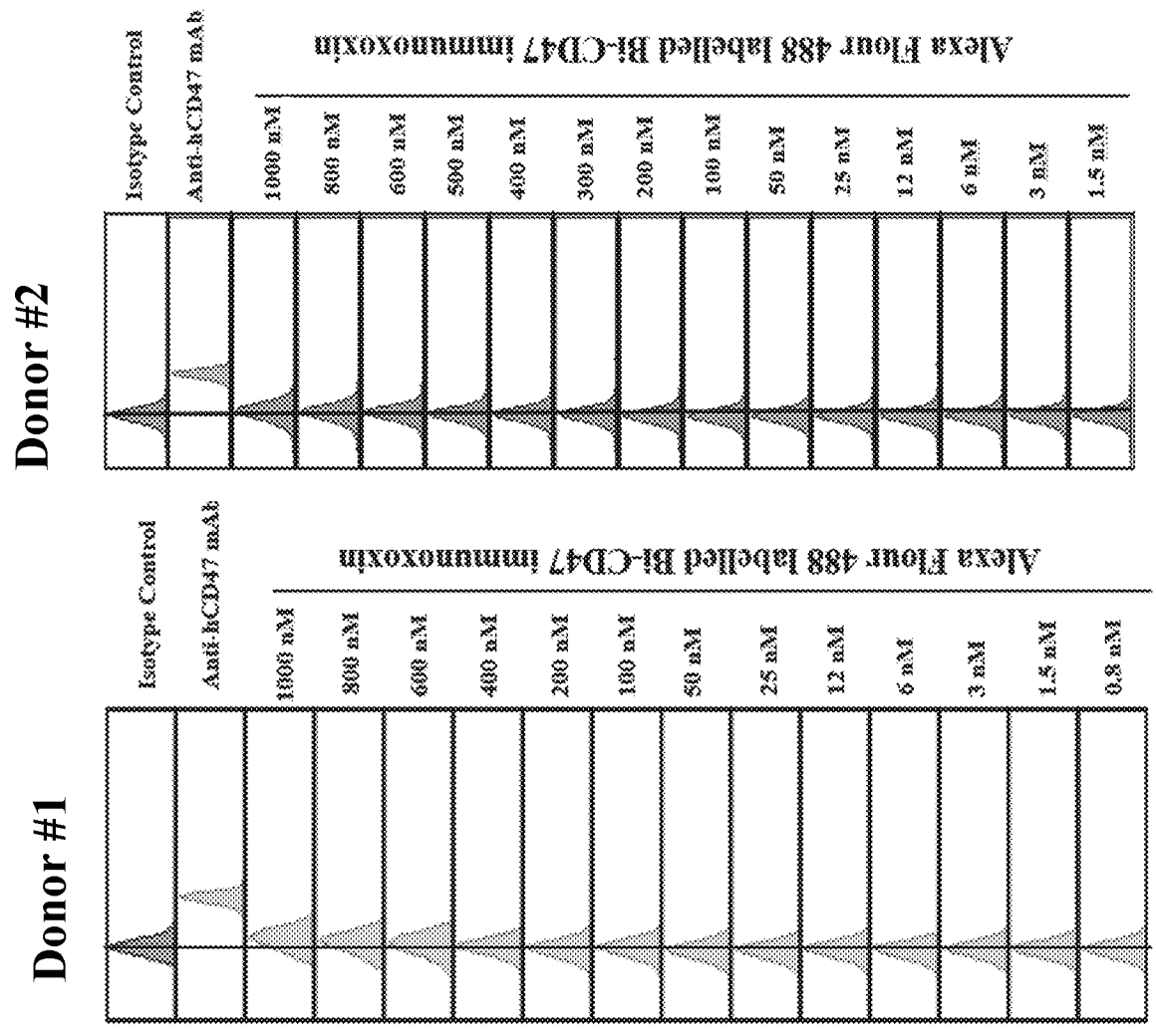

Donor #1
Isotype Control
Anti-hCD47 mAb
Alexa Flour 488 labelled Bi-CD47 immunoxin
1000 nM
800 nM
600 nM
400 nM
200 nM
100 nM
50 nM
25 nM
12 nM
6 nM
3 nM
1.5 nM
0.8 nM
Donor #2
Isotype Control
Anti-hCD47 mAb
Alexa Flour 488 labelled Bi-CD47 immunoxin
1000 nM
800 nM
600 nM
500 nM
400 nM
300 nM
200 nM
100 nM
50 nM
25 nM
12 nM
6 nM
3 nM
1.5 nM

FIG. 7A

FIG. 7B

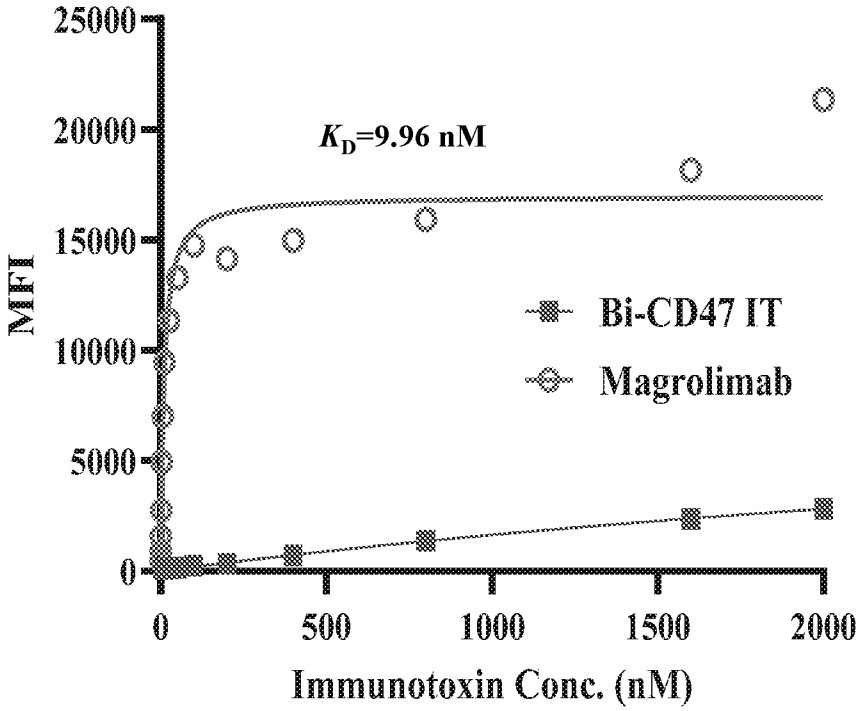

25000
20000
15000
10000
5000
0
MFI
K_D=9.96 nM
Bi-CD47 IT
Magrolimab
0
500
1000
1500
2000
Immunotoxin Conc. (nM)

FIG. 7C

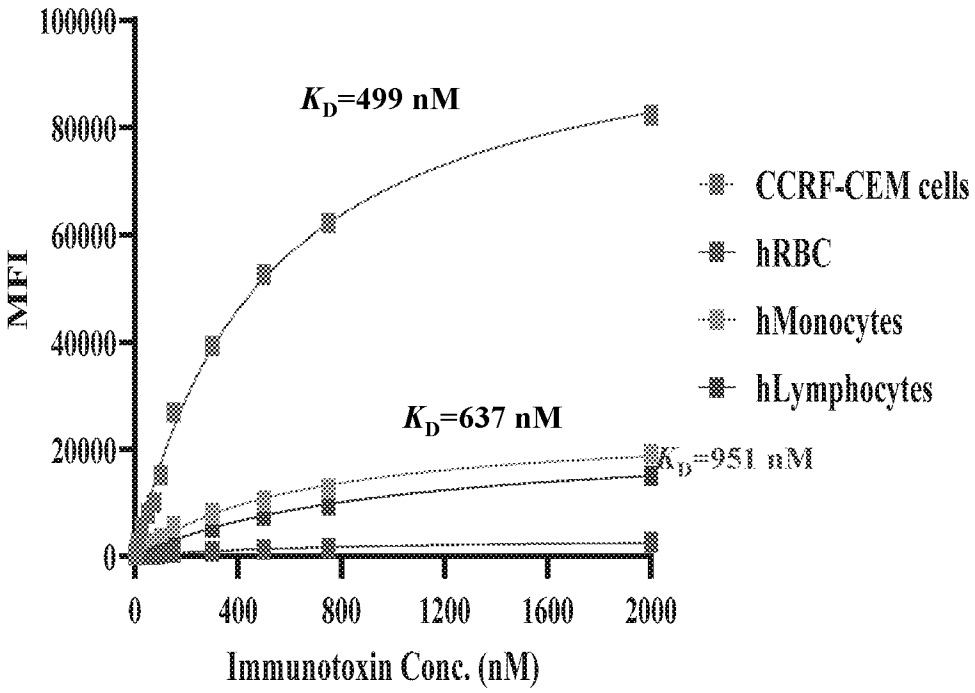

100000
80000
60000
40000
20000
0
MFI
K_D=499 nM
K_D=637 nM
K_D=951 nM
CCRF-CEM cells
hRBC
hMonocytes
hLymphocytes
0
400
800
1200
1600
2000
Immunotoxin Conc. (nM)

FIG. 7D

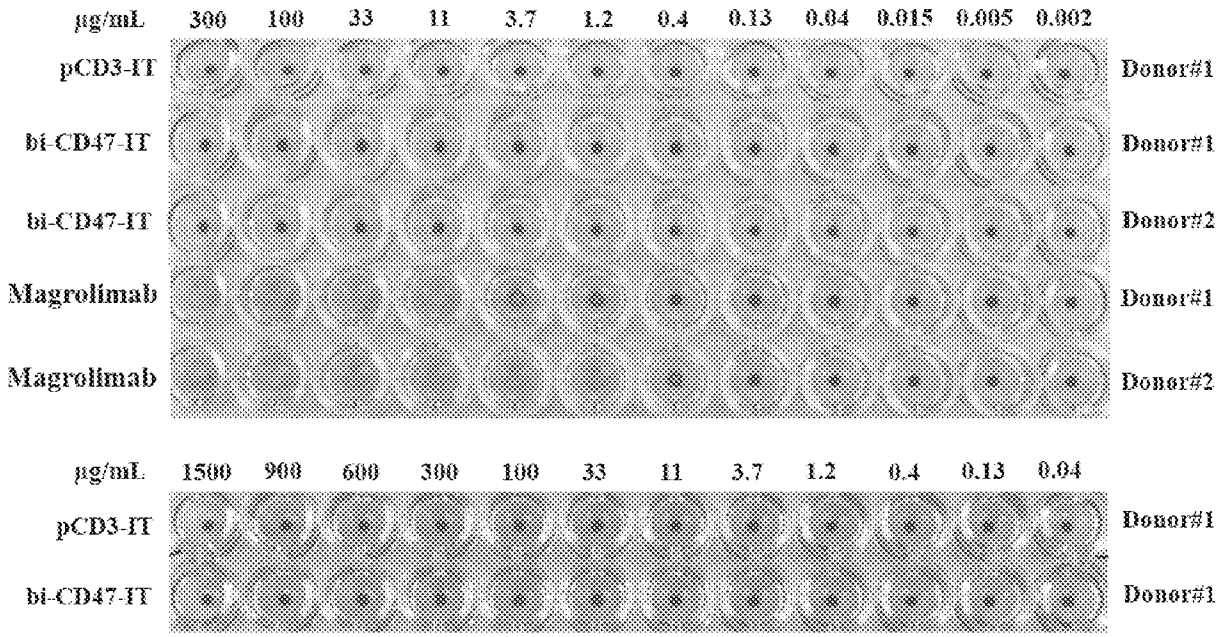

μg/mL 300 100 33 11 3.7 1.2 0.4 0.13 0.04 0.015 0.005 0.002
pCD3-IT Donor#1
bi-CD47-IT Donor#1
bi-CD47-IT Donor#2
Magrolimab Donor#1
Magrolimab Donor#2
μg/mL 1500 900 600 300 100 33 11 3.7 1.2 0.4 0.13 0.04
pCD3-IT Donor#1
bi-CD47-IT Donor#1

FIG. 7E

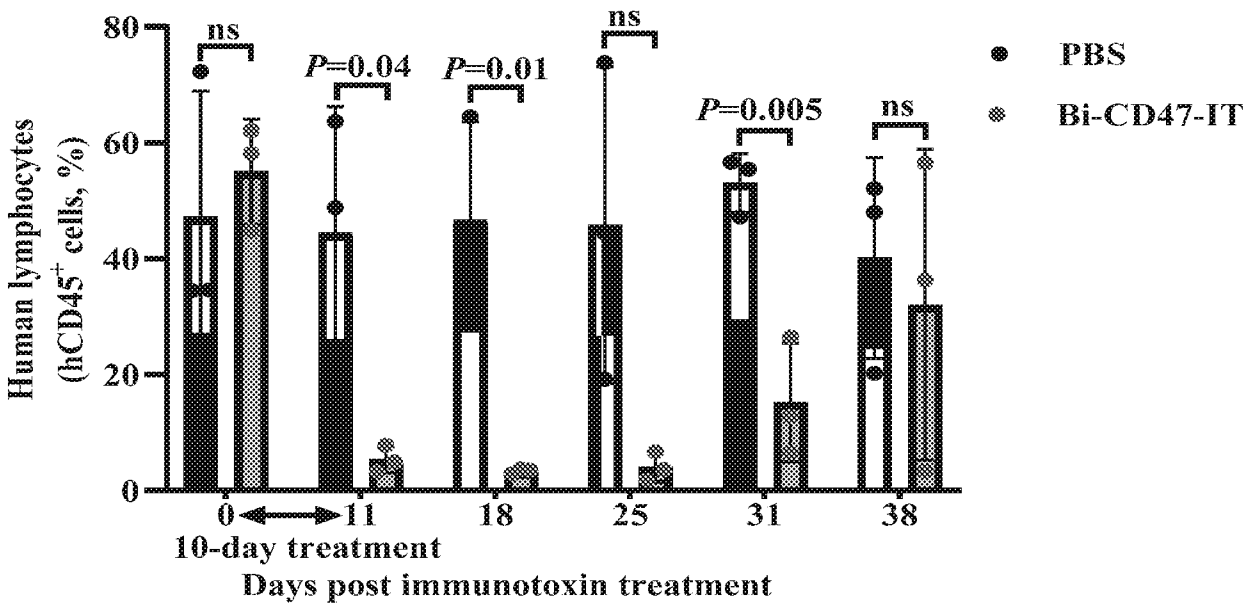

Human lymphocytes (hCD45+ cells, %)
80
60
40
20
0
ns
P=0.04
P=0.01
ns
P=0.005
ns
0
11
18
25
31
38
10-day treatment
Days post immunotoxin treatment
PBS
Bi-CD47-IT

FIG. 8A

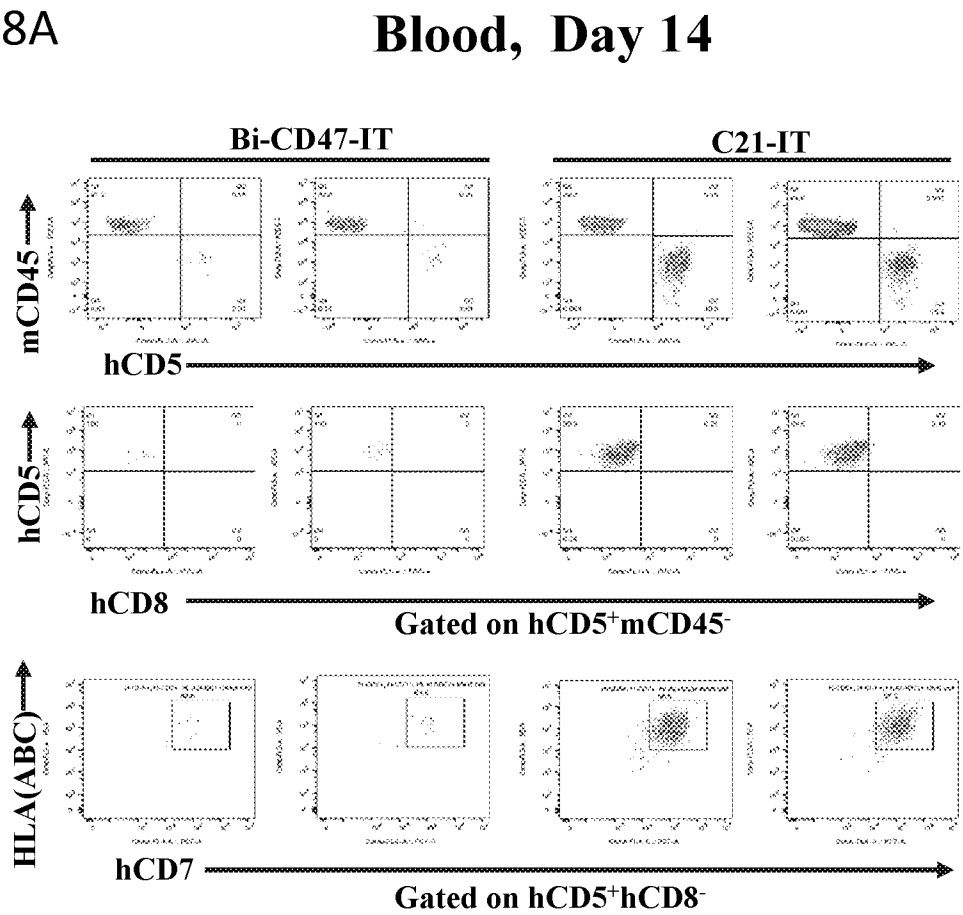

Blood, Day 14
Bi-CD47-IT
C21-IT
mCD45
hCD5
hCD5
hCD8
Gated on hCD5+mCD45-
HLA(ABC)
hCD7
Gated on hCD5+hCD8-

FIG. 8B

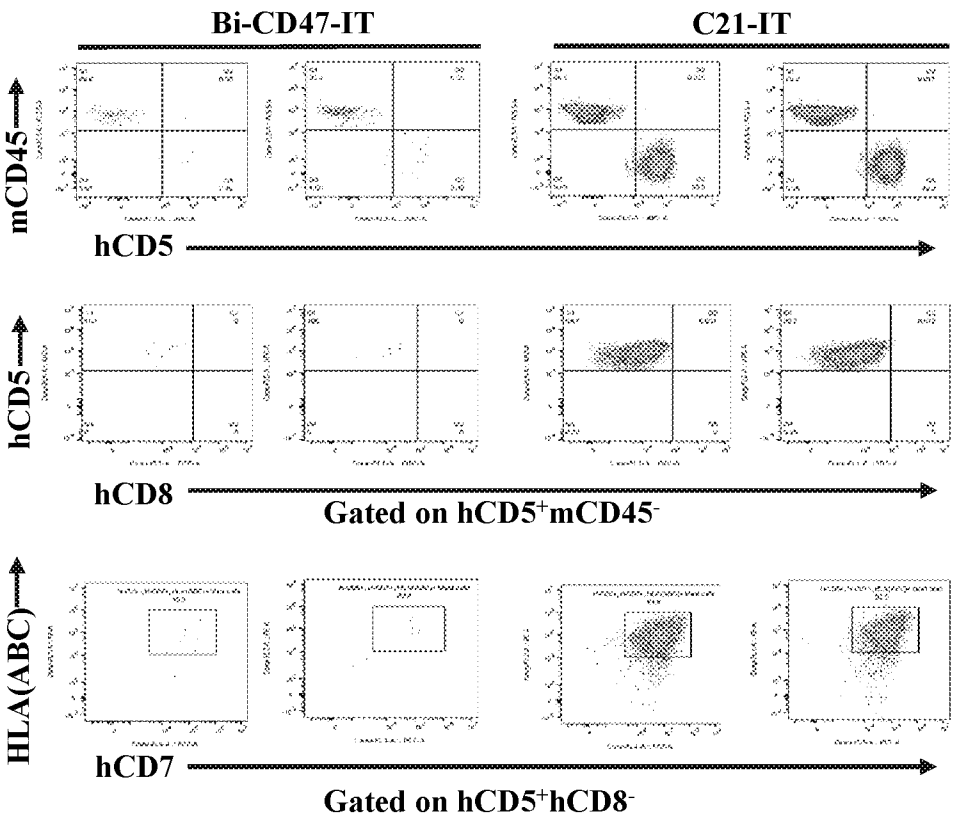

Blood, Day 21
Bi-CD47-IT
C21-IT
mCD45
hCD5
hCD5
hCD8
Gated on hCD5+mCD45-
HLA(ABC)
hCD7
Gated on hCD5+hCD8-

FIG. 8C

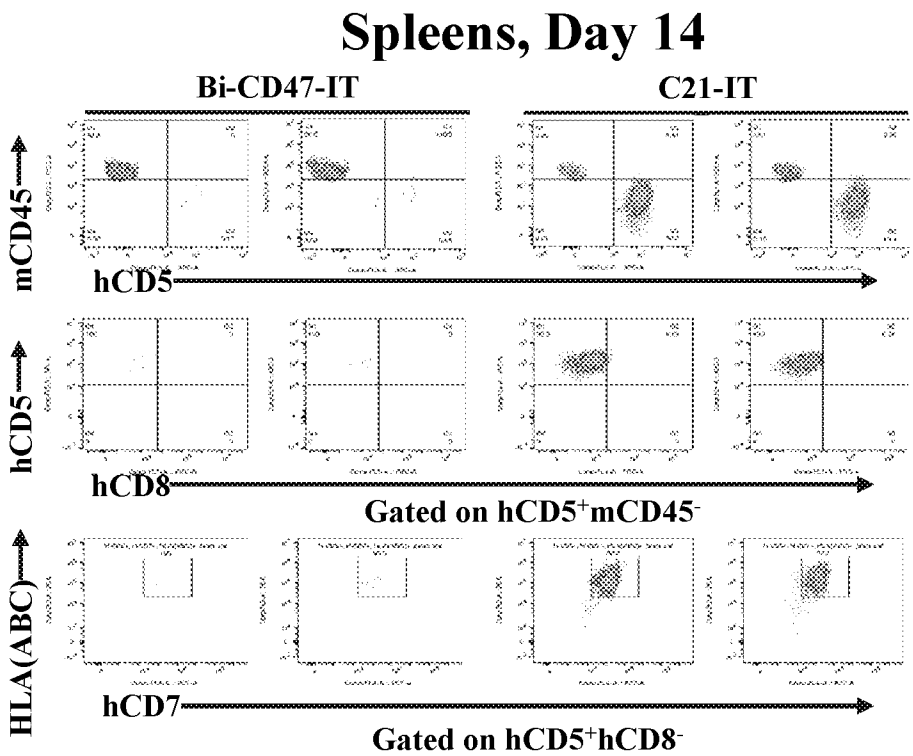

Spleens, Day 14
Bi-CD47-IT
C21-IT
mCD45
hCD5
hCD5
hCD8
Gated on hCD5+mCD45-
HLA(ABC)
hCD7
Gated on hCD5+hCD8-

FIG. 8D

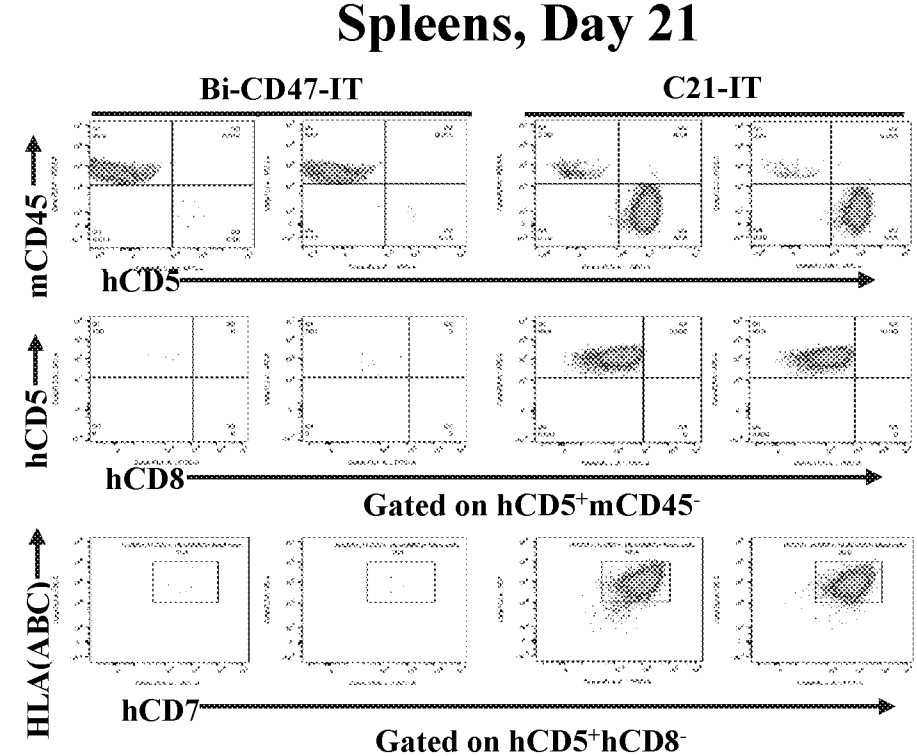

Spleens, Day 21
Bi-CD47-IT
C21-IT
mCD45
hCD5
hCD5
hCD8
Gated on hCD5+mCD45-
HLA(ABC)
hCD7
Gated on hCD5+hCD8-

FIG. 8E

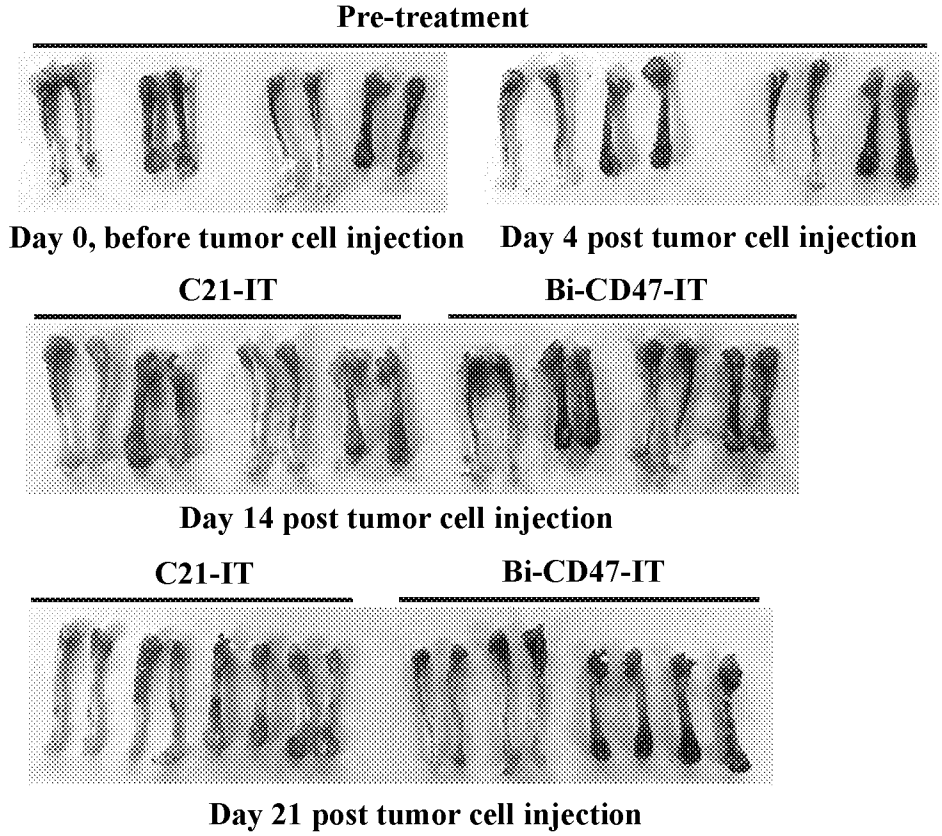

Pre-treatment
Day 0, before tumor cell injection
Day 4 post tumor cell injection
C21-IT
Bi-CD47-IT
Day 14 post tumor cell injection
C21-IT
Bi-CD47-IT
Day 21 post tumor cell injection

FIG. 8F

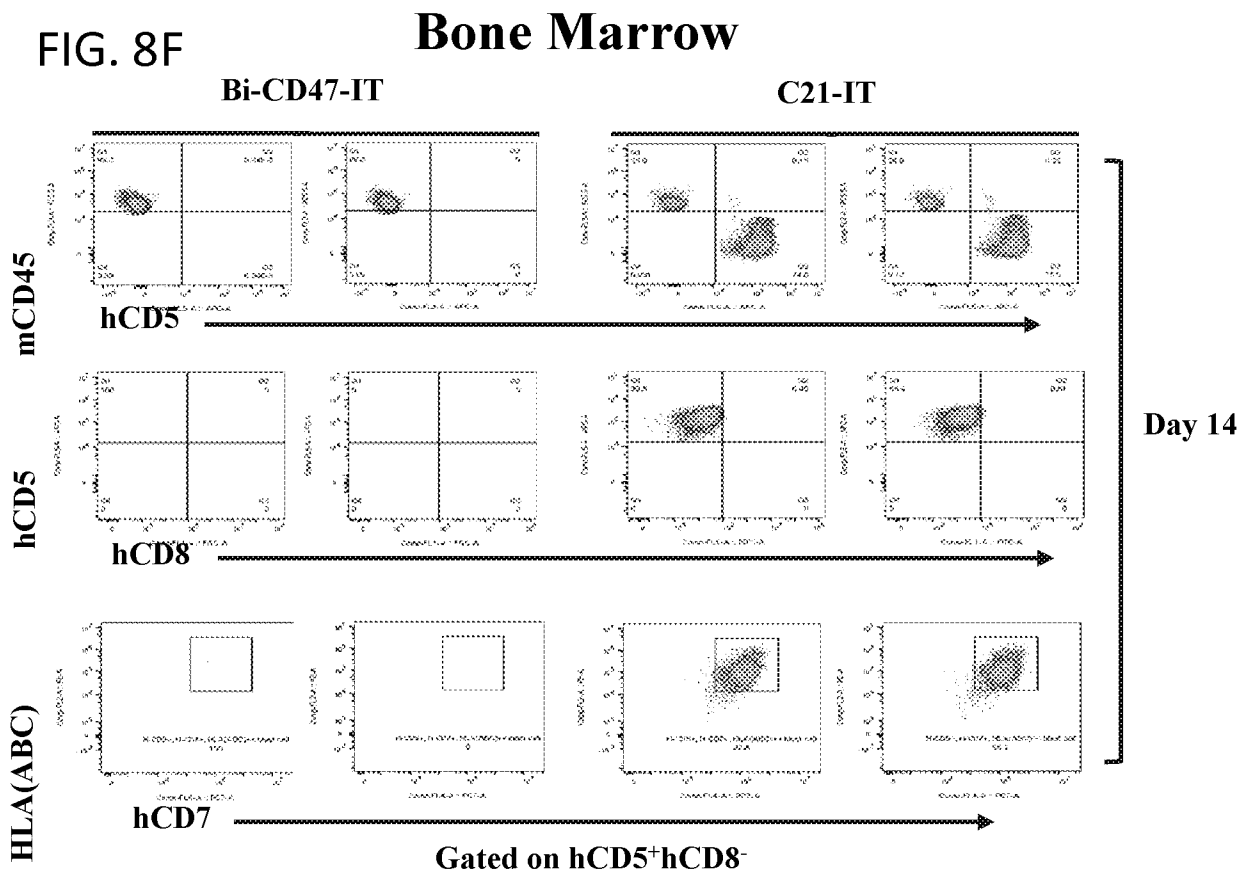

Bone Marrow
Bi-CD47-IT
C21-IT
mCD45
hCD5
hCD5
hCD8
HLA(ABC)
hCD7
Day 14
Gated on hCD5+hCD8-

FIG. 8G

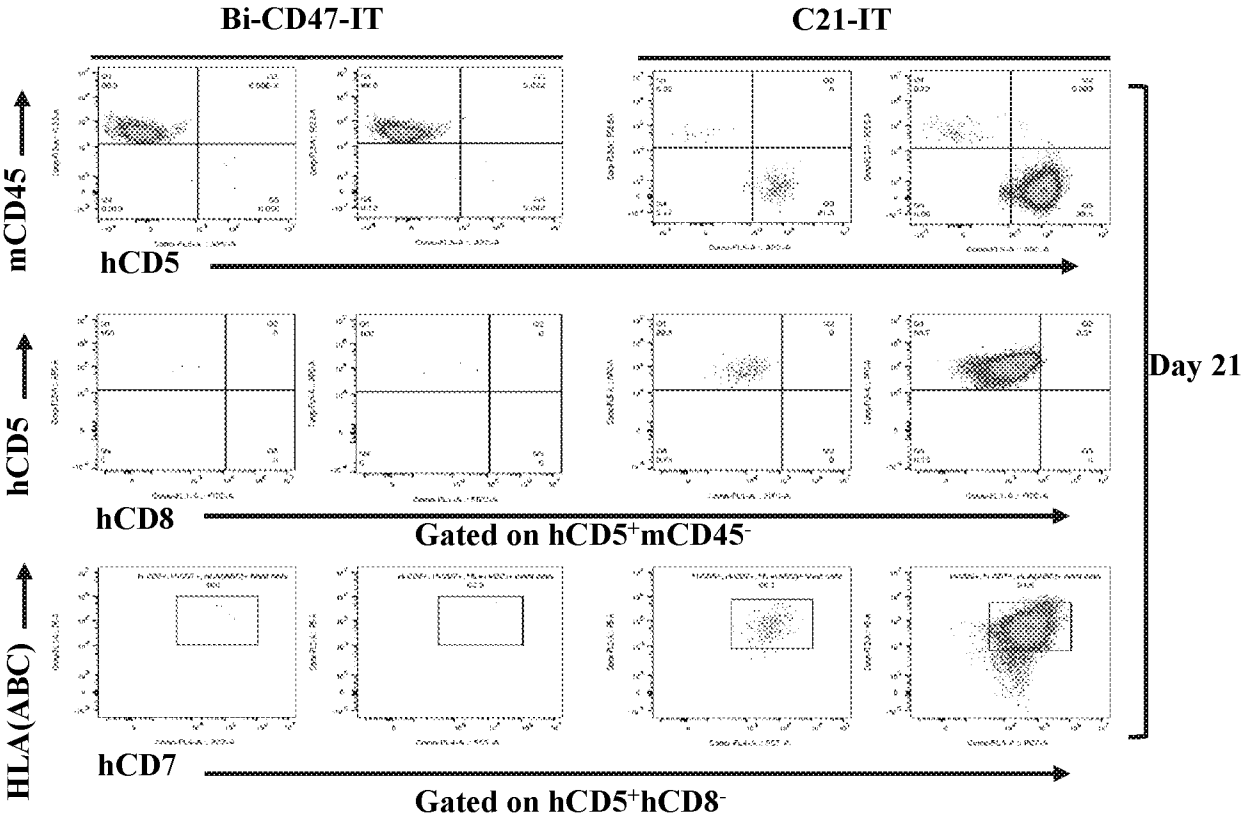

Bi-CD47-IT
C21-IT
mCD45
hCD5
hCD5
hCD8
Gated on hCD5+mCD45-
Day 21
HLA(ABC)
hCD7
Gated on hCD5+hCD8-

FIG. 8H

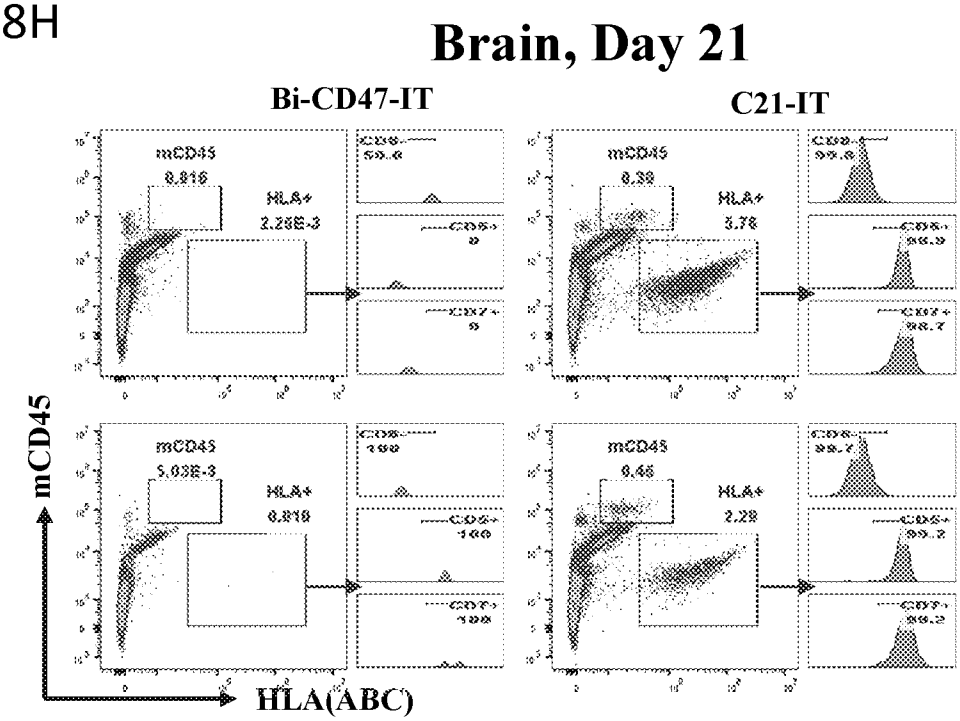

Brain, Day 21
Bi-CD47-IT
C21-IT
mCD45
HLA+
HLA(ABC)

FIG. 8I

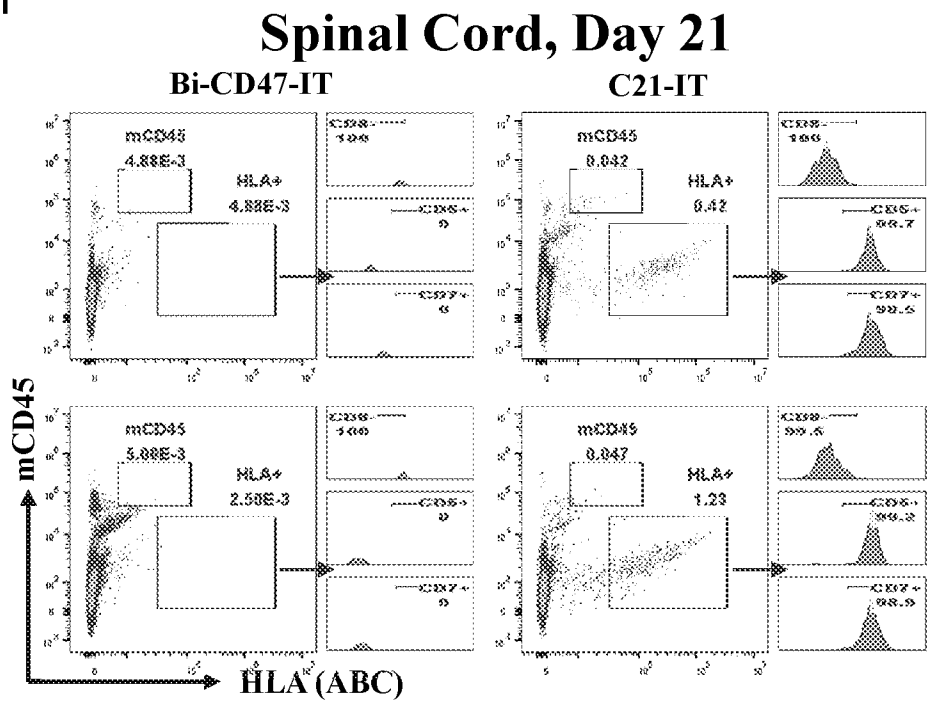
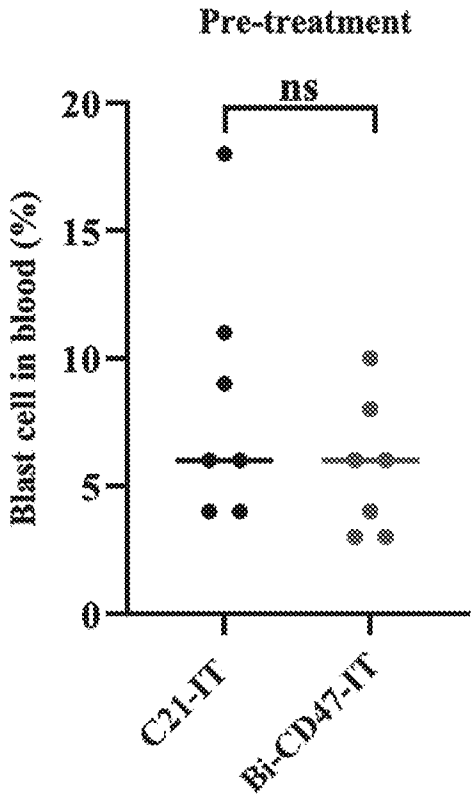

Spinal Cord, Day 21
Bi-CD47-IT
C21-IT
mCD45
HLA (ABC)
mCD45
4.88E-3
HLA+
4.88E-3
mCD45
8.042
HLA+
0.42
mCD45
5.08E-3
HLA+
2.58E-3
mCD45
0.047
HLA+
1.23

FIG. 9A


Pre-treatment
ns
Blast cell in blood (%)
20
15
10
5
0
C21-IT
Bi-CD47-IT

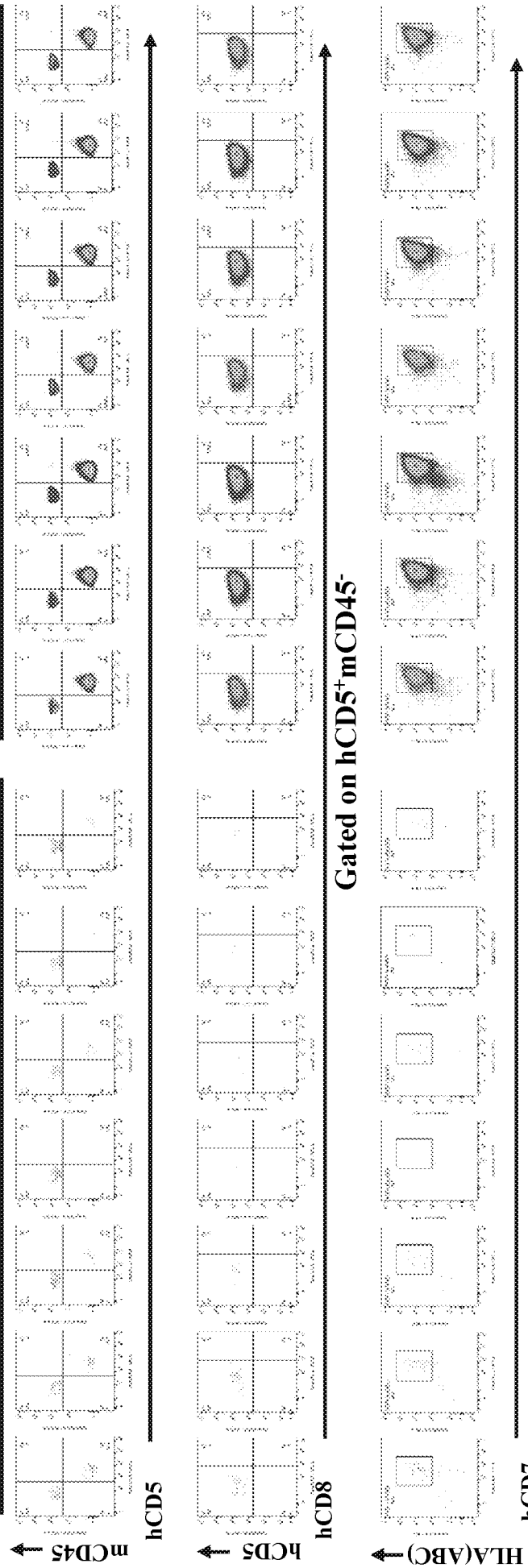

Bi-CD47-IT, Day 21
C21-IT, Day 21
Gated on hCD5+mCD45-
Gated on hCD5+hCD8-
mCD45
hCD5
hCD5
hCD8
HLA(ABC)
hCD7

FIG. 9B

FIG. 9C

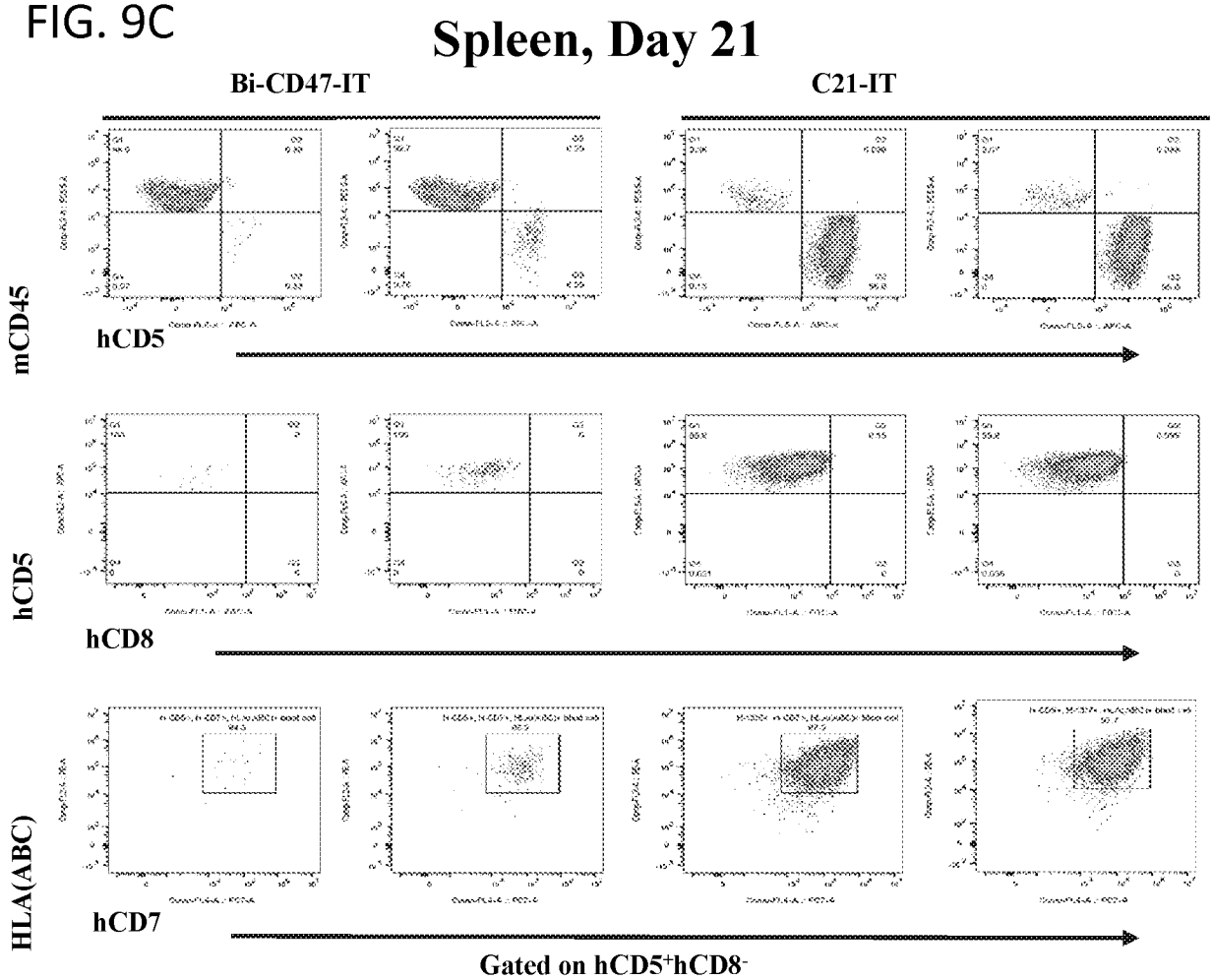

Spleen, Day 21
Bi-CD47-IT
C21-IT
mCD45
hCD5
hCD5
hCD8
HLA(ABC)
hCD7
Gated on hCD5+hCD8-

FIG. 9D

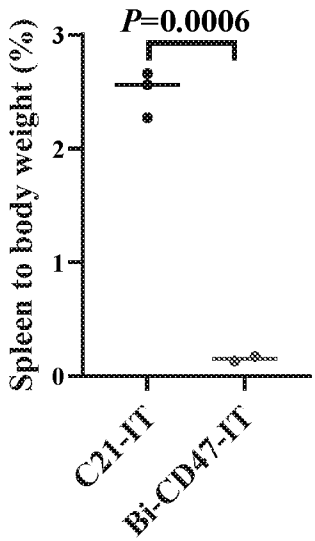

P=0.0006
Spleen to body weight (%)
3
2
1
0
C21-IT
Bi-CD47-IT

FIG. 9E

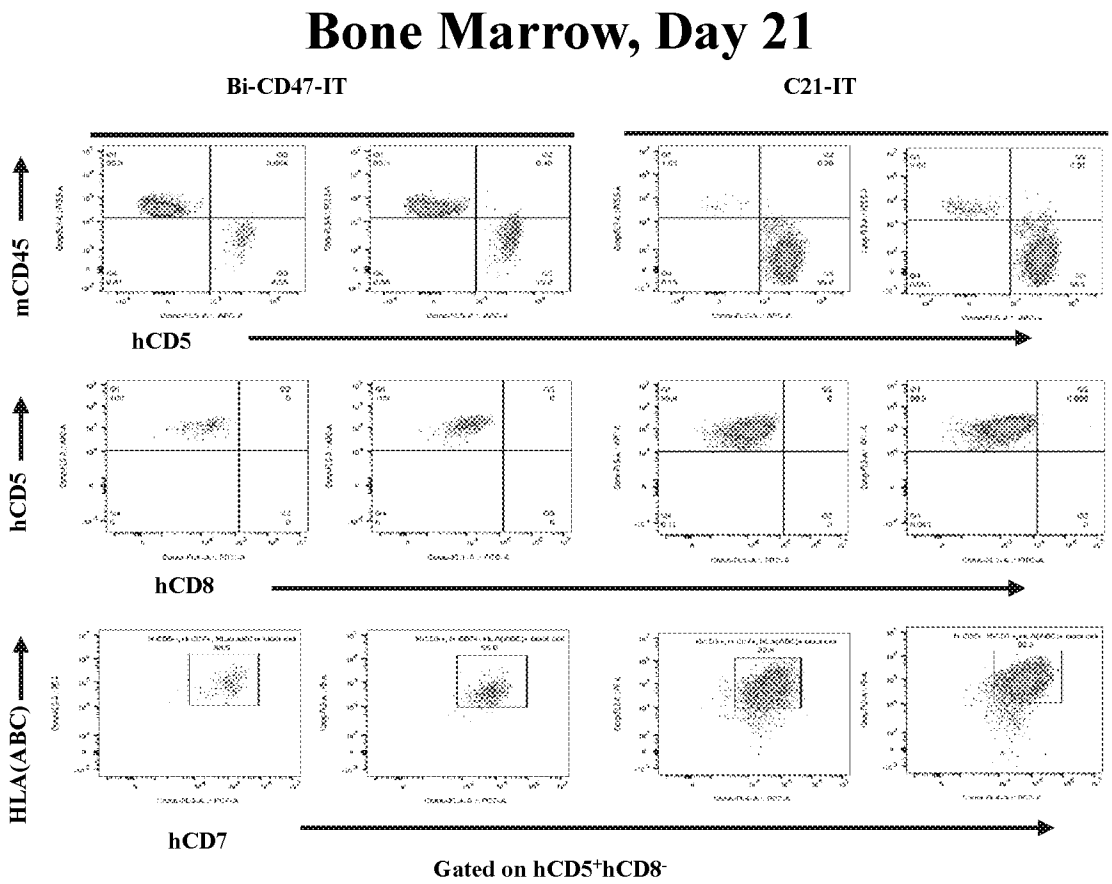

Bone Marrow, Day 21
Bi-CD47-IT
C21-IT
mCD45
hCD5
hCD5
hCD8
HLA(ABC)
hCD7
Gated on hCD5+hCD8-

FIG. 9F

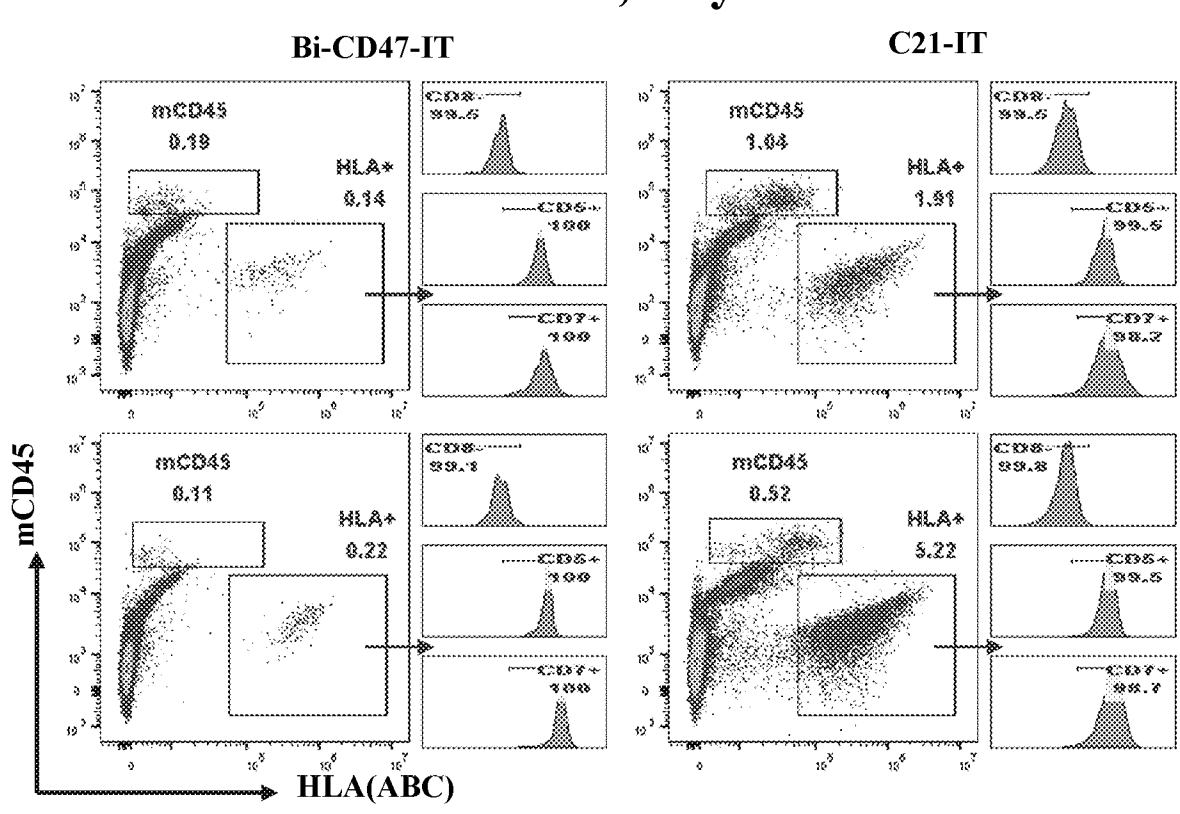

Brain, Day 21
Bi-CD47-IT
C21-IT
mCD45
0.19
HLA+
0.14
mCD45
1.04
HLA+
1.91
mCD45
0.11
HLA+
0.22
mCD45
0.52
HLA+
5.22
mCD45
HLA(ABC)

FIG. 9G

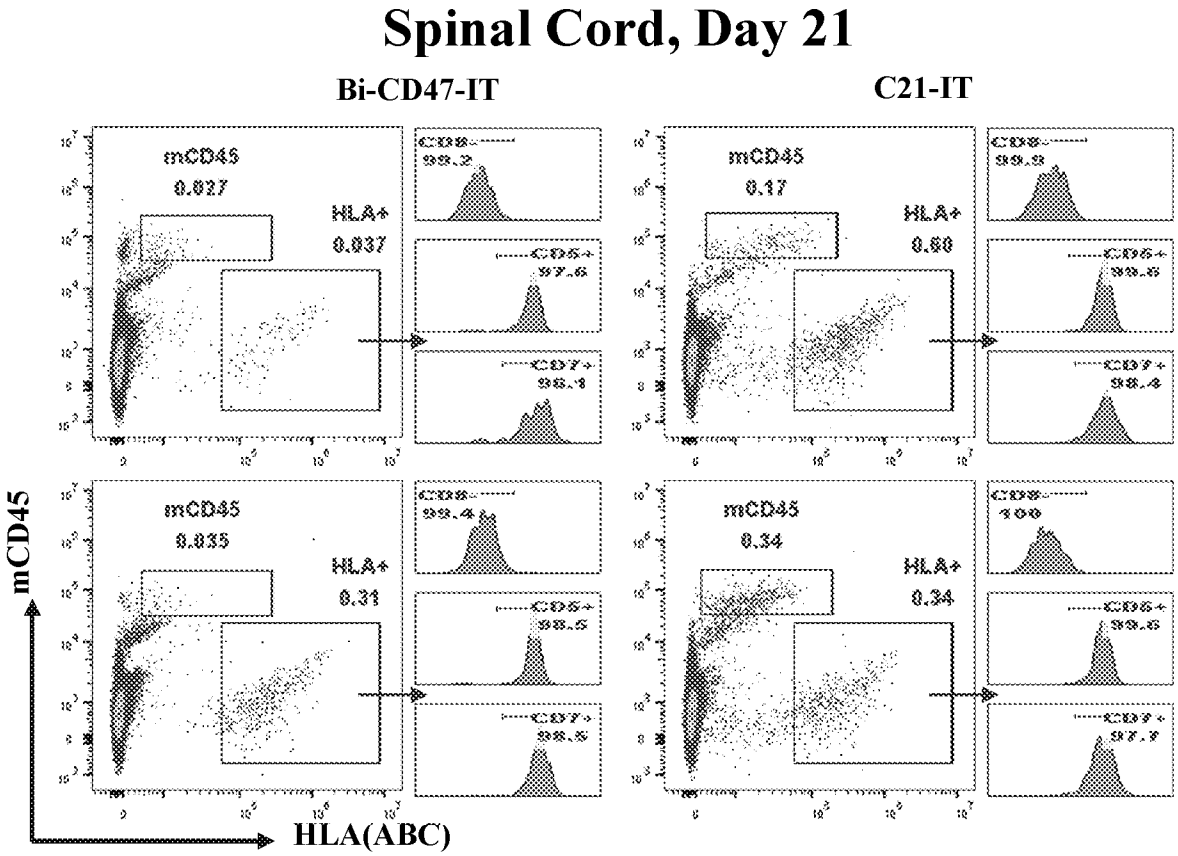
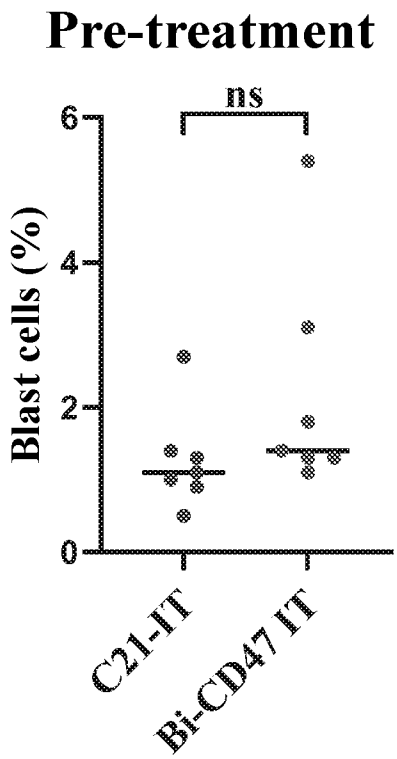


FIG. 10A



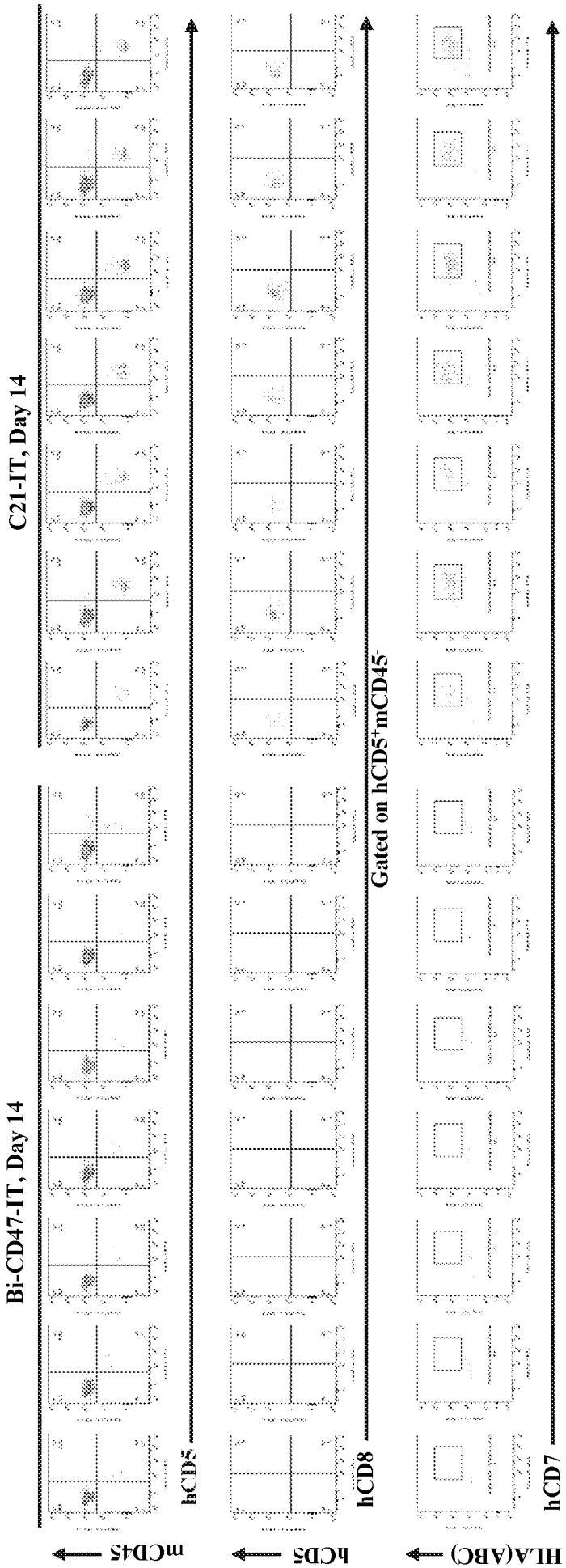

Bi-CD47-IT, Day 14
C21-IT, Day 14
Gated on hCD5+mCD45-
Gated on hCD5+hCD8-
mCD45
hCD5
HLA(ABC)
hCD8
hCD7

FIG. 10B

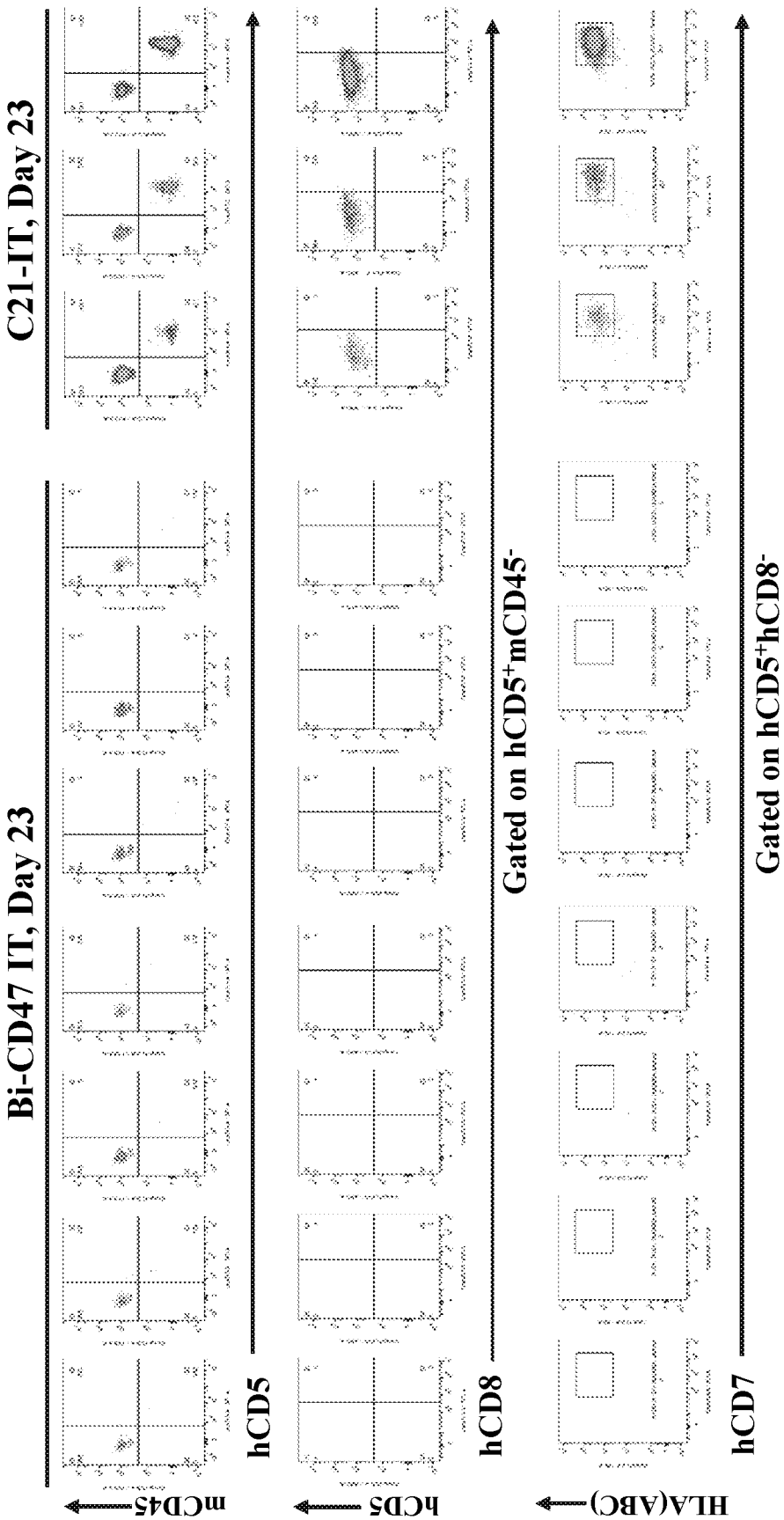

Bi-CD47 IT, Day 23
C21-IT, Day 23
mCD45
hCD5
hCD5
hCD8
HLA(ABC)
hCD7
Gated on hCD5+mCD45-
Gated on hCD5+hCD8-

FIG. 10C

FIG. 10D

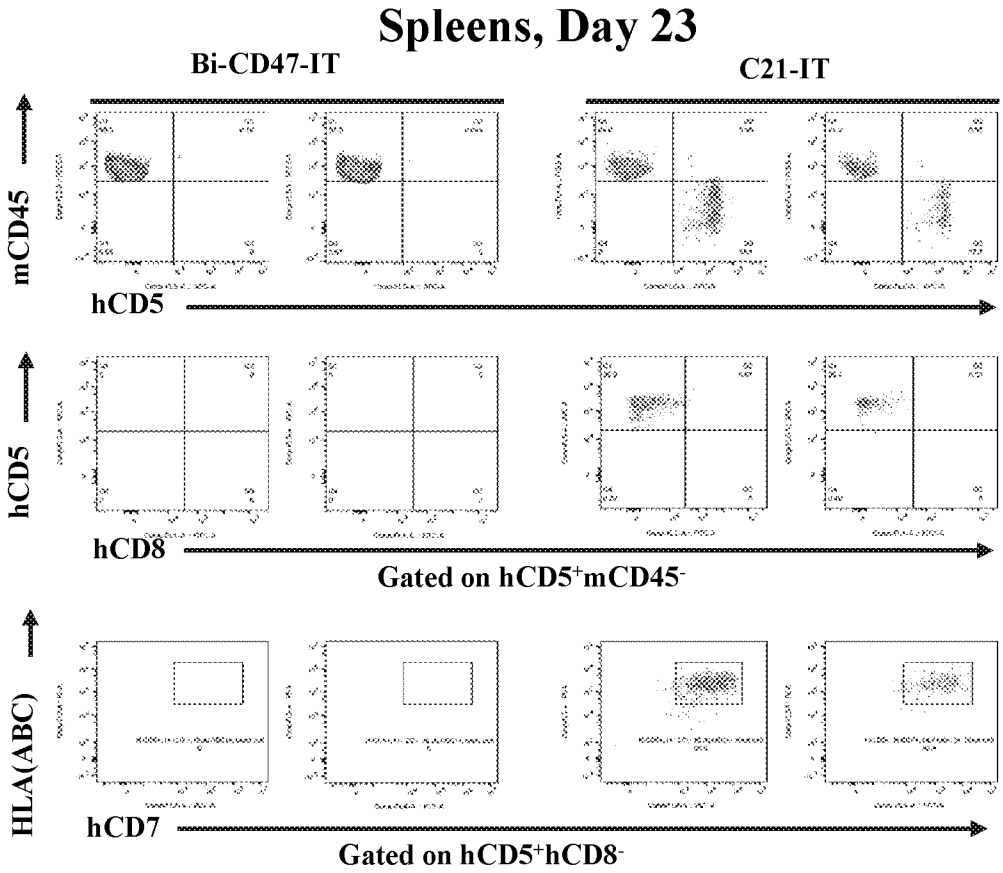


FIG. 10E

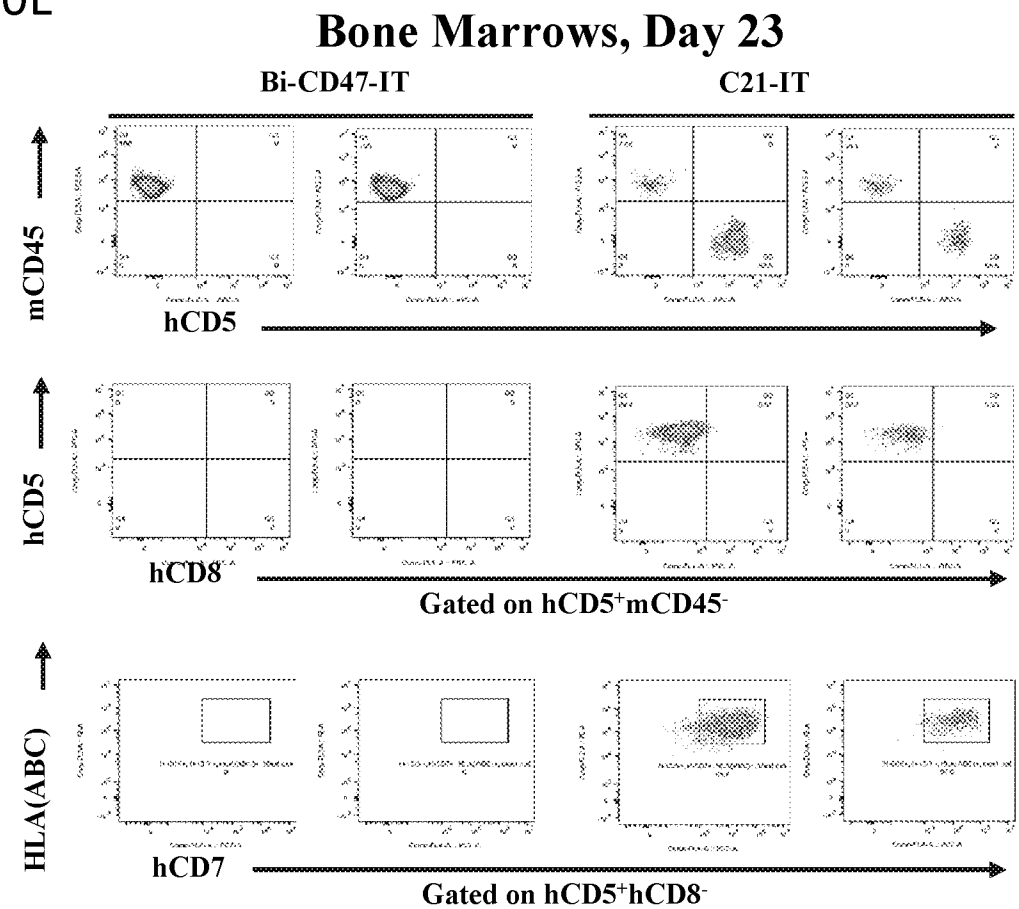


FIG. 10F

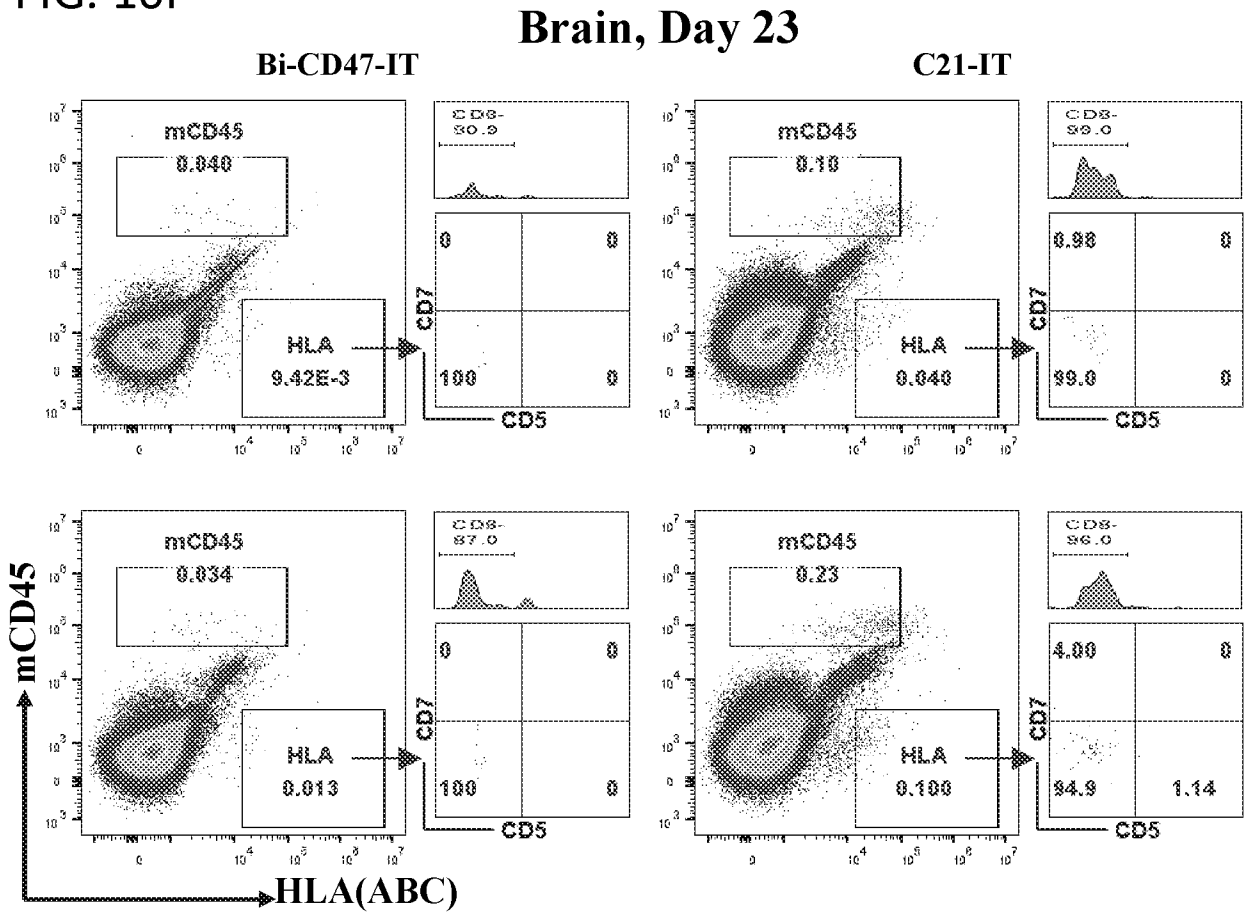
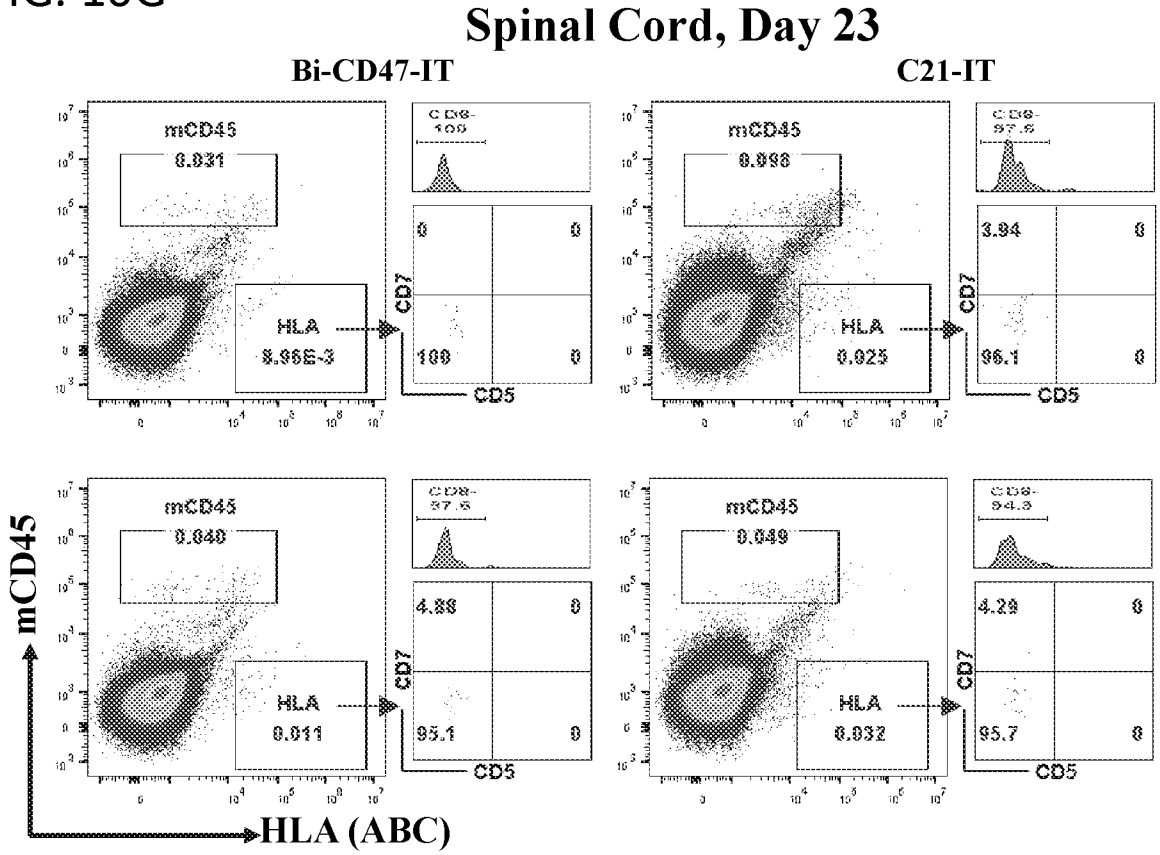


FIG. 10G



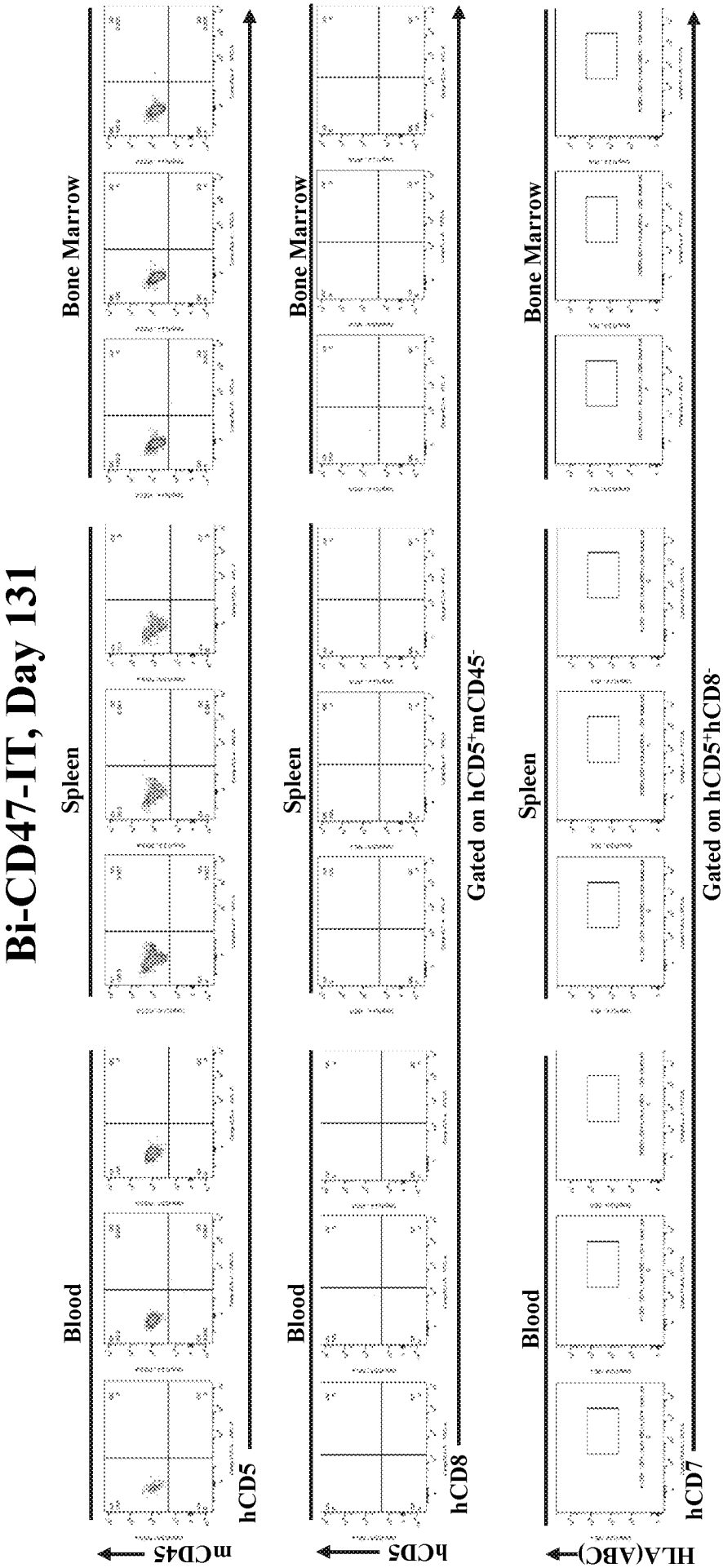

Bi-CD47-IT, Day 131
Blood
Spleen
Bone Marrow
mCD45
hCD5
hCD5
hCD8
HLA(ABC)
hCD7
Gated on hCD5+mCD45-
Gated on hCD5+hCD8-

FIG. 10H

FIG. 11A

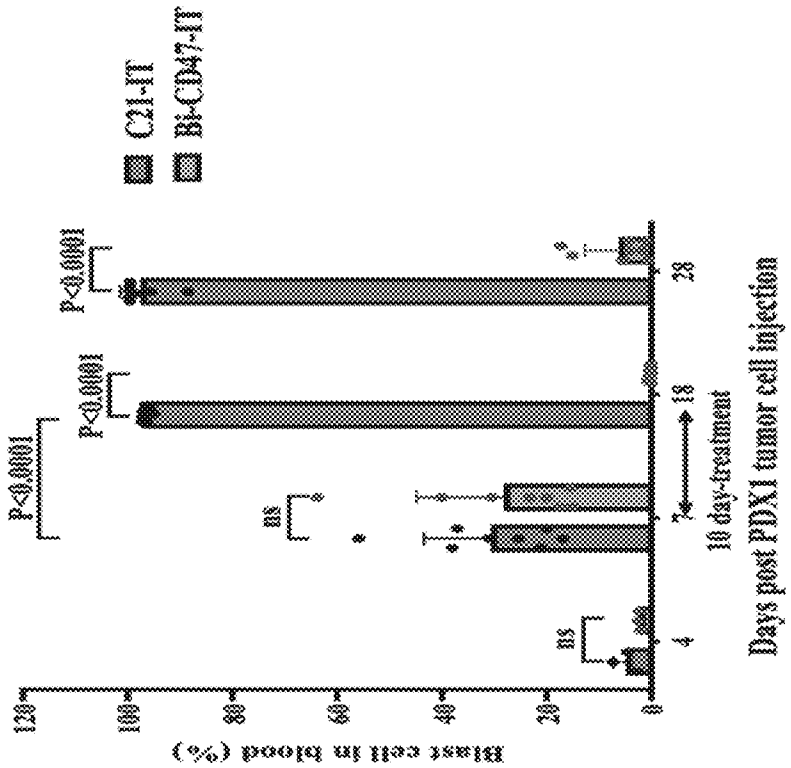
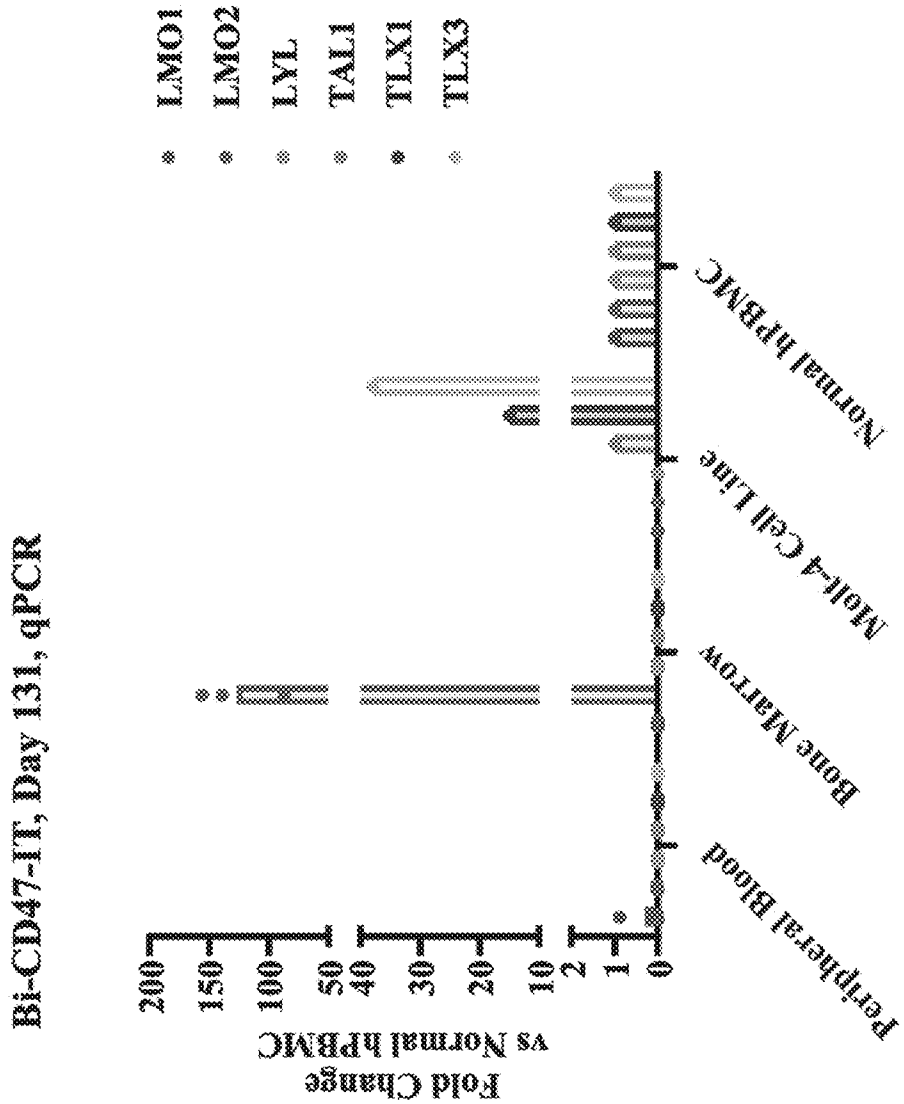


FIG. 10I



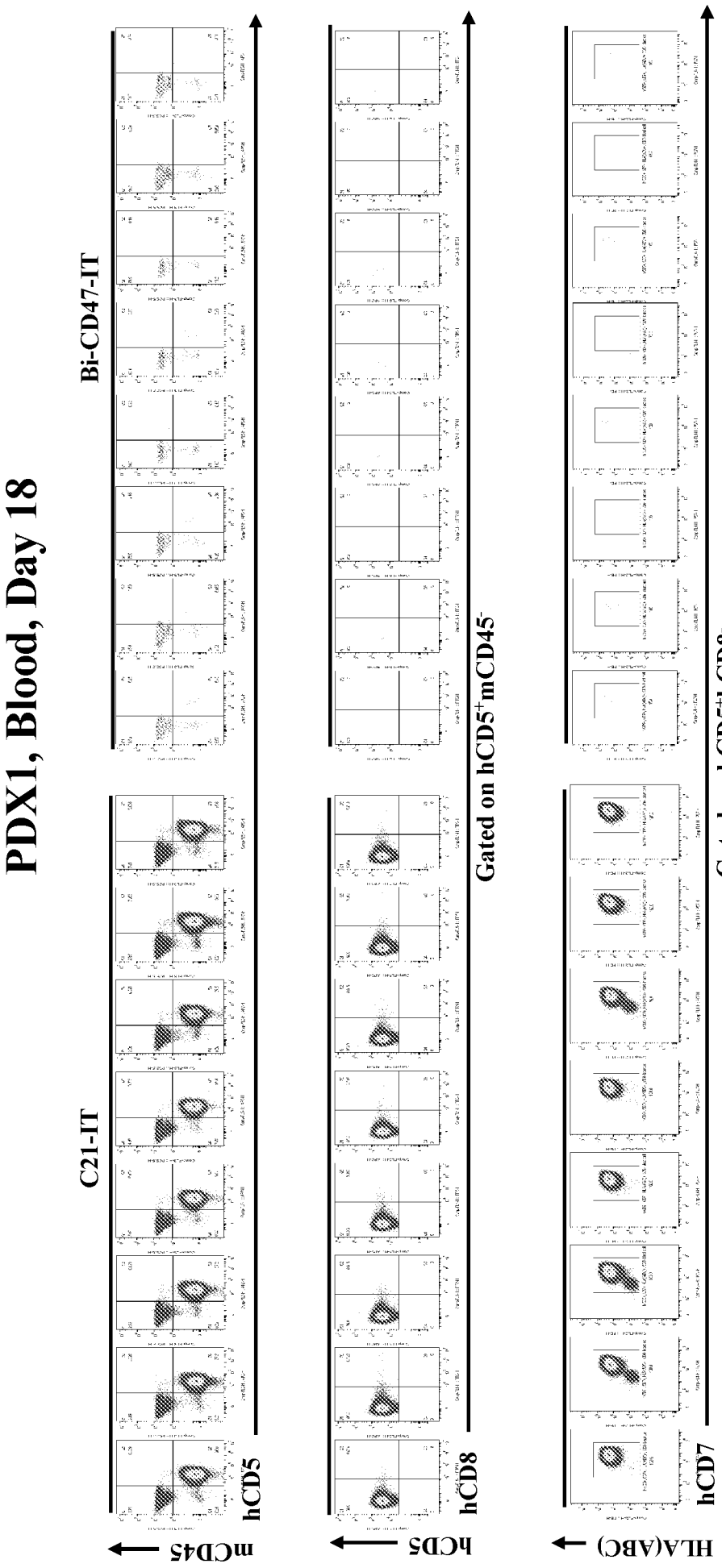

PDX1, Blood, Day 18
C21-IT
Bi-CD47-IT
mCD45
hCD5
hCD8
hCD5
Gated on hCD5+mCD45-
HLA(ABC)
hCD7
Gated on hCD5+hCD8-

FIG. 11B

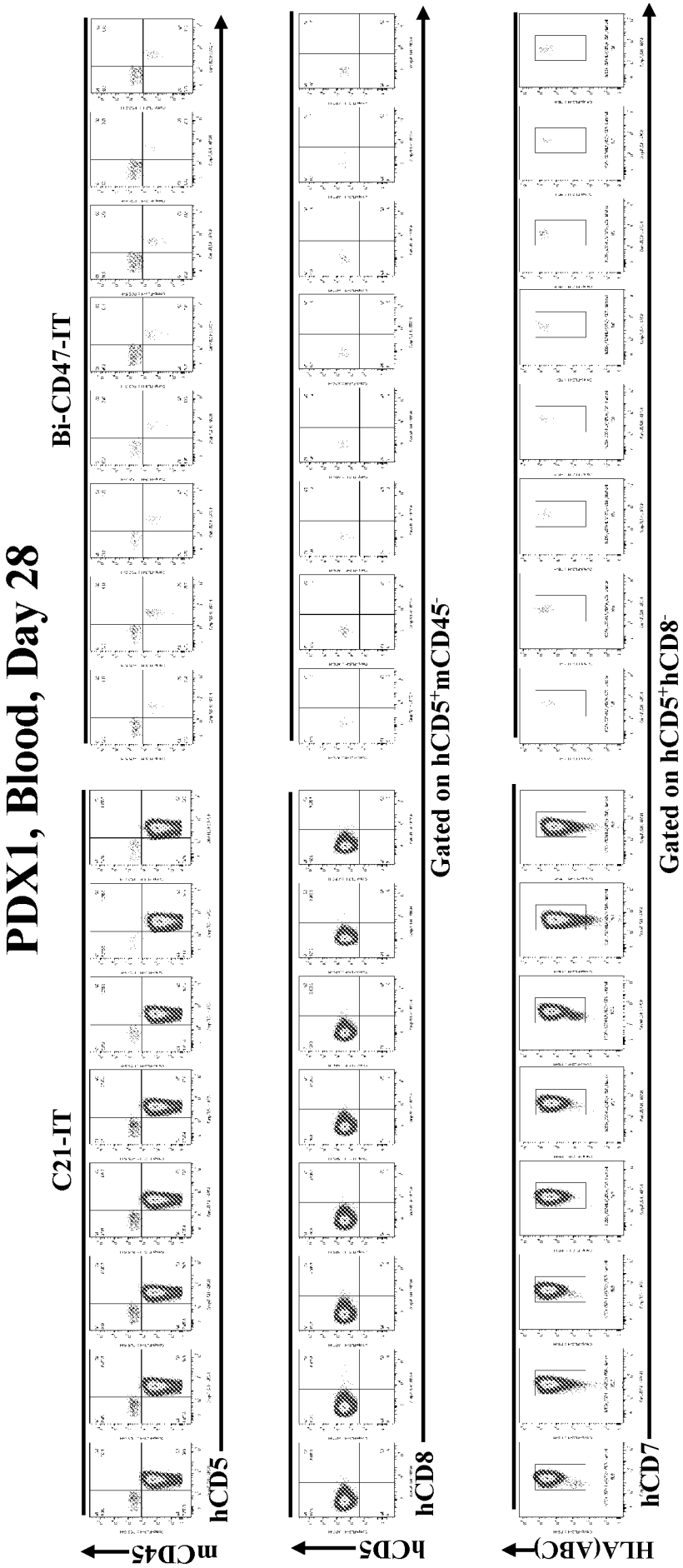

PDX1, Blood, Day 28
C21-IT
Bi-CD47-IT
mCD45
hCD5
hCD5
hCD8
Gated on hCD5+mCD45-
HLA(ABC)
hCD7
Gated on hCD5+hCD8-

FIG. 11C

FIG. 11D

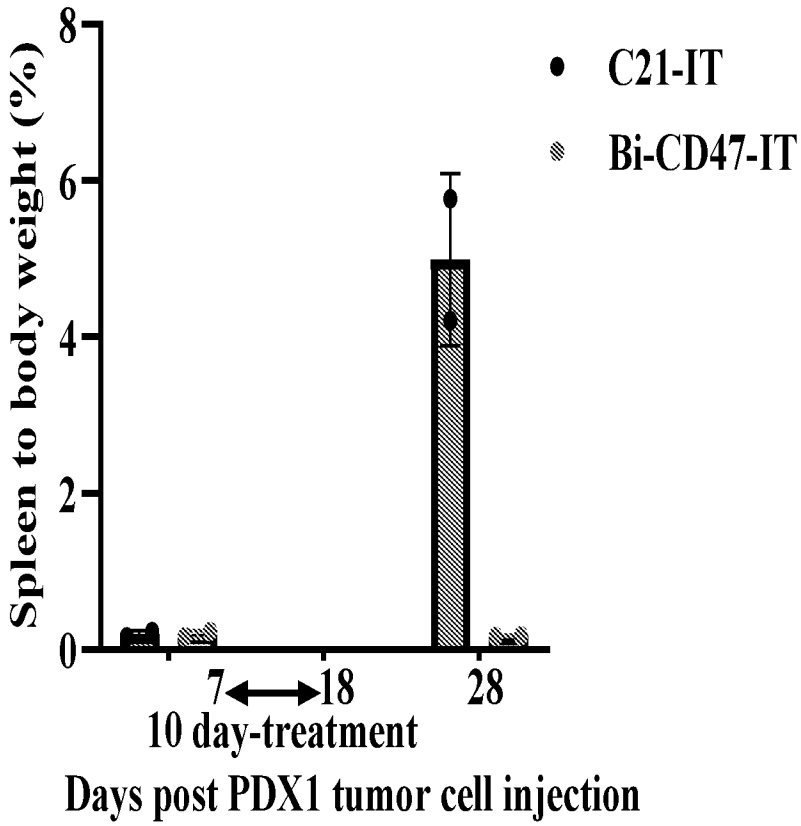

Spleen to body weight (%)
8
6
4
2
0
C21-IT
Bi-CD47-IT
7
18
28
10 day-treatment
Days post PDX1 tumor cell injection

FIG. 11E

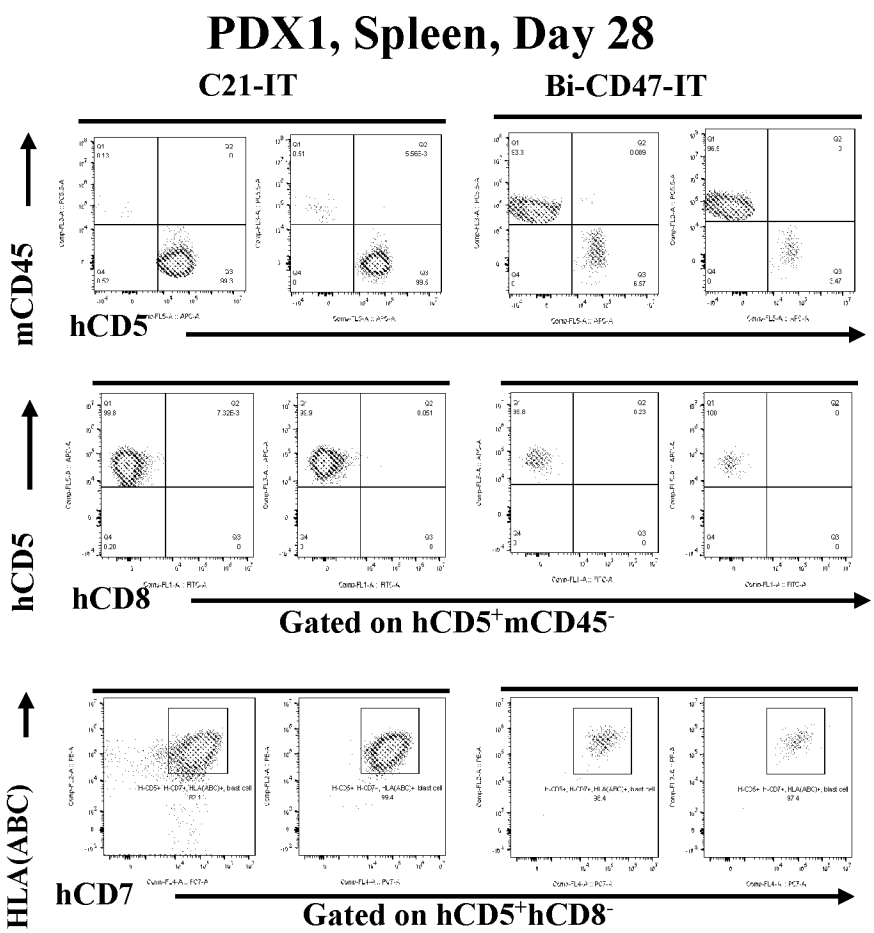


FIG. 11F

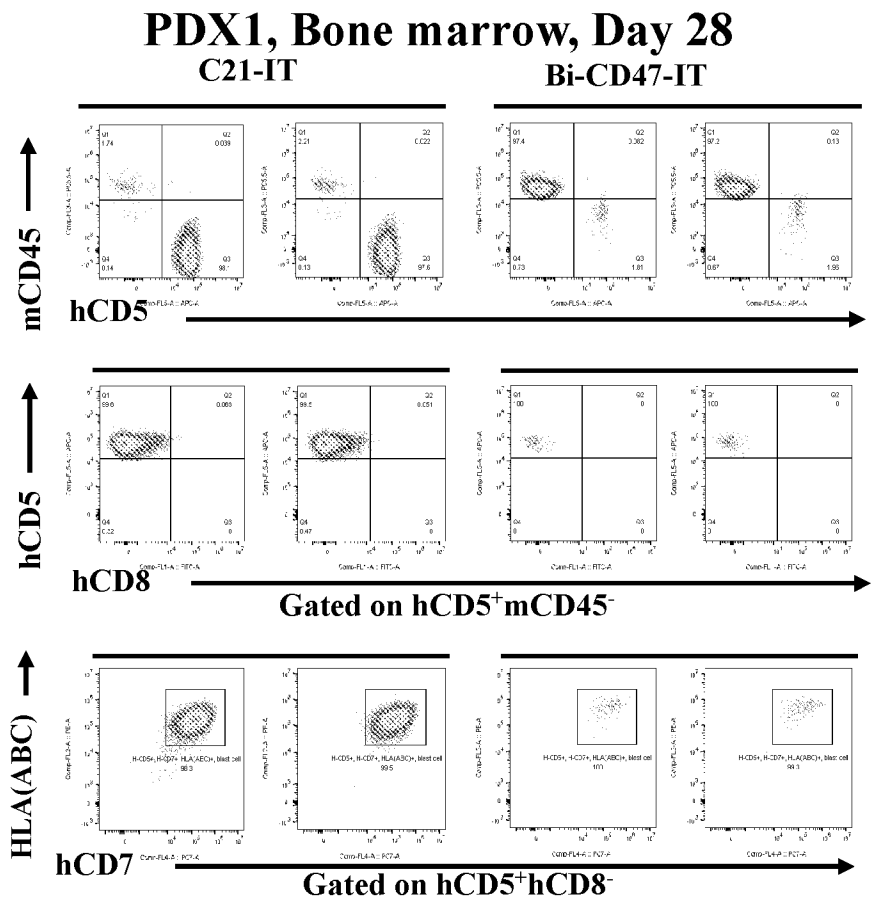


FIG. 11G

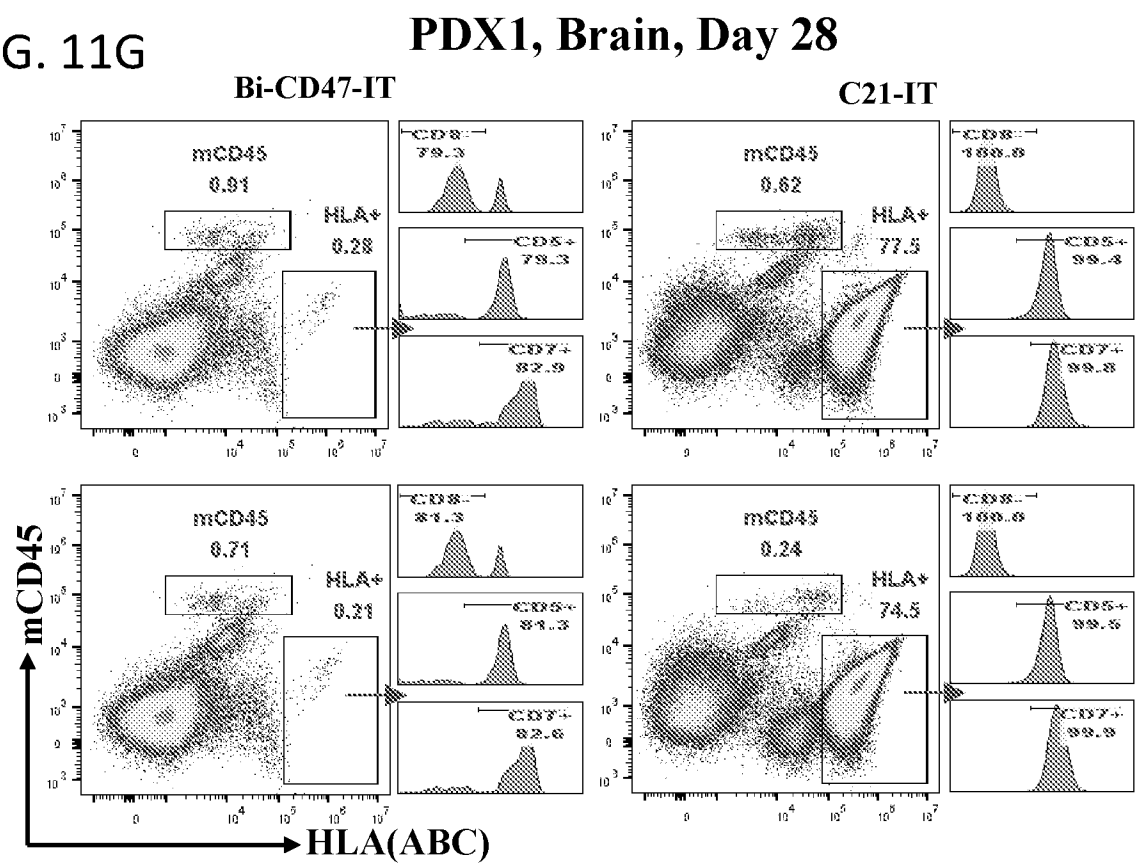
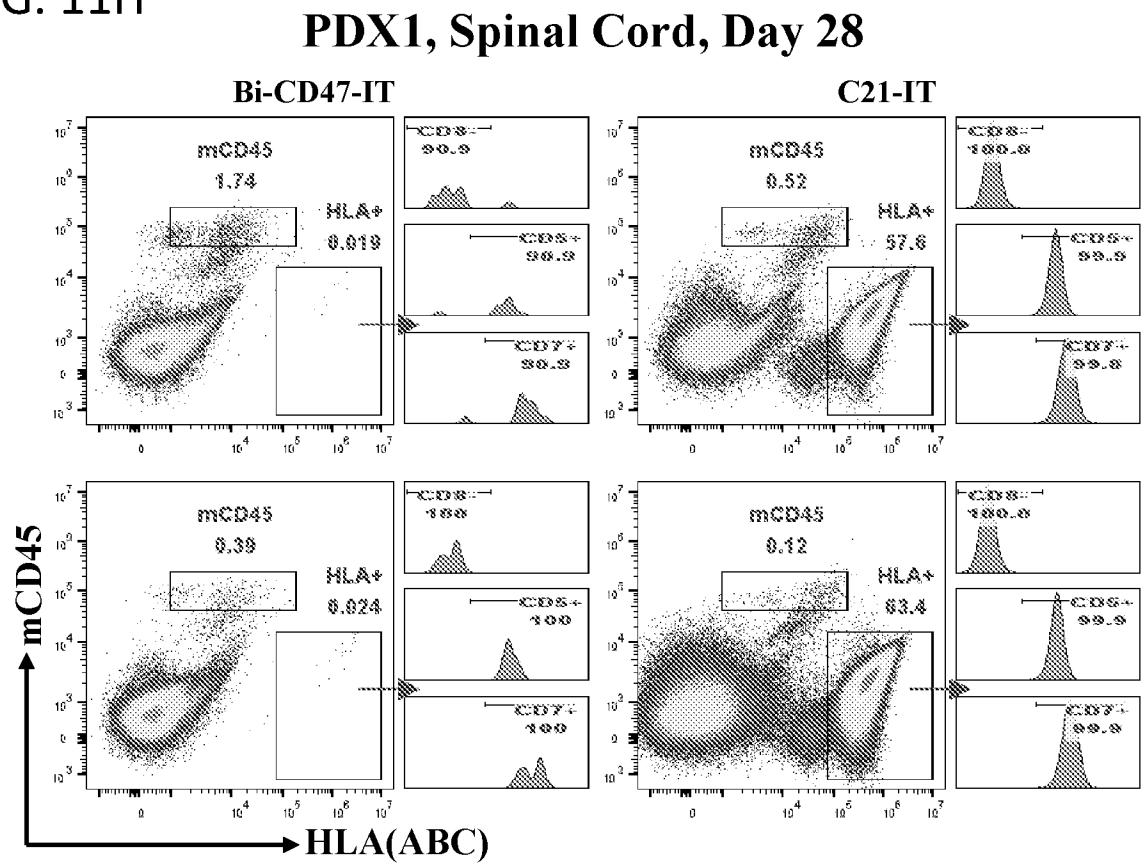


FIG. 11H



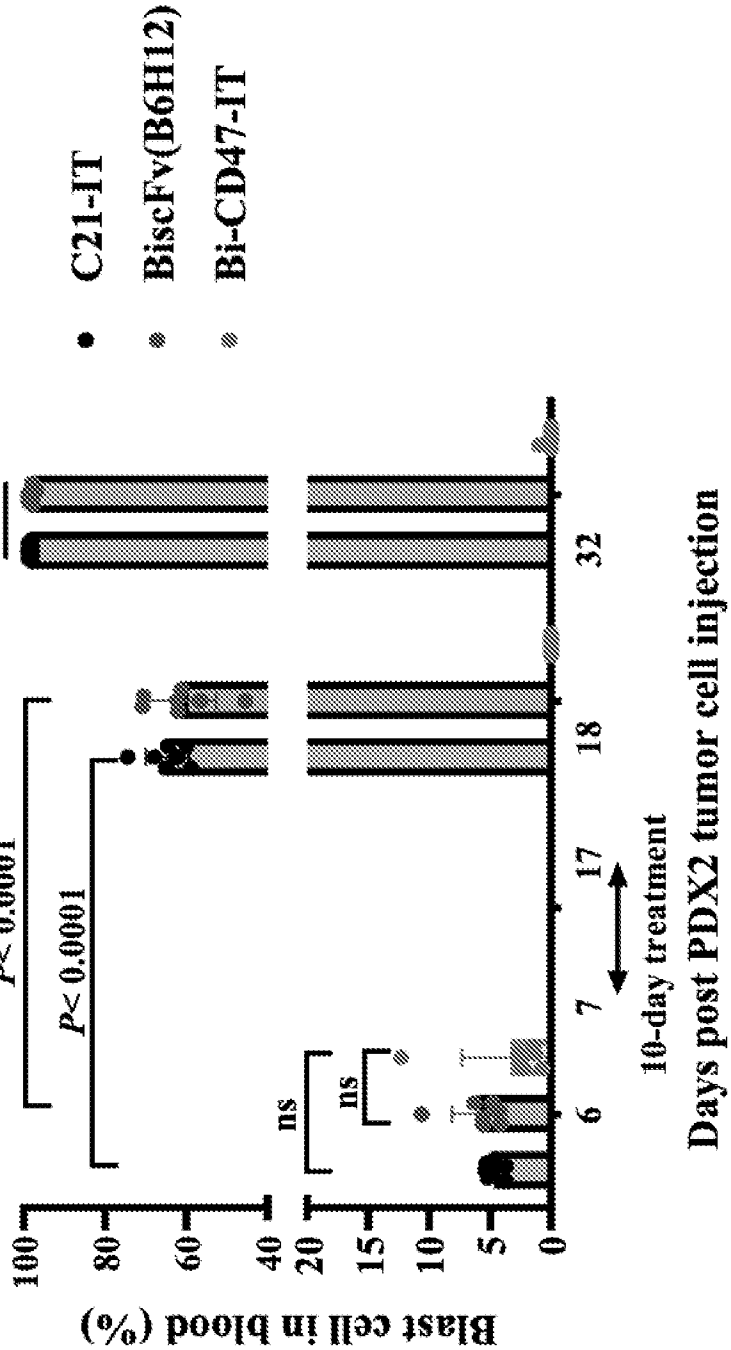

C21-IT
BiscFv(B6H12)
Bi-CD47-IT
P < 0.0001
P< 0.0001
P< 0.0001
ns
ns
Blast cell in blood (%)
100
80
60
40
20
15
10
5
0
6
7
17
18
32
10-day treatment
Days post PDX2 tumor cell injection

FIG. 11I

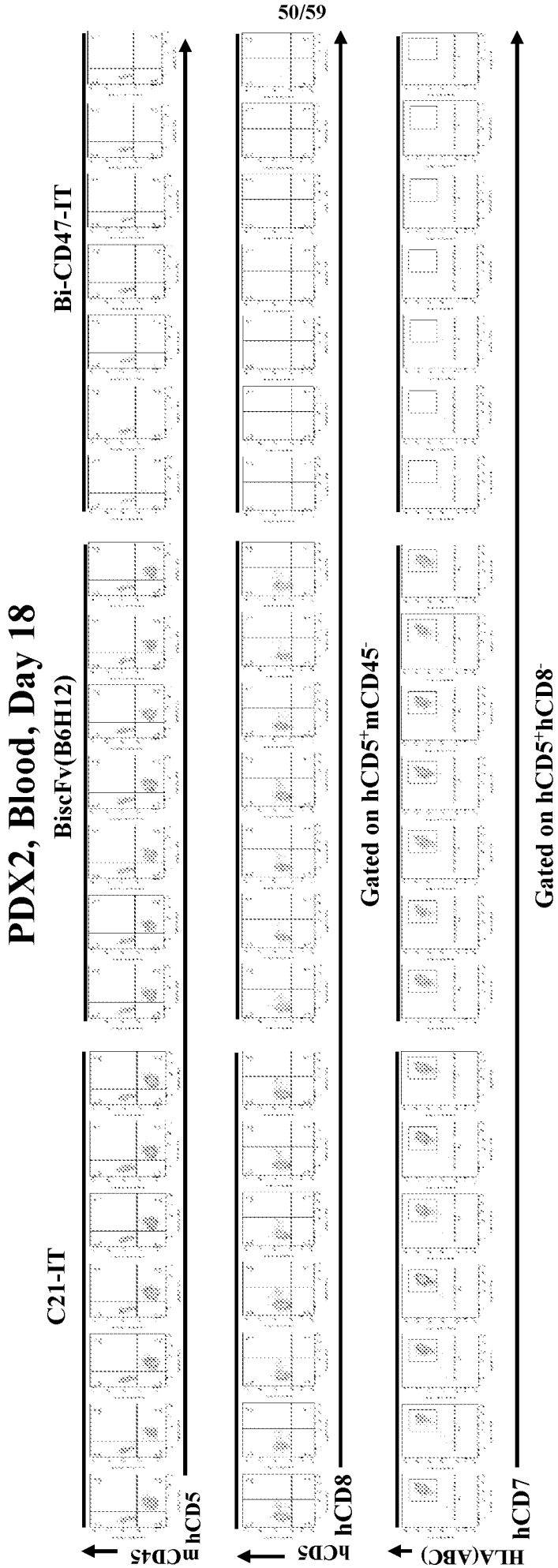

PDX2, Blood, Day 18
C21-IT
BiscFv(B6H12)
Bi-CD47-IT
Gated on hCD5+mCD45-
Gated on hCD5+hCD8-
mCD45
hCD5
hCD5
hCD8
HLA(ABC)
hCD7

FIG. 11J

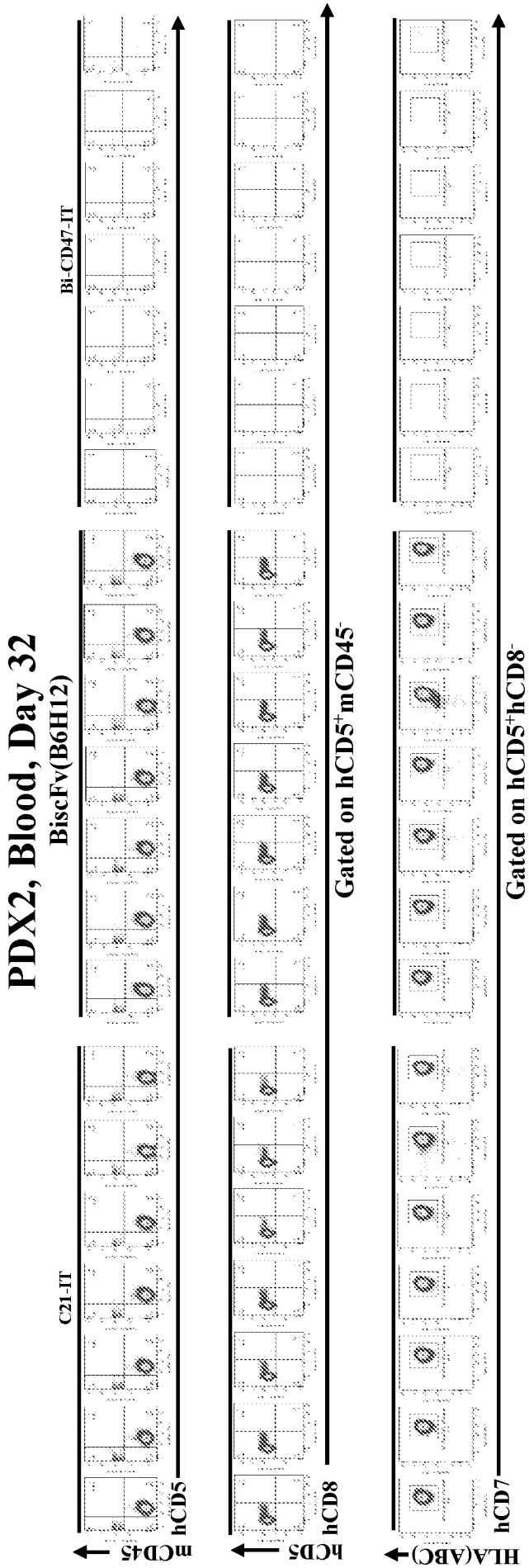

PDX2, Blood, Day 32
BiscFv(B6H12)
C21-IT
Bi-CD47-IT
mCD45
hCD5
hCD8
HLA(ABC)
hCD7
Gated on hCD5+mCD45-
Gated on hCD5+hCD8-

FIG. 11K

FIG. 11L

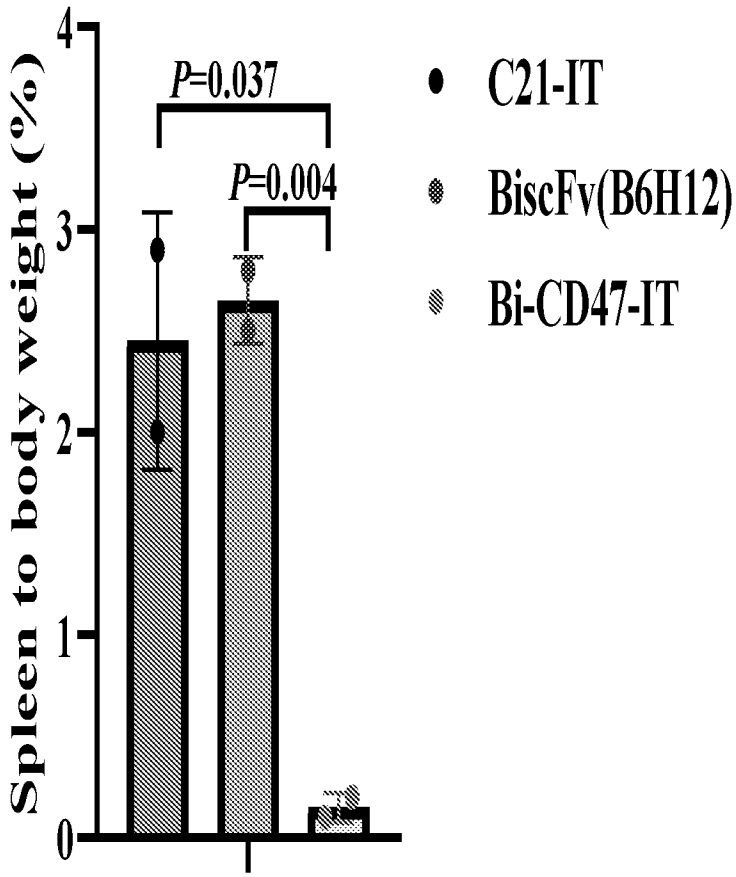

Spleen to body weight (%)
0
1
2
3
4
P=0.037
P=0.004
C21-IT
BiscFv(B6H12)
Bi-CD47-IT

FIG. 11M

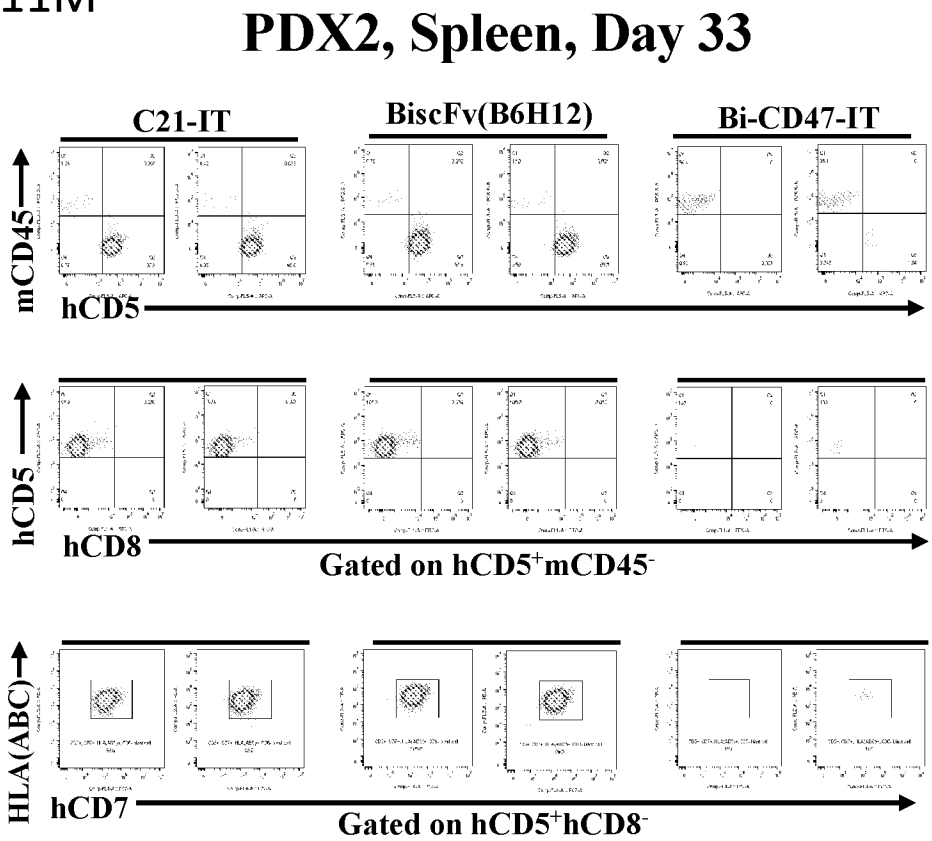
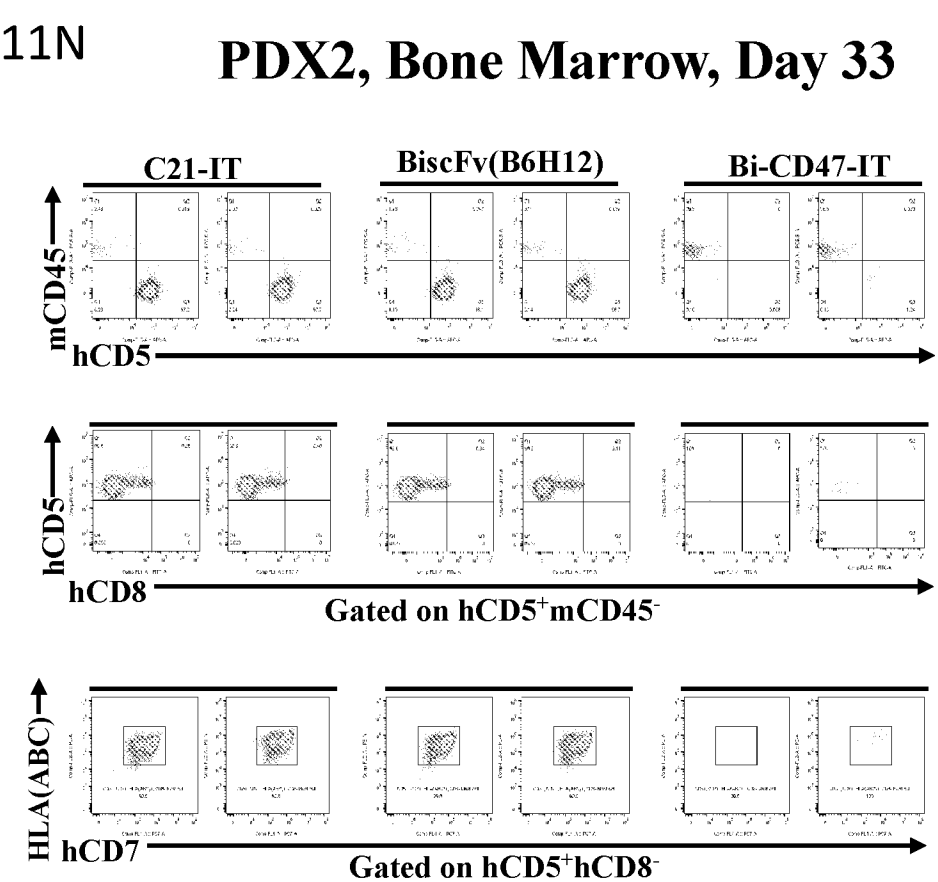


FIG. 11N



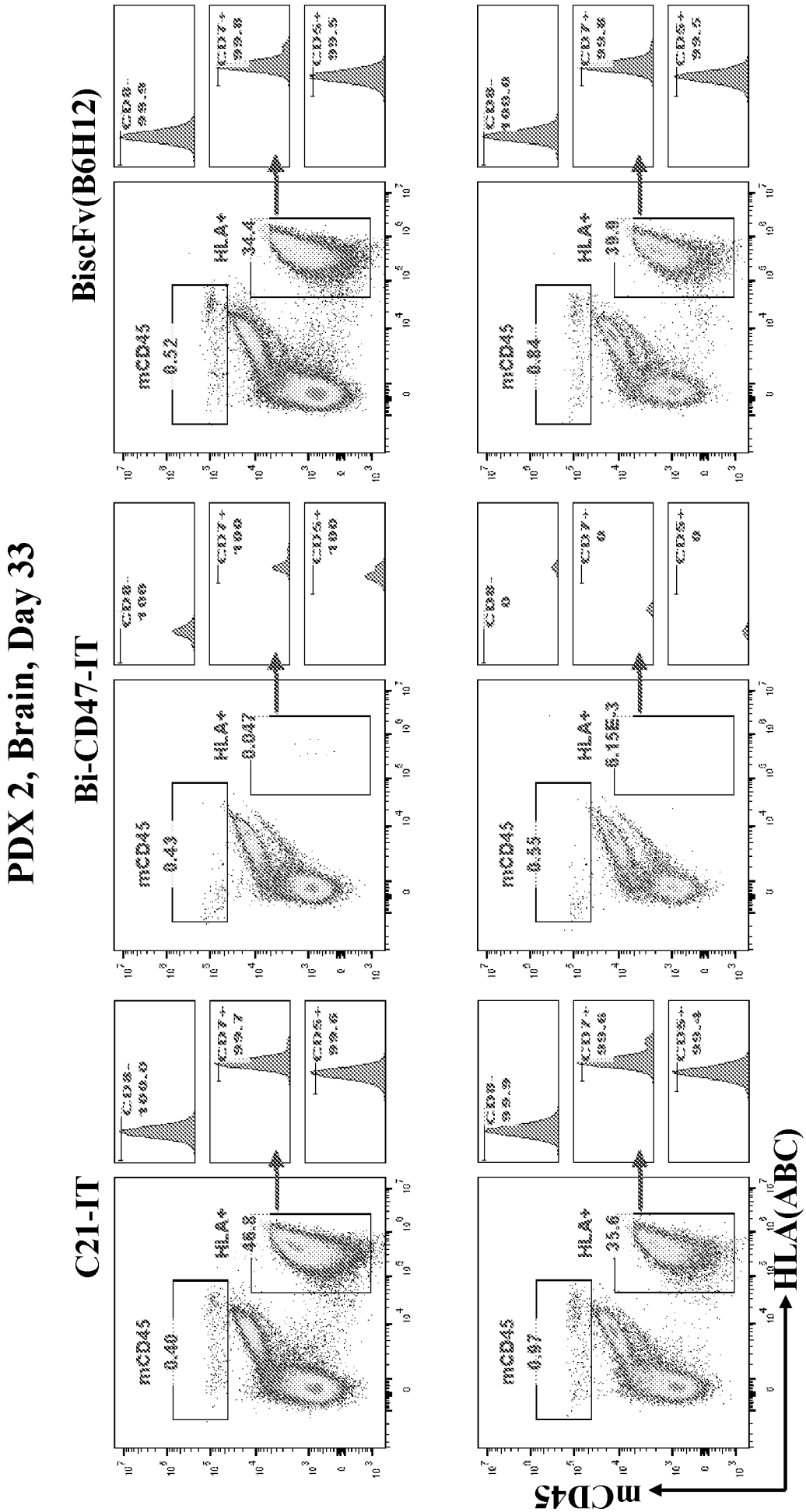


FIG. 11O

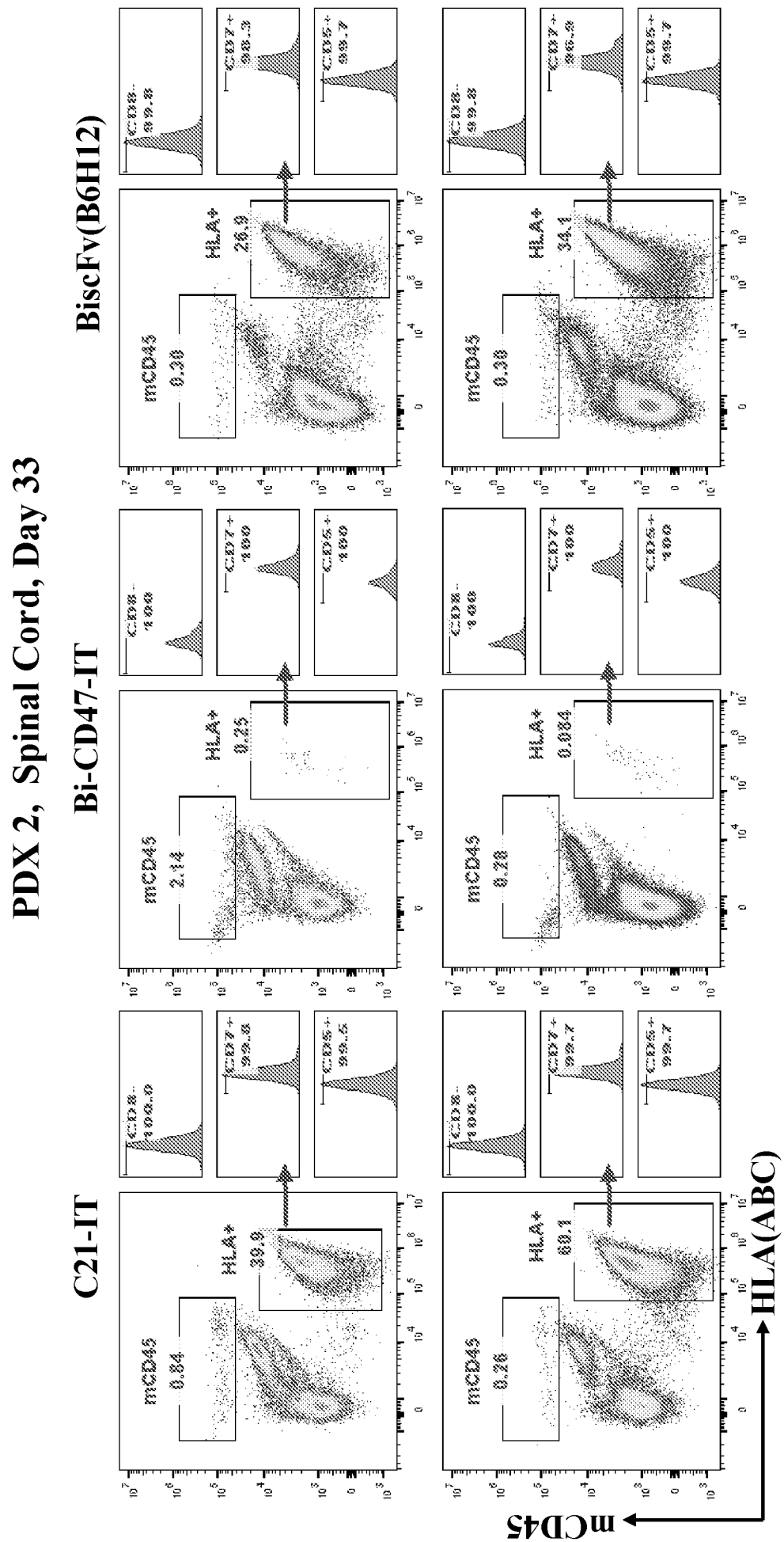

PDX 2, Spinal Cord, Day 33
C21-IT
Bi-CD47-IT
BiscFv(B6H12)
mCD45
HLA(ABC)
mCD45
0.84
HLA+
39.9
CD8-
100.0
CD7+
99.8
CD5+
99.5
mCD45
2.14
HLA+
0.25
CD8-
100
CD7+
100
CD5+
100
mCD45
0.30
HLA+
26.9
CD8-
99.8
CD7+
98.3
CD5+
99.7
mCD45
0.26
HLA+
69.1
CD8-
100.0
CD7+
99.7
CD5+
99.7
mCD45
0.28
HLA+
0.084
CD8-
100
CD7+
100
CD5+
100
mCD45
0.30
HLA+
34.1
CD8-
99.8
CD7+
96.8
CD5+
99.7

FIG. 11P

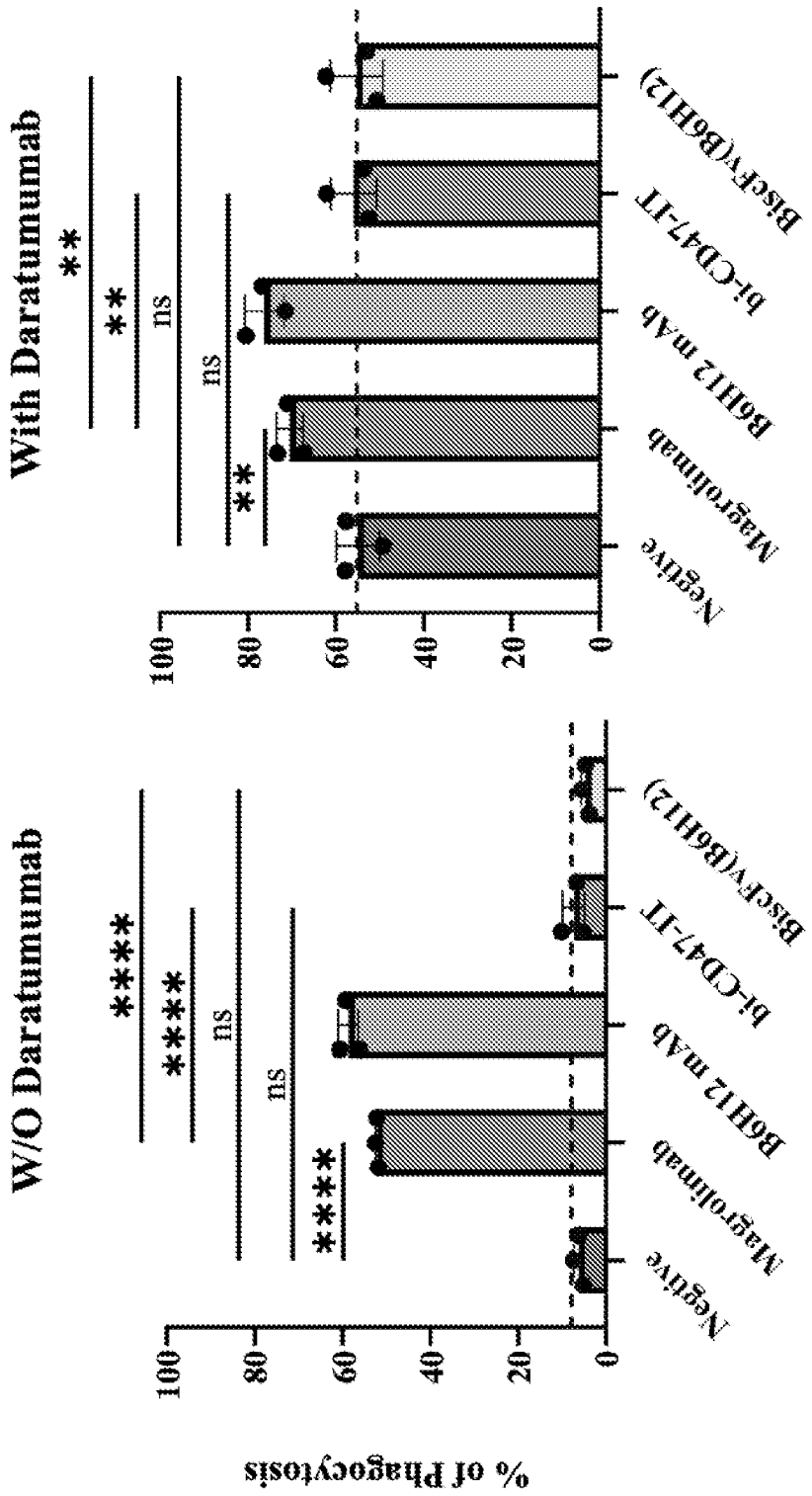
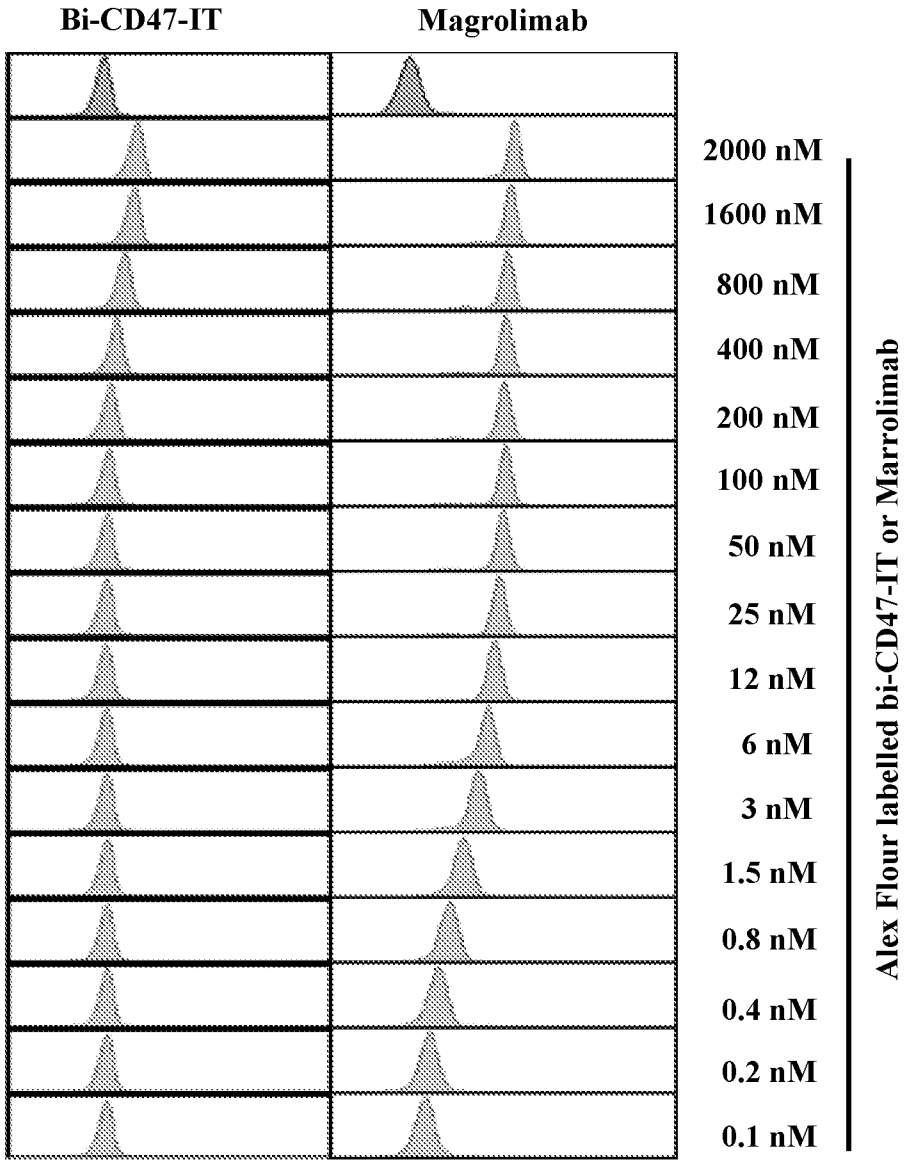

W/O Daratumumab
With Daratumumab
% of Phagocytosis
0
20
40
60
80
100
****
****
ns
ns
****
**
**
ns
**
Negtive
Magrolimab
B6H12 mAb
bi-CD47-IT
BiscFv(B6H12)

FIG. 12

FIG. 13A


Bi-CD47-IT
Magrolimab
2000 nM
1600 nM
800 nM
400 nM
200 nM
100 nM
50 nM
25 nM
12 nM
6 nM
3 nM
1.5 nM
0.8 nM
0.4 nM
0.2 nM
0.1 nM
Alex Flour labelled bi-CD47-IT or Marrolimab

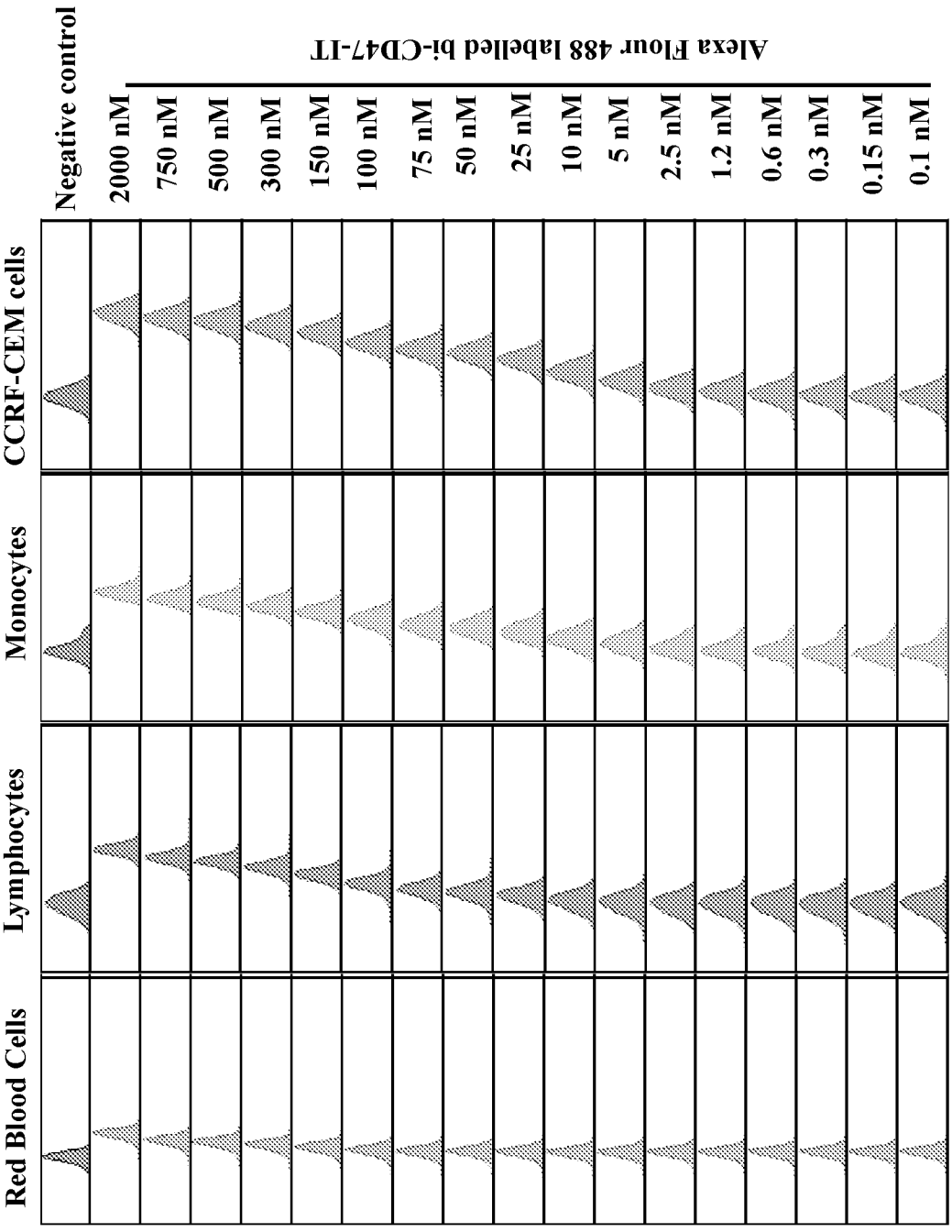

Red Blood Cells
Lymphocytes
Monocytes
CCRF-CEM cells
Negative control
2000 nM
750 nM
500 nM
300 nM
150 nM
100 nM
75 nM
50 nM
25 nM
10 nM
5 nM
2.5 nM
1.2 nM
0.6 nM
0.3 nM
0.15 nM
0.1 nM
Alexa Flour 488 labelled bi-CD47-1T

FIG. 13B

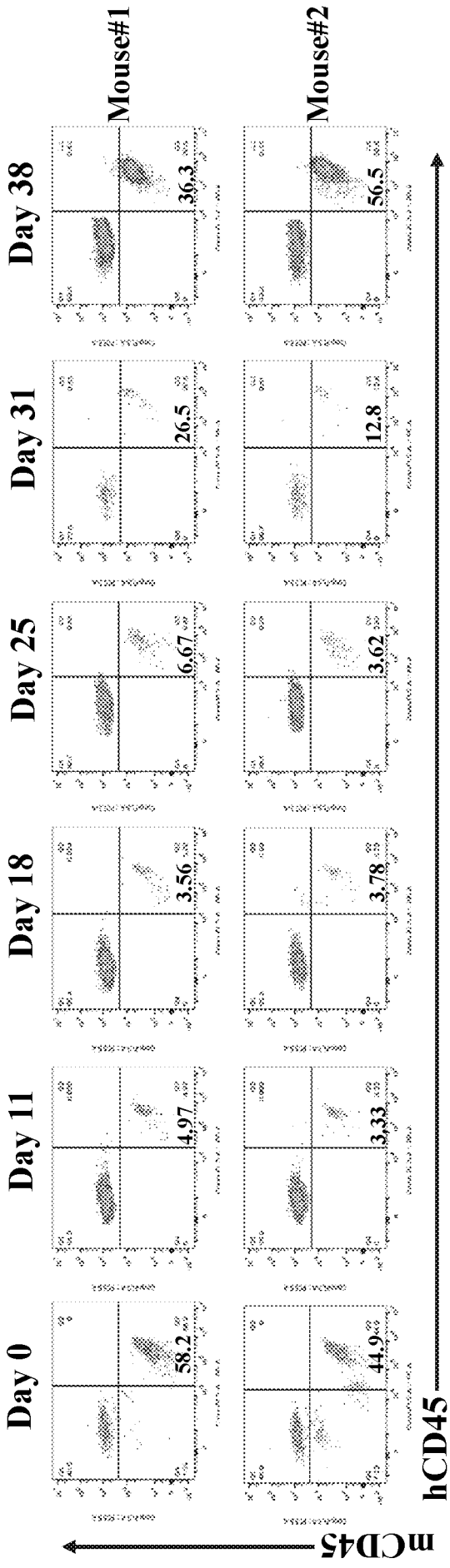

Mouse#1
Mouse#2
Day 0
Day 11
Day 18
Day 25
Day 31
Day 38
58.2
4.97
3.56
6.67
26.5
36.3
44.9
3.33
3.78
3.62
12.8
56.5
hCD45
mCD45

FIG. 13C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/038173

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***A61K 47/68*** (2024.01); ***A61P 35/00*** (2024.01); ***C07K 16/28*** (2024.01); ***C07K 16/46*** (2024.01); ***C12N 1/15*** (2024.01); ***C12N 15/63*** (2024.01); ***G01N 33/574*** (2024.01); ***A61K 39/395*** (2024.01)

CPC: ***A61K 47/6829; A61K 39/39558; G01N 33/574; C07K 16/28; A61P 35/00; C12N 1/16; C12N 15/63;*** *C07K 2317/21*

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)

See Search History Document

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

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO 2021/078219 A1 (WUXI BIOLOGICS (SHANGHAI) CO. LTD. et al.) 29 April 2021 (29.04.2021)	
X	entire document	1, 15-17, 19, 20
Y	entire document	1-3
	US 2017/0121419 A1 (THE GENERAL HOSPITAL CORPORATION) 04 May 2017 (04.05.2017)	
Y	entire document	1-3
	US 2020/0079869 A1 (CHANGCHUN GENESCIENCE PHARMACEUTICALS CORP.) 12 March 2020 (12.03.2020)	
A	entire document	1-3, 15-17, 19, 20
	US 2022/0144943 A1 (INTEGRITY BIOVENTURES INC.) 12 May 2022 (12.05.2022)	
A	entire document	1-3, 15-17, 19, 20

☐ Further documents are listed in the continuation of Box C. ☐ 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
"D" document cited by the applicant in the international application
"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
06 September 2024 (06.09.2024)	**24 September 2024 (24.09.2024)**

Name and mailing address of the ISA/US	Authorized officer
Mail Stop PCT, Attn: ISA/US **Commissioner for Patents** **P.O. Box 1450, Alexandria, VA 22313-1450**	**MATOS** **TAINA**
Facsimile No. **571-273-8300**	Telephone No. **571-272-4300**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/038173

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/038173

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.:
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:

3. ☑ Claims Nos.: **4-14, 18**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).