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(54) Title: CAR T-CELLS FOR THE TREATMENT OF CD1A-POSITIVE CANCER

(57) Abstract: Relapsed/refractory T-cell acute lymphoblastic leukemia (T-ALL) has a dismal outcome, and no effective targeted immunotherapies for T-ALL exist. The extension of chimeric antigen receptor T-cells (CARTs) to T-ALL remains challenging because the shared expression of target antigens between CARTs and T-ALL blasts leads to CARTs fratricide. CD la is exclusively expressed in cortical T-ALLs, a major subset of T-ALL. The expression of CD la is restricted to cortical thymocytes and neither CD34+ progenitors nor T-cells express CD la during ontogeny, confining the risk of on-target/off-tumor toxicity. The present invention provides CARs comprising a CD la-targeting moiety which may be transduced or transformed into T cells. The resultant CARTs are suitable for the treatment of cortical T-ALLs.

CAR T-cells for the treatment of CD1a-positive cancer

Technical Field

The present invention provides therapeutics for the treatment of CD1a-positive cancers such as T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma. In particular, the present invention provides chimeric antigen receptor (CAR) T-cells that can target CD1a.

Background art

T-cell lineage acute lymphoblastic leukemia (T-ALL) is a malignant disorder resulting from leukemic transformation of thymic T-cell precursors [1]. T-ALL is phenotypically and genetically heterogeneous, and is commonly associated with genetic alterations/mutations in transcription factors involved in hematopoietic stem/progenitor cell (HSPC) homeostasis and in master regulators of T-cell development[2]. T-ALL comprises 10-15% and 20-25% of all acute leukemias diagnosed in children and adults, respectively [3,4] with a median diagnostic age of 9 years [5-7]. Intensive chemotherapy regimens have led to the improved survival of patients with T-ALL. However, the event-free (EFS) and overall (OS) survival remains <70%, and relapsed/refractory (R/R) T-ALL has a particularly poor outcome. There are currently no potential curative options beyond hematopoietic cell transplantation and conventional chemotherapy, which is linked to large trade-offs in toxicities [4,8], reinforcing the need for novel targeted therapies. T-cell lymphoblastic lymphomas (TCL) are etiologically and pathogenically different fromT-ALL but phenotypically very similar. The main difference is that TCLs are found extramedullary while T-ALL is a bone marrow disease.

Immunotherapy has generated unprecedented expectations in cancer treatment and relies on the immune system as a powerful weapon against cancer. In recent years, adoptive cellular immunotherapy based on chimeric antigen receptors (CARs) has shown great potential. CAR therapy redirects genetically modified T-cells to specifically recognize and eliminate specific antigen-expressing tumor cells in a major histocompatibility complex-independent fashion [9,10]. The success of CAR T-cells (CARTs) re-directed against CD19 or CD22 is now indisputable for B-cell malignancies (mainly B-ALL) [11-14]. But, strategies targeting T-cell malignancies using CARTs remain challenging because of the shared expression of target antigens between CARTs and T-lineage tumoral cells. In this regard, CARTs against pan T-cell antigens have two major drawbacks: i) CARTs self-targeting/fratricide and, ii) T-cell aplasia, leading to life-threating immunodeficiency [15-17].

Recent elegant studies demonstrated that T-cells transduced with either CD7, CD3, CD5 or TCR CARs, the most expressed pan-T-cell antigens, efficiently eliminate T-ALL blasts *in vitro* and are able to control the disease *in vivo* [15-20]. Yet, approaches still far from the clinic, such as CRISPR/Cas9 genome

editing or protein expression blockers, were required for disruption of the target antigen in T-cells prior to CAR transduction, to avoid extensive self-antigen driven fratricide [15-17,19].

Thus, there remains a need for a therapy that can successfully treat T-ALL. The present invention aims to provide a therapy for treating CD1a-positive T-ALL.

Figures

Figure 1. CD1a expression in T-ALL and normal hematopoiesis and thymopoiesis. (A) Immunophenotype of *de novo* T-ALL samples (n=38) for the indicated markers. Upper and intermediate curly brackets identify $CD1a^{+/++}$ and $CD1a^{low/+}$ coT-ALL patients, respectively. Black circles at bottom depict non-coTALL patients. **(B)** Representative FACS dot plot of a coT-ALL patient. CD7+CD1a+ cells are coT-ALL blasts and CD3+CD7+CD1a- (either CD4+ or CD8+) are normal mature T-cell present in the diagnostic sample. **(C)** CD1a is retained at relapse (n=5 diagnostic-relapse coT-ALL pairs). Data shown as CD1a expression in relapse samples relative to the diagnostic-matched samples (diagnostic shown as 100% expression). **(D)** T-cells and CD34+ HSPCs do not express CD1a across ontogeny. **(E)** Scheme depicting the phenotype of developing thymic T-cell populations. **(F)** Representative FACS for pre-cortical ($CD34^{high}$CD7++CD1a-) and cortical (CD34+CD7++CD1a+) thymocytes. DX: diagnostic. RX: relapse.

Figure 2. CD1a CARTs specifically target and eliminate CD1a+ T-ALL cell lines *in vitro*. (A) Scheme of the CD1aCAR construct used. **(B)** CAR detection in 293T cells using an anti-scFv MoAb and GFP. **(C)** Representative CAR transduction and detection in CD4+ and CD8+ T-cells (n=6). **(D)** Proper T-cell activation (n=3). **(E)** Robust expansion of activated T-cells transduced with either mock or CD1a CAR reveals no signs of fratricide (n=4). **(F)** Surface expression of CD1a (black line) in Jurkat, MOLT4 and NALM6 cell lines. **(G)** CD1a antigen density in cell lines, primary coT-ALL samples and primografts. **(H)** Cytotoxicity of CD1a CARTs and MOCK T-cells against coT-ALL and B-ALL cell lines at the indicated E:T ratios in 16h assays (n=4). **(I)** Absolute counts of alive eFluor+ target cells measured by FACS in 72h cytotoxicity assays at 1:1 E:T ratio. **(J)** Representative FACS analysis of cytotoxicity with target cells labeled with eFluor670. **(K)** ELISA showing high-level production of the inflammatory cytokines IL-2, TNFα and IFNγ by CD1a CARTs exposed to Jurkat and NALM6 (negative control) cells in 16h assays at 1:1 E:T ratio (n=4). *p<0.05, **p<0.01, ***p<0.001.

Figure 3. CD1a CARTs specifically target and eliminate *in vitro* CD1a+ T-ALL blasts from primary samples or PDX models. (A) Expression of CD1a vs CD7 in coT-ALL blasts from primary patients/primografts. The % of CD1a+ blasts is indicated. **(B)** Cytotoxicity (in absolute counts of eFluor+ cells) measured by FACS in 48h cytotoxicity assays at 4:1 E:T ratio (n=3). **(C)** Representative FACS analysis of CD1a within the eFluor-labeled target cells at the end of the cytotoxicity assay,

revealing specificity of CD1a CARTs (n=3). **(D)** High-level production of pro-inflammatory cytokines by CD1a CARTs analyzed by ELISA (n=3 independent supernatants) in 16h assays at 4:1 E:T ratio. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 4. CD1a CARTs fully control the progression of coT-ALL cells in a mouse xenograft setting. (A) Scheme of the xenograft model. NSG mice (n=6/group) were i.v. injected with 3×10^6 Luc-GFP-expressing Jurkat cells followed 3 days after by a single i.v. injection of 5×10^6 mock or CD1a CARTs. Tumor burden was monitored every 4–6 days by bioluminescence (BLI) using IVIS imaging. When MOCK-treated animals were fully leukemic, one-half of the CD1a CARTs-treated animals were sacrificed and analyzed by FACS (BM, PB and spleen) for leukemic burden and CARTs persistence. The remaining animals were re-challenged 6 weeks after with 1.5×10^6 Luc-Jurkat and were followed-up as before. **(B)** IVIS imaging of tumor burden monitored by BLI at the indicated timepoints. **(C)** Total radiance quantification (p/sec/cm^2/sr) at the indicated timepoints. †: sacrifice. **(D)** Circulating Jurkat cells in PB 17 days after CARTs infusion. **(E)** T-cell persistence in PB at day 17, and spleen and BM at sacrifice. Data is shown as mean±SD (n=6 mice/group). *p<0.05, **p<0.01, ***p<0.001.

Figure 5. CD1a CARTs fully abolish the progression of primary CD1a+ coT-ALL blasts in a PDX setting. (A) Scheme of the PDX model. NSG mice (n=5–6/group) were i.v. injected with 1×10^6 primary coT-ALL cells followed three days after by a single i.v. injection of 1×10^6 mock or CD1a CARTs. Tumor burden was monitored by FACS every other week by bleeding and BM aspirate after 6 and 9 weeks. **(B,C)** Frequency of leukemic mice and levels of leukemia in BM **(B)** and PB **(C)** 6 and 9 weeks after infusion of CARTs. The left panels show representative FACS plots. Primary CD1a+ T-ALL blasts are shown inside the box (grey). Effector T-cells are shown outside the box in grey. Mouse cells are shown in black. **(D)** 9-week OS of coT-ALL primografts receiving either CD1a CARTs or MOCK T-cells. **(E)** Effector T-cell persistence overtime in PB (week 2 towards week 9) and BM (week 6 and 9). Each dot represents an independent mouse. **p<0.01, Malcolm-Cox test.

Figure 6. CD1a CARTs retain the ability to control progression of CD1a+ cell lines and coT-ALL primary samples in a re-challenge PDX setting. (A) IVIS imaging of Jurkat cells burden in the re-challenged mice. (B) Total radiance quantification (p/sec/cm^2/sr) overtime in the mice re-challenged with Jurkat cells. (C) Circulating Jurkat cells in PB 16 days after re-challenge. (D) Robust effector T-cell persistence in PB, BM and spleen at sacrifice of the re-challenged animals. (E) Scheme of the re-challenge PDX experiments using coT-ALL primary samples. CARTs-bearing PDX mice were re-challenged with 1×10^6 primary CD1a+ T-ALL seven weeks after initial CARTs infusion. (F) Secondary coT-ALL burden in engrafted PB (left panel) and BM (right panel) 6 weeks after leukemia re-challenge. (G) Effector T-cell persistence overtime in PB (week 2, 4 and 6) from PDXs re-challenged with coT-

ALL primary samples. Each dot represents an independent mouse. *p<0.05, **p<0.01, ***p<0.001, ****<0.0001.

Figure 7. CD1a CARTs derived from coT-ALL patients at presentation specifically lyse autologous CD1a+ T-ALL blasts. (A) Scheme depicting the experimental design for the autologous cytotoxic assay. Mature (normal) CD3+CD1a$^-$ T-cells were FACS-purified from the PB of a coT-ALL patient, infected with CD1a CAR, expanded, and exposed to autologous total PBMCs. **(B)** FACS analysis of autologous cytotoxic 48h-assay at 1:1 and 4:1 E:T. eFluor670-labeled total PBMC target population contains CD1a+ T-ALL blasts (upper box) and mature CD3+CD1a- T-cells (bottom box). **(C)** Quantification of CD1a CARTs-mediated specific lysis for coT-ALL blasts (upper panel) and CD3+CD1a- mature T-cells (bottom panel). **(D)** ELISpot showing the number of IFNγ SFC from mock *versus* CD1a CARTs stimulated with a pool of peptides from CMV, EBV and Flu (CEF). Staphylococcal enterotoxin B (SEB) was used as positive control.

Figure 8. Immunophenotype for each individual CD1a++ coT-ALL patient presented in this study. (A) Gating strategy distinguishing mature normal T-cells (CD3++CD1a- either CD4+ or CD8+) and coT-ALLs blasts (CD7+CD1a+). Note that coT-ALL blasts commonly have aberrant expression for CD3 and/or CD4/CD8). **(B)** CD7/CD3 *vs* CD1a FACS dot plots for n=16 available CD1a++ coT-ALL patients showing the percentage of mature normal T-cells (left quadrant) and coT-ALLs blasts (right quadrant).

Figure 9. *In vitro* specificity of CD1a CARTs. (A) Scheme of the CD1aCAR, CD22CAR and MOCK constructs used in the present study. (B) CD1a CARTs but not CD22 CARTs lyse the T-ALL cell line Jurkat. CD22 CARTs but not CD1a CARTs lyse the B-ALL line NALM6. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 10. *In vivo* cytotoxicity of CD1a CARTs is dose-dependent. (A) Tumor burden monitored by BLI at the beginning of the experiment (scale: 3×10^4 to 1×10^5 p/sec/cm^2/sr) confirming early and efficient T-ALL engraftment. **(B)** IVIS imaging of tumor burden monitored by BLI at the indicated time points for CARTs doses of 2×10^6 and 5×10^6 p/sec/cm^2/sr. **(C)** Total radiance quantification (p/sec/cm^2/sr) at the indicated time points for CARTs doses of 2×10^6 and 5×10^6. N=3-4 mice/group. †: sacrifice. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 11. CD1a CARTs do not target CD7+CD1a- thymocytes. Cytotoxicity assays against fetal thymic cells were performed at 16h and 72h at 4:1 E:T for CD1a CARTs and MOCK T-cells (n=2).

Figure 12. The absolute number of CD1a- primary coT-ALL cells remains identical after either MOCK or CD1a CART exposure. This confirms that CD1a expression was not lost/downregulated by immune pressure.

Summary of the invention

The choice of the antigen against which we wish to re-direct T-cells represents a major advance to solve the problems associated with the shared expression of T-cell markers between normal and malignant T-cells. We identified that CD1a, a lipid-presenting molecule, is a suitable target for treating a large subset of T-ALL, i.e. cortical T-ALL.

We developed and functionally characterized CD1a-specific CARTs, which displayed robust cytotoxicity against T-ALL cell lines and primary cortical CD1a+ T-ALL cells both *in vitro* and *in vivo* in xenograft models. The CD1a CARTs continuously expanded 200-fold, similar to MOCK T-cells, demonstrating that redirecting CARTs against CD1a antigen does not induce T-cell fratricide. Also, the use of CD1a CARTs for cortical T-ALL bypasses the need for sophisticated genome editing-based disruption of target antigens in T-cells prior to CAR transduction as a strategy to avoid self-antigen-driven fratricide [15-17,19]. We further demonstrated that in steady-state hematopoiesis, CD1a is exclusively expressed in a subset of cortical CD34+CD7+ thymic T progenitors, whereas earlier $CD34^{high}CD7^{high}$ T-progenitors lack CD1a. In addition, neither normal CD34+ HSPCs nor mature T-cells from multiple tissues express CD1a during ontogeny, thereby minimizing the risk of on-target/off-tumor toxicity. Indeed, when human fetal thymus-derived CD7+ thymocytes were exposed to CD1a CARTs, only the CD1a+ cortical thymocytes were eliminated by the CD1a CARTs, while developmentally earlier and later thymic T-lineage populations (CD34+ and CD34-) were not targeted, limiting the on-target/off-tumor effects to a developmentally transient thymic population of cortical thymocytes and further confirming the fratricide resistant nature of CD1a CARTs.

The exclusive thymic localization of cortical thymocytes, and the fact that thymic subpopulations of CD34+CD7+CD1a- T-cell progenitors physiologically/constantly maturing into functional T cells reside upstream of CD1a+ cortical thymocytes, provides an additional level of safety for the use of CD1a CARTs in patients with R/R T-ALL. We do not expect irreversible toxicities or severe T-cell aplasia attributed to CD1a CARTs for the following reasons: i) the CD1a+ thymocyte population is a transient thymic T-cell fraction, eventually regenerated by upstream CD1a- T-cell progenitors; ii) CD1a CARTs themselves respond normally to viral antigens and therefore are likely to be protective against pathogens; iii) the clinical use of specific antibodies against CD5 or CD7 [42] did not reveal severe or irreversible toxicities; iv) there are multiple studies that demonstrate extrathymic maturation of T-cells and a balance between the innate and adaptive immune system that may, at least in part, guarantee immunological protection in patients who have undergone partial or total thymectomy [45-47].

Thus, in one aspect, the present invention provides a chimeric antigen receptor (CAR) comprising an extracellular domain comprising a CD1a targeting-moiety, a transmembrane domain, and an intracellular signaling domain.

The present invention also provides a nucleic acid encoding the CAR of the present invention. Further, the present invention provides a cell comprising the nucleic acid and/or CAR of the present invention. And, the present invention provides a pharmaceutical composition comprising a plurality of cells in accordance with the present invention and a pharmaceutically acceptable carrier or diluent.

The cell of the present invention or pharmaceutical composition of the present invention is provided for use as a medicament. In particular, the present invention provides a method of treating a CD1a-positive cancer comprising administering the cell of the present invention or the pharmaceutical composition of the present invention to a patient in need thereof.

Detailed description of the invention

Definitions

"Administering" or "administration of" a medicament to a patient (and grammatical equivalents of this phrase) refers to direct administration, which may be administration to a patient by a medical professional or may be self-administration, and/or indirect administration, which may be the act of prescribing a drug. E.g., a physician who instructs a patient to self-administer a medicament or provides a patient with a prescription for a drug is administering the drug to the patient.

The term "affibody" refers to a protein that is derived from the Z domain of protein A and that been engineered to bind to a specific target (see Frejd & Kim, 2017. Exp Mol Med. 49(3): e306).

The term "antibody" refers to a molecule comprising at least one immunoglobulin domain that binds to, or is immunologically reactive with, a particular target. The term includes whole antibodies and any antigen binding portion or single chains thereof and combinations thereof; for instance, the term "antibody" in particular includes bivalent antibodies and bivalent bispecific antibodies.

A typical type of antibody comprises at least two heavy chains ("HC") and two light chains ("LC") interconnected by disulfide bonds.

Each "heavy chain" comprises a "heavy chain variable domain" (abbreviated herein as "VH") and a "heavy chain constant domain" (abbreviated herein as "CH"). The heavy chain constant domain typically comprises three constants domains, CH1, CH2, and CH3.

Each "light chain" comprises a "light chain variable domain" (abbreviated herein as "VL") and a "light chain constant domain" ("CL"). The light chain constant domain (CL) can be of the kappa type or of the lambda type. The VH and VL domains can be further subdivided into regions of hypervariability, termed Complementarity Determining Regions ("CDR"), interspersed with regions that are more conserved, termed "framework regions" ("FW").

Each VH and VL is composed of three CDRs and four FWs, arranged from amino-terminus to carboxy-terminus in the following order: FW1, CDR1, FW2, CDR2, FW3, CDR3, FW4. The present disclosure inter alia presents VH and VL sequences as well as the subsequences corresponding to CDR1, CDR2, and CDR3.

The precise amino acid sequence boundaries of a given CDR can be determined using any of a number of well-known schemes, including those described by Kabat et al. (1991), “Sequences of Proteins of Immunological Interest,” 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (“Kabat” numbering scheme), Al-Lazikani et al., (1997) JMB 273,927-948 (“Chothia” numbering scheme).

Accordingly, a person skilled in the art would understand that the sequences of FW1, FW2, FW3 and FW4 are equally disclosed. For a particular VH, FW1 is the subsequence between the N-terminus of the VH and the N-terminus of H-CDR1, FW2 is the subsequence between the C-terminus of H-CDR1 and the N-terminus of H-CDR2, FW3 is the subsequence between the C-terminus of H-CDR2 and the N-terminus of H-CDR3, and FW4 is the subsequence between the C-terminus of H-CDR3 and the C-terminus of the VH. Similarly, for a particular VL, FW1 is the subsequence between the N-terminus of the VL and the N-terminus of L-CDR1, FW2 is the subsequence between the C-terminus of L-CDR1 and the N-terminus of L-CDR2. FW3 is the subsequence between the C-terminus of L-CDR2 and the N-terminus of L-CDR3, and FW4 is the subsequence between the C-terminus of L-CDR3 and the C-terminus of the VL.

The variable domains of the heavy and light chains contain a region that interacts with a binding target, and this region interacting with a binding target is also referred to as an “antigen-binding site” or “antigen binding site” herein. The constant domains of the antibodies can mediate the binding of the antibody to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system. Exemplary antibodies of the present disclosure include typical antibodies, but also bivalent fragments and variations thereof such as a F(ab’)2.

As used herein, the term "antibody" encompasses intact polyclonal antibodies, intact monoclonal antibodies, bivalent antibody fragments (such as F(ab')2), multispecific antibodies such as bispecific antibodies, chimeric antibodies, humanized antibodies, human antibodies, and any other modified immunoglobulin molecule comprising an antigen binding site.

An antibody can be of any the five major classes (isotypes) of immunoglobulins: IgA, IgD, IgE, IgG, and IgM, or subclasses thereof (e.g. IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), based on the identity of their heavy-chain constant domains referred to as alpha, delta, epsilon, gamma, and mu, respectively. The different classes of immunoglobulins have different and well known subunit structures and three-dimensional configurations. Antibodies can be naked or conjugated to other molecules such as therapeutic agents or diagnostic agents to form immunoconjugates.

The term “anticalin” refers to a protein that is derived from the lipocalin and that been engineered to bind to a specific target (see Skerra, 2008. FEBS J. 275(11):2677-83).

The term “antigen-binding fragment” or “Fab” refers to an antibody fragment comprising one constant and one variable domain of each of the heavy and light chain. A Fab fragment may be obtained by digesting an intact monoclonal antibody with papain.

The term “cancer” refers to a group of diseases, which can be defined as any abnormal benign or malignant new growth of tissue that possesses no physiological function and arises from uncontrolled usually rapid cellular proliferation and has the potential to invade or spread to other parts of the body.

The term “CD1a” refers to a non-polymorphic MHC Class 1 related cell surface glycoprotein, expressed in association with β-2-microglobulin. CD1a is expressed by cortical thymocytes, Langerhans cells and by interdigitating cells. CD1a is also expressed by some malignancies of T cell lineage and in Langerhans cell histiocytosis. CD1a is expressed on cortical thymocytes, epidermal Langerhans cells, dendritic cells, on certain T-cell leukemias, and in various other tissues. CD1a is structurally related to the major histocompatibility complex (MHC) proteins and form heterodimers with β-2-microglobulin. Exemplary sequence and data related to human CD1a has been deposited in the UniProtKB database under ID number P06126.

“CD1a-positive” cancer, including a “CD1a-positive” cancerous disease, is one comprising cells, which have CD1a present at their cell surface. The term “CD1a-positive” also refers to a cancer that produces sufficient levels of CD1a at the surface of cells thereof, such that a CAR-comprising cell of the present invention has a therapeutic effect, mediated by the binding of the CAR to CD1a. In some embodiments,

the CD1a-positive cancer is cortical T-cell acute lymphoblastic leukemia, and T-cell lymphoblastic lymphoma or Langerhans cell histiocytosis (LCH).

The term "CD1a-targeting moiety" refers to a substance that is able to bind CD1a. Within the context of a CAR, a CD1a-targeting moiety targets T cells to a CD1a-positive cell, preferably a cancer cell. Within the context of a CAR, it is to be understood that the CD1a-targeting moiety is genetically encodable.

The term "chimeric antigen receptor" or "CAR" refers to a synthetic receptor that targets T cells to a chosen antigen and reprograms T cell function, metabolism and persistence (see Rivière & Sadelain, 2017. *Mol Ther*. 25(5):1117-1124). Similarly, the term "CART" refers to a T cell that comprises a CAR.

"Combination therapy", "in combination with" or "in conjunction with" as used herein denotes any form of concurrent, parallel, simultaneous, sequential or intermittent treatment with at least two distinct treatment modalities (i.e., compounds, components, targeted agents or therapeutic agents). As such, the terms refer to administration of one treatment modality before, during, or after administration of the other treatment modality to the subject. The modalities in combination can be administered in any order. The therapeutically active modalities are administered together (e.g., simultaneously in the same or separate compositions, formulations or unit dosage forms) or separately (e.g., on the same day or on different days and in any order as according to an appropriate dosing protocol for the separate compositions, formulations or unit dosage forms) in a manner and dosing regimen prescribed by a medical care taker or according to a regulatory agency. In general, each treatment modality will be administered at a dose and/or on a time schedule determined for that treatment modality. Optionally, three or more modalities may be used in a combination therapy. Additionally, the combination therapies provided herein may be used in conjunction with other types of treatment. For example, other anti-cancer treatment may be selected from the group consisting of chemotherapy, surgery, radiotherapy (radiation) and/or hormone therapy, amongst other treatments associated with the current standard of care for the subject.

A "complete response" or "complete remission" or "CR" indicates the disappearance of all target lesions as defined in the RECIST v1.1 guideline. This does not always mean the cancer has been cured.

The term "costimulatory signaling domain" refers to a signaling moiety that provides to T cells a signal which, in addition to the primary signal provided by for instance the CD3ζ chain of the TCR/CD3 complex, mediates a T cell response, including, but not limited to, activation, proliferation, differentiation, cytokine secretion, and the like. A co-stimulatory domain can include all or a portion of, but is not limited to, CD27, CD28, 4-1BB (CD137), OX40 (CD134), CD30, CD40, 1COS, lymphocyte

function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that specifically binds with CD83. In some embodiments, the co-stimulatory signaling domain is an intracellular signaling domain that interacts with other intracellular mediators to mediate a cell response including activation, proliferation, differentiation and cytokine secretion, and the like.

The term "designed ankyrin repeat proteins" or "DARPin" refers to a protein that is derived from an ankyrin repeat that has been engineered to bind to a specific target (see Plückthun, 2015. Annu Rev Pharmacol Toxicol. 55:489-511).

"Disease free survival" (DFS) refers to the length of time during and after treatment that the patient remains free of disease.

As used herein, the term "effective amount" of an agent, e.g., a therapeutic agent such as a CART, is that amount sufficient to effect beneficial or desired results, for example, clinical results, and, as such, an "effective amount" depends upon the context in which it is being applied. For example, in the context of administering a therapeutic agent that treats T-ALL, an effective amount can reduce the number of cancer cells; reduce the tumor size or burden; inhibit (i.e., slow to some extent and in a certain embodiment, stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and in a certain embodiment, stop) tumor metastasis; inhibit, to some extent, tumor growth; relieve to some extent one or more of the symptoms associated with the cancer; and/or result in a favorable response such as increased progression-free survival (PFS), disease-free survival (DFS), or overall survival (OS), complete response (CR), partial response (PR), or, in some cases, stable disease (SD), a decrease in progressive disease (PD), a reduced time to progression (TTP) or any combination thereof. The term "effective amount" can be used interchangeably with "effective dose," "therapeutically effective amount," or "therapeutically effective dose".

The term "fynomer" refers to a protein that is derived from the SH3 domain of human Fyn kinase that has been engineered to bind to a specific target (see Bertschinger et al., 2007. Protein Eng Des Sel. 20(2):57-68).

The terms "individual", "patient" or "subject" are used interchangeably in the present application to designate a human being and are not meant to be limiting in any way. The "individual", "patient" or "subject" can be of any age, sex and physical condition. The term "patient in need thereof" usually refers to a patient who suffers from a CD1a-positive cancer.

"Infusion" or "infusing" refers to the introduction of a therapeutic agent-containing solution into the body through a vein for therapeutic purposes. Generally, this is achieved via an intravenous bag.

"Intracellular signaling domain" as used herein refers to all or a portion of one or more domains of a molecule (here the chimeric receptor molecule) that provides for activation of a lymphocyte. Intracellular domains of such molecules mediate a signal by interacting with cellular mediators to result in proliferation, differentiation, activation and other effector functions. Examples of intracellular signaling domains for use in a CAR of the invention include the intracellular sequences of the CD3ζ chain, and/or co-receptors that act in concert to initiate signal transduction following CAR engagement, as well as any derivative or variant of these sequences and any synthetic sequence that has the same functional capability. T cell activation can be said to be mediated by two distinct classes of cytoplasmic signaling sequence: those that initiate antigen-dependent primary activation and provide a T cell receptor like signal (primary cytoplasmic signaling sequences) and those that act in an antigen- independent manner to provide a secondary or co-stimulatory signal (secondary cytoplasmic signaling sequences). Primary cytoplasmic signaling sequences that act in a stimulatory manner may contain signaling motifs which are known as receptor tyrosine-based activation motifs or ITAMs. Examples of ITAM containing primary cytoplasmic signaling sequences include those derived from CD3ζ, FcRγ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b, and CD66d.

The term “monobody” refers to a protein that is derived from a fibronectin type III domain that has been engineered to bind to a specific target (see Koide et al., 2013. J Mol Biol. 415(2):393-405).

The term “nanobody” refers to a protein comprising the soluble single antigen-binding V-domain of a heavy chain antibody, preferably a camelid heavy chain antibody (see Bannas et al., 2017. Front Immunol. 8:1603).

“Overall Survival” (OS) refers to the time from patient enrollment to death or censored at the date last known alive. OS includes a prolongation in life expectancy as compared to naive or untreated individuals or patients. Overall survival refers to the situation wherein a patient remains alive for a defined period of time, such as one year, five years, etc., e.g., from the time of diagnosis or treatment.

A “partial response” or “PR” refers to at least a 30% decrease in the sum of diameters of target lesions, taking as reference the baseline sum diameter, in response to treatment, as defined in the RECIST v1.1 guideline.

The term “peptide aptamer” refers to a short, 5-20 amino acid residue sequence that can bind to a specific target. Peptide aptamers are typically inserted within a loop region of a stable protein scaffold (see Reverdatto et al., 2015. Curr Top Med Chem. 15(12):1082-101).

As used herein, "pharmaceutically acceptable carrier" or “pharmaceutically acceptable diluent” means any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed and, without limiting the scope of the present invention, include: additional buffering agents; preservatives; co-solvents; antioxidants, including ascorbic acid and methionine; chelating agents such as EDTA; metal complexes (e.g., Zn-protein complexes); biodegradable polymers, such as polyesters; salt-forming counterions, such as sodium, polyhydric sugar alcohols; amino acids, such as alanine, glycine, glutamine, asparagine, histidine, arginine, lysine, ornithine, leucine, 2-phenylalanine, glutamic acid, and threonine; organic sugars or sugar alcohols, such as lactitol, stachyose, mannose, sorbose, xylose, ribose, ribitol, myoinisitose, myoinisitol, galactose, galactitol, glycerol, cyclitols (e.g., inositol), polyethylene glycol; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, [alpha]-monothioglycerol, and sodium thio sulfate; low molecular weight proteins, such as human serum albumin, bovine serum albumin, gelatin, or other immunoglobulins; and hydrophilic polymers, such as polyvinylpyrrolidone. Other pharmaceutically acceptable carriers, excipients, or stabilizers, such as those described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980) may also be included in a pharmaceutical composition described herein, provided that they do not adversely affect the desired characteristics of the pharmaceutical composition.

“Progressive disease” or “disease that has progressed” refers to the appearance of one more new lesions or tumors and/or the unequivocal progression of existing non-target lesions as defined in the RECIST v1.1 guideline. Progressive disease or disease that has progressed can also refer to a tumor growth of more than 20 percent since treatment began, either due to an increase in mass or in spread of the tumor.

“Progression free survival” (PFS) refers to the time from enrollment to disease progression or death. PFS is generally measured using the Kaplan-Meier method and Response Evaluation Criteria in Solid Tumors (RECIST) 1.1 standards. Generally, progression free survival refers to the situation wherein a patient remains alive, without the cancer getting worse.

The term “RECIST” means Response Evaluation Criteria in Solid Tumours. RECIST guideline, criteria, or standard, describes a standard approach to solid tumor measurement and definitions for objective assessment of change in tumor size for use in adult and pediatric cancer clinical trials. RECIST v1.1 means version 1.1 of the revised RECIST guideline and it is published in European Journal of Cancers 45 (2009) 228-247.

The term "repebody" refers to a protein that is derived from a leucine-rich repeat module and that been engineered to bind to a specific target (see Lee et al., 2012. PNAS. 109(9): 3299-3304).

The term "respond favorably" generally refers to causing a beneficial state in a subject. With respect to cancer treatment, the term refers to providing a therapeutic effect on the subject. Positive therapeutic effects in cancer can be measured in a number of ways (See, Weber, 2009. J Nucl Med. 50 Suppl 1:1S-10S). For example, tumor growth inhibition, molecular marker expression, serum marker expression, and molecular imaging techniques can all be used to assess therapeutic efficacy of an anti-cancer therapeutic. With respect to tumor growth inhibition, according to NCI standards, a T/C $\leq$ 42% is the minimum level of anti-tumor activity. A T/C <10% is considered a high anti-tumor activity level, with T/C (%) = Median tumor volume of the treated / Median tumor volume of the control x 100. A favorable response can be assessed, for example, by increased progression-free survival (PFS), disease-free survival (DFS), or overall survival (OS), complete response (CR), partial response (PR), or, in some cases, stable disease (SD), a decrease in progressive disease (PD), a reduced time to progression (TTP) or any combination thereof.

The term "sequence identity" refers to a percentage value obtained when two sequences are compared using a pairwise sequence alignment tool. In the present case, the sequence identity is obtained using the global alignment tool "EMBOSS Needle" using the default settings (Rice et al., 2000. Trends Genet. 16(6):276-7; Li et al., 2015. Nucleic Acids Res. 43(W1):W580-4). The global alignment tool is available at: https://www.ebi.ac.uk/Tools/psa/ .

The term "single-chain antigen-binding fragment" or "scFab" refers to a fusion protein comprising one variable and one constant domain of the light chain of an antibody attached to one variable and one constant domain of the heavy chain of an antibody, wherein the heavy and light chains are linked together through a short peptide.

The term "single-chain variable fragment" or "scFv" refers to a fusion protein comprising the variable domains of the heavy chain and light chain of an antibody linked to one another with a peptide linker. The term also includes a disulfide stabilized Fv (dsFv). Methods of stabilizing scFvs with disulfide bonds are disclosed in Reiter et al., 1996. Nat Biotechnol. 14(10):1239-45.

"Stable disease" refers to disease without progression or relapse as defined in the RECIST v1.1 guideline. In stable disease there is neither sufficient tumor shrinkage to qualify for partial response, nor sufficient tumor increase to qualify as progressive disease.

"Time to Tumor Progression" (TTP) is defined as the time from enrollment to disease progression. TTP is generally measured using the RECIST v1.1 criteria.

The terms "treatment" and "therapy", as used in the present application, refer to a set of hygienic, pharmacological, surgical and/or physical means used with the intent to cure and/or alleviate a disease and/or symptoms with the goal of remediating the health problem. The terms "treatment" and "therapy" include preventive and curative methods, since both are directed to the maintenance and/or reestablishment of the health of an individual or animal. Regardless of the origin of the symptoms, disease and disability, the administration of a suitable medicament to alleviate and/or cure a health problem should be interpreted as a form of treatment or therapy within the context of this application.

Chimeric antigen receptor

In one aspect, the present invention provides a chimeric antigen receptor (CAR) comprising an extracellular domain comprising a CD1a targeting-moiety, a transmembrane domain, and an intracellular signaling domain.

CD1a targeting-moiety

In some embodiments, the CD1a-targeting moiety is an antibody, anticalin, repebody, monobody, scFv, Fab, scFab, affibody, fynomer, DARPin, nanobody, or peptide aptamer that specifically binds to CD1a.

Binding molecules that bind specifically to CD1a may be very useful in the diagnosis and treatment of the disorders mentioned above. Several murine monoclonal antibodies against CD1a are known in the field (Kelly (1994), Amiot et al. (1986), Furue et al. (1992)). However, murine antibodies are limited for *in vivo* use due to issues associated with the administration of murine antibodies to humans, such as short serum half-life, the inability to trigger certain human effector functions and the generation of an undesired immune response against the murine antibody (Van Kroonenburgh and Pauwels (1988)). New human antibodies have been developed (Bechan (2012), and Gitanjali (2005) in recent years overcoming these previously mentioned drawbacks. Besides NA1/34.HLK , other hybridomas are commercially available, e.g. OKT6 (IgG1 isotype), from SIGMA ALDRICH.

Please refer to:

- Amiot M., Bernard A., Raynal B., Knapp W., Deschildre C. and Boumsell L. (1986), *J. Immunol.* 136:1752-1757.
- Furue M., Nindl M., Kawabe K., Nakamura K., Ishibashi Y. and Sagawa K. (1992), *J. Am. Acad. Dermatol.* 27:419-42
- Kelly K. M., Beverly P. C., Chu A. C., Davenport V., Gordon I., Smith M. and Pritchard J. (1994), *J. Pediatr.* 125:717-722
- Van Kroonenburgh M. J. and Pauwels E. K. (1988), *Nucl. Med. Commun.* 9:919-930.

- Gitanjali Bechan, David W. Lee, R. Maarten Egeler and Robert J. Arceci
  Blood 2005 106:4815
  Bechan, G. I., Lee, D. W., Zajonc, D. M., Heckel, D. , Xian, R. , Throsby, M. , Meijer, M. , Germeraad, W. T., Kruisbeek, A. M., Maarten Egeler, R. and Arceci, R. J. (2012), Br J Haematol, 159: 299-310.

Phage display and combinatorial methods for generating antibodies are known in the art (as described in, e.g., Ladner et al. U.S. Patent No.5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication WO 93/01288; McCafferty et al. International Publication No. WO 92/01047; Garrard et al. International Publication No. WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) Bio/Technology 9:1370-1372; Hay et al. (1992) Hum Antibod Hybridomas 3:81-85; Huse et al. (1989) Science 246:1275-1281; Griffths et al. (1993) EMBO J 12:725-734; Hawkins et al. (1992) J Mol Biol 226:889-896; Clackson et al. (1991) Nature 352:624-628; Gram et al. (1992) PNAS 89:3576-3580; Garrad et al. (1991) Bio/Technology 9:1373-1377; Hoogenboom et al. (1991) Nuc Acid Res 19:4133-4137; and Barbas et al. (1991) PNAS 88:7978-7982, the contents of all of which are incorporated by reference herein).

Further, methods of generating and selecting non-immunglobulin scaffolds that bind to a particular target are known in the art (see, for example, Škrlec, *et al.*, 2015. *Trends Biotechnol.* 33(7):408-18).

In some embodiments, the CD1a-targeting moiety is an antibody, scFv, Fab, or scFab comprising a VL domain and VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and LCDR1 consists of [QDINKY] (SEQ ID NO: 1), LCDR2 consists of [YTS], LCDR3 consists of [LHYDNLPWT] (SEQ ID NO: 3), HCDR1 consists of [GYAFSTYT] (SEQ ID NO: 4), HCDR2 consists of [INPNSAST] (SEQ ID NO: 5), and HCDR3 consists of [ARGFYTMDY] (SEQ ID NO: 6).

In some embodiments, the CD1a-targeting moiety is a scFv comprising a VL domain and VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and LCDR1 consists of [QDINKY] (SEQ ID NO: 1), LCDR2 consists of [YTS], LCDR3 consists of [LHYDNLPWT] (SEQ ID NO: 3), HCDR1 consists of [GYAFSTYT] (SEQ ID NO: 4), HCDR2 consists of [INPNSAST] (SEQ ID NO: 5), and HCDR3 consists of [ARGFYTMDY] (SEQ ID NO: 6).

In some embodiments, the CD1a-targeting moiety is an antibody, scFv, Fab, or scFab comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO: 7 and the VH domain consists of SEQ ID NO: 8.

In some embodiments, the CD1a-targeting moiety is a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO: 7 and the VH domain consists of SEQ ID NO: 8.

VL domain (SEQ ID NO: 7)
[RDIQMTQSPSSLSASLGGKVTITCQASQDINKYIAWYQFKPGKGPRLLIHYTSTLQPAIPSRFS
GSGSGREYSFSISNLEPEDIATYYCLHYDNLPWTFGGGTKLEIKRA]

VH domain (SEQ ID NO: 8)
[QVQLQQSGAELARPGASVKMSCKASGYAFSTYTMHWVKQRPRQGLEWIGYINPNSASTSY
NENFKDKATLTADKSSNTAYMHLSSLTSEDSAVYYCARGFYTMDYWGQGTSVTVSS]

In some embodiments, the CD1a-targeting moiety is a scFv comprising or consisting of SEQ ID NO: 9.

scFv derived from clone NA1/34.HLK (SEQ ID NO: 9)
[QVQLQQSGAELARPGASVKMSCKASGYAFSTYTMHWVKQRPRQGLEWIGYINPNSASTSY
NENFKDKATLTADKSSNTAYMHLSSLTSEDSAVYYCARGFYTMDYWGQGTSVTVSSGGGGS
GGGGSGGGGSGGGGSRDIQMTQSPSSLSASLGGKVTITCQASQDINKYIAWYQFKPGKGPRLL
IHYTSTLQPAIPSRFSGSGSGREYSFSISNLEPEDIATYYCLHYDNLPWTFGGGTKLEIKRA]

Transmembrane domain

The transmembrane domain may be derived either from a natural or a synthetic source. When the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions may comprise at least the transmembrane region(s) of the α-, β- or ζ- chain of CD28, CD3, CD45, CD4, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, or CD154.

A transmembrane domain may be synthetic or a variant of a naturally occurring transmembrane domain. In some embodiments, synthetic or variant transmembrane domains comprise predominantly hydrophobic residues such as leucine and valine.

In some embodiments, the transmembrane domain comprises the transmembrane domain of CD28, CD3, CD45, CD4, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, or a variant thereof, wherein the variant thereof has a 95% sequence identity.

In some embodiments, the transmembrane domain comprises the transmembrane domain of CD28, CD3, CD45, CD4, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, CD154, or a variant thereof, wherein the variant thereof has a 98% sequence identity.

In some embodiments, the transmembrane domain comprises the transmembrane domain of CD28, CD3, CD45, CD4, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, or CD154.

In some embodiments, the transmembrane domain comprises the transmembrane domain of CD8 or a variant thereof, wherein the variant thereof has a 95% sequence identity.

In some embodiments, the transmembrane domain comprises the transmembrane domain of CD8 or a variant thereof, wherein the variant thereof has a 98% sequence identity.

In some embodiments, the transmembrane domain comprises the transmembrane domain of CD8.

In some embodiments, the transmembrane domain comprises SEQ ID NO: 10 or a sequence that has 95% sequence identity to SEQ ID NO: 10.

In some embodiments, the transmembrane domain comprises SEQ ID NO: 10 or a sequence that has 98% sequence identity to SEQ ID NO: 10.

In some embodiments, the transmembrane domain comprises SEQ ID NO: 10. In some embodiments, the transmembrane domain consists of SEQ ID NO: 10.

Transmembrane domain derived from CD8 (SEQ ID NO: 10)
[TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLV
ITLYC]

Intracellular signaling domain

The intracellular signaling domain provides for the activation of at least one function of the cell expressing the CAR upon binding to the ligand expressed on tumor cells. In some embodiments, the intracellular signaling domain contains one or more intracellular signaling domains. In some embodiments, the intracellular signaling domain is a portion of and/or a variant of an intracellular signaling domain that provides for activation of at least one function of the CAR-comprising cell.

In some embodiments, the intracellular signaling domain comprises the intracellular domain of CD3ζ, FcRγ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b, CD66b, or a variant thereof, wherein the variant thereof has a 95% sequence identity.

In some embodiments, the intracellular signaling domain comprises the intracellular domain of CD3ζ, FcRγ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b, CD66b, or a variant thereof, wherein the variant thereof has a 98% sequence identity.

In some embodiments, the intracellular signaling domain comprises the intracellular domain of CD3ζ, FcRγ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b or CD66b.

In some embodiments, the intracellular signaling domain comprises the intracellular domain of CD3ζ or a variant thereof, wherein the variant thereof has a 95% sequence identity.

In some embodiments, the intracellular signaling domain comprises the intracellular domain of CD3ζ or a variant thereof, wherein the variant thereof has a 98% sequence identity.

In some embodiments, the intracellular signaling domain comprises the intracellular domain of CD3ζ.

In some embodiments, the intracellular signaling domain comprises SEQ ID NO: 11 or a sequence that has 95% sequence identity to SEQ ID NO: 11.

In some embodiments, the intracellular signaling domain comprises SEQ ID NO: 11 or a sequence that has 98% sequence identity to SEQ ID NO: 11.

In some embodiments, the intracellular signaling domain comprises SEQ ID NO: 11 or a sequence that has 99% sequence identity to SEQ ID NO: 11.

In some embodiments, the intracellular signaling domain comprises SEQ ID NO: 11. In some embodiments, the intracellular signaling domain consists of SEQ ID NO: 11.

Intracellular signaling domain derived from CD3ζ (SEQ ID NO: 11)
[RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLY
NELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR]

Costimulatory signaling domain

In some embodiments, the CAR may further comprise a costimulatory signaling domain. In some embodiments, the costimulatory signaling domain comprises the intracellular domain of CD27, CD28, CD137, CD134, CD30, CD40, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, CD276 or a variant thereof, wherein the variant thereof has a 95% sequence identity.

In some embodiments, the costimulatory signaling domain comprises the intracellular domain of CD27, CD28, CD137, CD134, CD30, CD40, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, CD276 or a variant thereof, wherein the variant thereof has a 98% sequence identity.

In some embodiments, the costimulatory signaling domain comprises the intracellular domain of CD27, CD28, CD137, CD134, CD30, CD40, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, or CD276.

In some embodiments, the costimulatory signaling domain comprises the intracellular domain of CD137 or a variant thereof, wherein the variant thereof has a 95% sequence identity.

In some embodiments, the costimulatory signaling domain comprises the intracellular domain of CD137 or a variant thereof, wherein the variant thereof has a 98% sequence identity.

In some embodiments, the costimulatory signaling domain comprises the intracellular domain of CD137.

In some embodiments, the costimulatory signaling domain comprises SEQ ID NO: 12 or a sequence that has 95% sequence identity to SEQ ID NO: 12.

In some embodiments, the costimulatory signaling domain comprises SEQ ID NO: 12 or a sequence that has 98% sequence identity to SEQ ID NO: 12.

In some embodiments, the costimulatory signaling domain comprises SEQ ID NO: 12 or a sequence that has 99% sequence identity to SEQ ID NO: 12.

In some embodiments, the costimulatory signaling domain comprises SEQ ID NO: 12. In some embodiments, the costimulatory signaling domain consists of SEQ ID NO: 12.

Costimulatory signaling domain derived from CD137 (SEQ ID NO: 12)
[KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL]

Full sequence CARs according to the present invention

In some embodiments, the CAR comprises:

(i) a scFv comprising a VL domain and VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and LCDR1 consists of [QDINKY] (SEQ ID NO: 1), LCDR2 consists of [YTS], LCDR3 consists of [LHYDNLPWT] (SEQ ID NO: 3), HCDR1 consists of [GYAFSTYT] (SEQ ID NO: 4), HCDR2 consists of [INPNSAST] (SEQ ID NO: 5), and HCDR3 consists of [ARGFYTMDY] (SEQ ID NO: 6);

(ii) a transmembrane domain comprising SEQ ID NO: 10 or a sequence that has 95% sequence identity to SEQ ID NO: 10;

(iii) an intracellular signaling domain comprising SEQ ID NO: 11 or a sequence that has 95% sequence identity to SEQ ID NO: 11; and

(iv) a costimulatory signaling domain comprising SEQ ID NO: 12 or a sequence that has 95% sequence identity to SEQ ID NO: 12.

In some embodiments, the CAR comprises:

(i) a scFv comprising a VL domain and VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and LCDR1 consists of [QDINKY] (SEQ ID NO: 1), LCDR2 consists of [YTS], LCDR3 consists of [LHYDNLPWT] (SEQ ID NO: 3), HCDR1 consists of [GYAFSTYT] (SEQ ID NO: 4), HCDR2 consists of [INPNSAST] (SEQ ID NO: 5), and HCDR3 consists of [ARGFYTMDY] (SEQ ID NO: 6);

(ii) a transmembrane domain comprising SEQ ID NO: 10 or a sequence that has 98% sequence identity to SEQ ID NO: 10;

(iii) an intracellular signaling domain comprising SEQ ID NO: 11 or a sequence that has 98% sequence identity to SEQ ID NO: 11; and

(iv) a costimulatory signaling domain comprising SEQ ID NO: 12 or a sequence that has 98% sequence identity to SEQ ID NO: 12.

In some embodiments, the CAR comprises:

(i) a scFv comprising a VL domain and VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and LCDR1 consists of [QDINKY] (SEQ ID NO: 1), LCDR2 consists of [YTS], LCDR3 consists of [LHYDNLPWT] (SEQ ID NO: 3), HCDR1 consists of [GYAFSTYT] (SEQ ID NO: 4), HCDR2 consists of [INPNSAST] (SEQ ID NO: 5), and HCDR3 consists of [ARGFYTMDY] (SEQ ID NO: 6);

(ii) a transmembrane domain comprising SEQ ID NO: 10 or a sequence that has 98% sequence identity to SEQ ID NO: 10;

(iii) an intracellular signaling domain comprising SEQ ID NO: 11 or a sequence that has 99% sequence identity to SEQ ID NO: 11; and

(iv) a costimulatory signaling domain comprising SEQ ID NO: 12 or a sequence that has 99% sequence identity to SEQ ID NO: 12.

In some embodiments, the CAR comprises:

(i) a scFv comprising a VL domain and VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and LCDR1 consists of [QDINKY] (SEQ ID NO: 1), LCDR2 consists of [YTS], LCDR3 consists of [LHYDNLPWT] (SEQ ID NO: 3), HCDR1 consists of [GYAFSTYT] (SEQ ID NO: 4), HCDR2 consists of [INPNSAST] (SEQ ID NO: 5), and HCDR3 consists of [ARGFYTMDY] (SEQ ID NO: 6);

(ii) a transmembrane domain comprising SEQ ID NO: 10;

(iii) an intracellular signaling domain comprising SEQ ID NO: 11; and

(iv) a costimulatory signaling domain comprising SEQ ID NO: 12.

In some embodiments, the CAR comprises:

(i) a scFv comprising a VL domain and VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and LCDR1 consists of [QDINKY] (SEQ ID NO: 1), LCDR2 consists of [YTS], LCDR3 consists of [LHYDNLPWT] (SEQ ID NO: 3), HCDR1 consists of [GYAFSTYT] (SEQ ID NO: 4), HCDR2 consists of [INPNSAST] (SEQ ID NO: 5), and HCDR3 consists of [ARGFYTMDY] (SEQ ID NO: 6);

(ii) a transmembrane domain consisting of SEQ ID NO: 10;

(iii) an intracellular signaling domain consisting of SEQ ID NO: 11; and

(iv) a costimulatory signaling domain consisting of SEQ ID NO: 12.

In some embodiments, the CAR comprises:

(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO: 7 and the VH domain consists of SEQ ID NO: 8;

(ii) a transmembrane domain comprising SEQ ID NO: 10 or a sequence that has 95% sequence identity to SEQ ID NO: 10;

(iii) an intracellular signaling domain comprising SEQ ID NO: 11 or a sequence that has 95% sequence identity to SEQ ID NO: 11; and

(iv) a costimulatory signaling domain comprising SEQ ID NO: 12 or a sequence that has 95% sequence identity to SEQ ID NO: 12.

In some embodiments, the CAR comprises:
(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO: 7 and the VH domain consists of SEQ ID NO: 8;
(ii) a transmembrane domain comprising SEQ ID NO: 10 or a sequence that has 98% sequence identity to SEQ ID NO: 10;
(iii) an intracellular signaling domain comprising SEQ ID NO: 11 or a sequence that has 98% sequence identity to SEQ ID NO: 11; and
(iv) a costimulatory signaling domain comprising SEQ ID NO: 12 or a sequence that has 98% sequence identity to SEQ ID NO: 12.

In some embodiments, the CAR comprises:
(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO: 7 and the VH domain consists of SEQ ID NO: 8;
(ii) a transmembrane domain comprising SEQ ID NO: 10 or a sequence that has 98% sequence identity to SEQ ID NO: 10;
(iii) an intracellular signaling domain comprising SEQ ID NO: 11 or a sequence that has 99% sequence identity to SEQ ID NO: 11; and
(iv) a costimulatory signaling domain comprising SEQ ID NO: 12 or a sequence that has 99% sequence identity to SEQ ID NO: 12.

In some embodiments, the CAR comprises:
(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO: 7 and the VH domain consists of SEQ ID NO: 8;
(ii) a transmembrane domain comprising SEQ ID NO: 10;
(iii) an intracellular signaling domain comprising SEQ ID NO: 11; and
(iv) a costimulatory signaling domain comprising SEQ ID NO: 12.

In some embodiments, the CAR comprises:
(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO: 7 and the VH domain consists of SEQ ID NO: 8;
(ii) a transmembrane domain consisting of SEQ ID NO: 10;
(iii) an intracellular signaling domain consisting of SEQ ID NO: 11; and
(iv) a costimulatory signaling domain consisting of SEQ ID NO: 12.

In some embodiments, the CAR comprises or consists of SEQ ID NO: 2 or a sequence that has 95% sequence identity with SEQ ID NO: 2. In some embodiments, the CAR comprises or consists of SEQ ID NO: 2 or a sequence that has 98% sequence identity with SEQ ID NO: 2. In some embodiments, the CAR comprises or consists of SEQ ID NO: 2 or a sequence that has 99% sequence identity with SEQ ID NO: 2. In some embodiments, the CAR comprises or consists of SEQ ID NO: 2.

Full sequence of the CAR (SEQ ID NO: 2)
[MALPVTGLLLSLGLLLHAARPTGQVQLQQSGAELARPGASVKMSCKASGYAFSTYTMHWV
KQRPRQGLEWIGYINPNSASTSYNENFKDKATLTADKSSNTAYMHLSSLTSEDSAVYYCARG
FYTMDYWGQGTSVTVSSGGGGSGGGGSGGGGSGGGGSRDIQMTQSPSSLSASLGGKVTITCQ
ASQDINKYIAWYQFKPGKGPRLLIHYTSTLQPAIPSRFSGSGSGREYSFSISNLEPEDIATYYCL
HYDNLPWTFGGGTKLEIKRATTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFA
CDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEG
GCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQ
EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR]

Nucleic acid

In one aspect, the present invention provides a nucleic acid encoding any one of the CARs of the present invention, including any one of the CARs disclosed above. The nucleic acid sequence that encodes the chimeric receptor links together a number of modular components that can be excised and replaced with other components in order to customize the chimeric receptor for efficient T cell activation and recognition of CD1a.

In some embodiments, the nucleic acid is suitable for transducing or transforming a cell. In some embodiments, the nucleic acid is suitable for transducing or transforming a T cell for use in adoptive immunotherapy.

In some embodiments, the nucleic acid is codon optimized for expression in mammalian cells. Codon optimization methods are known in the art (see, for example, Parret et al., 2016. Curr Opin Struct Biol. 39: 155-162).

The nucleic acid of the present invention may be comprised in a γ-retroviral or lentiviral vector which can be used to transduce or transform a T cell (see Rivière & Sadelain, 2017. *Mol Ther*. 25(5):1117-1124). The nucleic acid may also be inserted into a cell through the use of DNA transposons, RNA transfection or genome editing techniques such as TALEN, ZFN and CRISPR/Cas9 (see Rivière & Sadelain, 2017. *Mol Ther*. 25(5):1117-1124).

Cells

In one aspect, the present invention provides a cell comprising the nucleic acid of the present invention and/or the CAR of the present invention. In some embodiments, the cell is a T-cell (referred to as a CART).

In some embodiments, the cell is a naïve T cell, memory stem T cell or central memory T cell. It is currently thought that these cells are better suited for adaptive immunotherapy (see Rivière & Sadelain, 2017. *Mol Ther*. 25(5):1117-1124).

In some embodiments, the cell is an autologous T cell. The term "autologous cell" refers to a cell obtained from the same patient that is to be treated using any one of the methods of the present invention. It is noted that flow cytometric analysis of peripheral blood obtained from 40 patients with active T-cell acute lymphoblastic leukemia revealed the presence of normal CD3+CD1a- T-cells in all the patients. Thus, it is entirely possible to treat a patient using an autologous T cell comprising the nucleic acid and/or CAR of the present invention.

In some embodiments, the cell is an allo-tolerant T cell. The term "allo-tolerant cell" refers to a cell that has been engineered to decrease the risk of a Graft-versus-host disease response. In some embodiments, this is achieved by genomic editing-mediated deletion of TCR and/or β2-microglobulin[15,19]. Allo-tolerant cells are known in the art (see section of allogeneic T cells in Rivière & Sadelain, 2017. *Mol Ther*. 25(5):1117-1124).

In some embodiments, the T cell is a CD3-positive and CD1a-negative T cell.

In some embodiments, the cell is a lymphoid precursor, embryonic stem cell or an induced pluripotent stem cell with the capacity to differentiate into a mature T cell (see Rivière & Sadelain, 2017. *Mol Ther*. 25(5):1117-1124).

Pharmaceutical composition

In one aspect, the present invention provides a pharmaceutical composition comprising a plurality of cells of the present invention and a pharmaceutically acceptable carrier or diluent.

A pharmaceutical composition as described herein may also contain other substances. These substances include, but are not limited to, cryoprotectants, surfactants, anti-oxidants, and stabilizing agents. The term "cryoprotectant" as used herein, includes agents which provide stability to the CARTs against freezing-induced stresses. Non-limiting examples of cryoprotectants include sugars, such as sucrose, glucose, trehalose, mannitol, mannose, and lactose; polymers, such as dextran, hydroxyethyl starch and

polyethylene glycol; surfactants, such as polysorbates (e.g., PS-20 or PS-80); and amino acids, such as glycine, arginine, leucine, and serine. A cryoprotectant exhibiting low toxicity in biological systems is generally used.

In some embodiments, the cells are formulated by first harvesting them from their culture medium, and then washing and concentrating the cells in a medium and container system suitable for administration (a "pharmaceutically acceptable" carrier) in a therapeutically effective amount. Suitable infusion medium can be any isotonic medium formulation, typically normal saline, Normosol R (Abbott) or Plasma-Lyte A (Baxter), but also 5% dextrose in water or Ringer's lactate can be utilized. The infusion medium can be supplemented with human serum albumin, fetal bovine serum or other human serum components.

In one aspect, the present invention provides a cell according to the present invention or a pharmaceutical composition according to the present invention for use as a medicament.

Methods of treatment

In one aspect, the present invention provides a method of treating a CD1a-positive cancer comprising administering the cell of the present invention or the pharmaceutical composition of the present invention to a patient in need thereof.

In some embodiments, the patient is administered a therapeutically effective amount of cells. In some embodiments, the patient is administered at least 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 or 10^{10} cells. The number of cells will depend upon the ultimate use for which the composition is intended as will the type of cells included therein. For example, if cells that are specific for a particular antigen are desired, then the population will contain greater than 70%, generally greater than 80%, 85% and 90-95% of such cells. For uses provided herein, the cells are generally in a volume of a liter or less, can be 500 ml or less, even 250 ml or less, or 100 ml or less. The clinically relevant number of cells can be apportioned into multiple infusions that cumulatively equal or exceed 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 or 10^{10} cells.

In some embodiments, the cell or pharmaceutical composition is administered intravenously, intraperitoneally, into the bone marrow, into the lymph node, and /or into cerebrospinal fluid.

In some embodiments, the method comprises a combination therapy. In some embodiments, the method comprises further administering an immune checkpoint inhibitor (see Lim & June, 2017. Cell. 168(4):724-740). In a further embodiment, the method comprises further administering an immune checkpoint inhibitor and/or an IAP inhibitor (see WO 2016/054555).

In some embodiments, the cell or pharmaceutical composition as described herein is administered in combination with chemotherapeutic agents and/or immunosuppressants. In an embodiment, a patient is first treated with a chemotherapeutic agent that inhibits or destroys other immune cells followed by the cell or pharmaceutical composition described herein. In some cases, chemotherapy may be avoided entirely.

In some embodiments, the CD1a-positive cancer is cortical T-cell acute lymphoblastic leukemia or Langerhans cell histiocytosis. In some embodiments, the CD1a-positive cancer is cortical T-cell acute lymphoblastic leukemia. In some embodiments, the CD1a-positive cancer is relapsed/refractory cortical T-cell acute lymphoblastic leukemia.

In general, the relapse of leukemia can manifest several months or years after the initial remission; however, most relapses occur within two years after the initial treatment. Refractoriness is a term that implies that the patient has no longer responded to at least one therapy strategy after a relapse.
There is a broad consensus in first-line trials for ALL, specifically in adults that a relapse is defined as "detection of more than 5% of blast cells in the bone marrow after a previous achievement of complete remission (CR) or unequivocal demonstration of extramedullary leukemia participation" (see Gökbuget (2017)). The European Working Group on Adult *ALL* (EWALL) has documented this statement in a consensus recommendation, (see Dohner (2010)) with the additional explanation that "in the case of 5 to 20% of cell blasts at some stage during the intensive treatment phase and / or during regeneration, the evaluation of the bone marrow should be repeated one week later to distinguish among bone marrow relapse and regeneration phenomenon ". The cited definition is based on international recommendations for outcome parameters in acute myeloid leukemia (see Cheson (2003) and Chantepie (213)); that has been extrapolated to several subtypes of ALL, as in the case of T-ALL.

More recently, some trials did not even define the concept of relapse. Therefore, studies with chimeric antigen receptor (CAR) T cells included patients with "measurable disease" and also included patients with haematological relapse (no additional specification) or minimal residual disease (MRE) (see Lee (2015) and Maude (2014) and Gökbuget (2017)). Please refer to:

- Dohner H, Estey EH, Amadori S, et al, Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European Leukemia Net. Blood 2010;115:453–74.
- Cheson BD, Bennett JM, Kopecky KJ, et al. Revised recommendations of the International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. J Clin Oncol 2003;21:4642–9.

- Chantepie SP, Cornet E, Salaun V, Reman O. Hematogones: an overview. Leuk Res 2013;37:1404–11.
- 13. Maude SL, Frey N, Shaw PA, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med 2014;371:1507–17.
- Gökbuget N, Dombret H, Bassan R, Wadleigh M, Doubek M, Ribera J. Inclusion and response criteria for clinical trials in relapsed/refractory acute lymphoblastic leukemia and usefulness of historical control trials. *Haematologica*. 2017;102(3):e118–e119.

In some embodiments, the patient to be treated with the method of the present invention is in complete or near-complete remission after treatment with another therapy. It may be preferable desirable to decrease the tumor burden before using the methods of the present invention because since there are several alternative effector T-cells in cases of patients with highly active relapsed/refractory cortical T-cell acute lymphoblastic leukemia. In some embodiments, the patient to be treated with the method of the present invention has previously been treated with another therapy which resulted in a partial response, complete response, stable disease, decrease in progressive disease, reduced time to tumor progression or any combination thereof.

Examples

Materials and methods

CD1a-specific scFv generation and CAR design

The CD1a-specific single-chain variable fragment (scFv) derived from the NA1/34.HLK clone of CD1a-specific antibody was obtained using commercial synthesis (Sigma-Aldrich) with the mouse IgG Library Primer Set (Progen), and was cloned into a pCCL lentiviral-based second-generation CAR backbone containing a human CD8 transmembrane (TM) domain, human CD137 and CD3ζ endodomains, and a T2A-GFP cassette. Identical lentiviral vectors expressing either GFP alone (mock vector) or CD22 CAR backbone were used as controls (Fig 1D & 8A).

CAR-expressing lentiviral production, T-cell transduction, activation and expansion

CAR-expressing viral particles pseudotyped with VSV-G were generated in 293T cells using a standard polyethylenimine transfection protocol, and were concentrated by ultracentrifugation as described elsewhere [27]. Viral titers were consistently in the range of 10^8 TU/mL. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats from healthy volunteers by Ficoll-Hypaque gradient centrifugation. Buffy coats were obtained from the Barcelona Blood and Tissue Bank (BST) upon IRB approval (HCB/2018/0030). T-cells were activated by plate-bound anti-CD3 (OKT3) and anti-CD28 antibodies (BD Biosciences) for 2 days and were then transduced with CAR-expressing lentivirus (MOI=10) in the presence of interleukin-7 (IL-7) and IL-15 (10 ng/mL, Mitenyi Biotec) [16,18]. The cell surface expression of CD1aCAR was traced by fluorescence-activated cell sorting (FACS) co-expression of GFP and was confirmed using an AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG

(H+L) (Jackson ImmunoResearch). Proper activation of CAR-transduced T cells was demonstrated by staining for CD25 and CD69 after 2-day expansion.

Immunophenotyping of healthy CD34+ progenitors, T-cells and primary T-ALL samples

The expression of CD1a antigen in CD34+ stem/progenitor cells (HSPCs), CD34+CD7+ thymic T-cell progenitors and CD3+ T-cells was prospectively analyzed in fresh human thymus, fetal liver and bone marrow (BM), cord blood and adult BM and peripheral blood (PB) (n=3). Fetal tissue was collected as previously described [28,29] from developing embryos aborted at 18-22 weeks of pregnancy, obtained from the MRC/Wellcome Trust Human Developmental Biology Resource upon informed consent and approval by our local ethics and biozahard board committee (CMRBCEIC-26/2013). Neonatal and adult tissues were obtained from the BST upon IRB approval (HCB/2018/0030). Primary T-ALL samples and diagnostic immunophenotyping data were obtained from the local hospitals *Sant Joan de Den, Germans Trias i Pujol,* and *Santa Creu i San Pau* (Barcelona, Spain). For immunophenotyping of T-ALL primary samples, the following fluorochrome-conjugated monoclonal antibodies (MoAb) were used: anti-CD2-PE, CD7-FITC/PE, CD13-PerCP-Cy5.5, CD34-APC, CD3-PE, CD5-FITC, CD4-BV-421, CD8-APC-Cy7, CD45-AmCyan, CD1 a-BV-421/APC/PE, CD33-APC and CD123-APC (BDBiosciencies or Miltenyi Biotec). Isotype-matched, non-reactive fluorochrome-conjugated MoAb were always used as a fluorescence reference. Briefly, PB mononuclear cells (PBMCs, ~$5x10^5$) were incubated with erythrocyte-lysing solution (BDBiosciencies) for 10 min and then stained with MoAb (20 min at 4°C in the dark). Stained cells were washed in phosphate buffered saline (PBS) and analyzed by FACS on a FACSCanto-II flow cytometer equipped with FACSDiva software (BDBiosciencies) [30-32].

In vitro cytotoxicity assays and cytokine release determination

Cell lines Jurkat, MOLT4 and NALM6 were purchased from DSMZ (Braunschweig, Germany) and expanded according to DSMZ recommendations. Luciferase (Luc)/GFP-expressing cells were stably generated by retroviral transduction and FACS purification of GFP+ cells [33]. Target cells (cell lines and primary T-ALL blasts) were labeled with 3 μM eFluor670 (eBioscience) and incubated with CD1a, CD22 or mock CARTs at different Effector:Target (E:T) ratios for the indicated time periods. CART-mediated cytotoxicity was determined by analyzing the residual alive (7-AAD-) eFluor670+ target cells at each time point and E:T ratio. Absolute cell counts were determined using Trucount absolute count beads (BD Biosciences). Additionally, FACS-sorted CD3+CD1a- mature T-cells from the PB of cortical T-ALL patients at presentation were activated, transduced with CD1a CAR and tested against their eFluor670-labeled autologous CD1a+ T-ALL blasts. The production of the pro-inflammatory cytokines IL-2, TNFα and IFNγ was measured by ELISA (Human ELISA SET, BD Biosciences) in supernatants harvested after 16 hours.

In vivo Jurkat and T-ALL patient-derived xenograft (PDX) models

Six- to 12-week-old nonobese diabetic (NOD)-Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice (The Jackson Laboratory) were bred and housed under pathogen-free conditions in the animal facility of the Barcelona Biomedical Research Park (PRBB). Mice were irradiated (2 Gy) and intravenously (i.v.) transplanted with $3x10^6$ Luc-GFP-expressing Jurkat cells or with $1x10^6$ primary cortical CD1a+ T-ALL blasts (primary and primograft-expanded)[34]. Between 1.5 $x10^6$ to $5x10^6$ CD1a or mock CARTs were i.v. infused 3 days later. When Luc-Jurkat cells were used, tumor burden was followed by bioluminescence (BLI) using the Xenogen IVIS 50 Imaging System (Perkin Elmer). To measure luminescence, mice received 150 mg/kg of D-luciferin intraperitoneally, and tumor burden was monitored at the indicated time points. Living Image software (Perkin Elmer) was used to visualize and calculate total luminescence. Tumor burden of primary T-ALL samples was followed-up by biweekly bleeding and FACS analysis. Mice were sacrificed when mock CARTs-treated animals were leukemic, and tumor burden (hHLA-ABC+hCD45+hCD1a+ graft) and CART persistence (hHLA-ABC+hCD45+hCD3+hCD1a-GFP+) was analysed in BM, PB and spleen by FACS. In re-challenge experiments, leukemia-free animals that had received an infusion of CD1a CARTs 5-6 weeks before were re-infused with either $1.5x10^6$ Luc-Jurkat cells or $1x10^6$ CD1a+ T-ALL primografts, and disease reappearance was followed-up by BLI and FACS, as above. All procedures were performed in compliance with the institutional animal care and usage committee of the PRBB (DAAM7393).

Enzyme-linked immunospot assay (ELISpot)

ELISpot plates (Millipore) were coated with anti-human IFNγ antibody (1-D1K, Mabtech) and kept overnight at 4°C. Plates were then washed six times with PBS containing 1% fetal calf serum and then cells from three independent donors were plated at $5x10^5$ to $1x10^6$ cells/well and cultured in triplicate for 20h at 37°C and 5% CO2. We measured IFNγ-secreting cells in response to CEF at 1 μg/mL, a peptide pool of T-cell epitopes of Cytomegalovirus (CMV), Epstein-Barr virus (EBV) and Flu and to staphylococcal enterotoxin B (SEB) at 1 μg/mL as a positive control. Plates were then revealed with biotinylated anti-human IFNγ, streptavidin-alkaline phosphatase (Mabtech), as previously described [35,36]. The frequency of IFNγ-secreting cells was quantified using ImmunoCapture and ImmunoSpot software to calculate the number of IFNγ Spot Forming Units per 10^5 (SFU).

Statistical analysis

Data from at least three individual donors are shown in all figures, and experimental duplicates were always performed. At least five animals were used in each *in vivo* condition. All p-values were calculated by unpaired two-tailed Student's t-test using Prism software (GraphPad). Event-free-survival (EFS) of mice was determined using a Mantle-Cox test. A p-value <0.05 was considered statistically significant.

Example 1: CD1a specifically marks cortical T-ALL blasts

The shared expression of target antigens between CARTs and T-lineage blasts has limited immunotherapy approaches in T-ALL due to CART-related fratricide and potential life-threating T-cell aplasia. However, CD1a antigen is expressed in cortical T-ALLs, a major subset of T-ALLs (Fig 1A,B), but is completely absent in functional T-cells in all extra-thymic tissues [25], and steady-state CD34+ HSPCs lack CD1a expression in multiple hematopoietic sites across ontogeny (Fig 1C). T-cell development is initiated within the thymus by a first colonizing $CD34^{high}CD7$-CD1a- primitive HSPC with lympho-myeloid potential, which then differentiate in response to the thymic microenvironment into $CD34^{high}$CD7+CD1a- early T-cell progenitors [37]. As they progress through thymic differentiation, T-cell progenitors maintain CD7 expression and gradually lose CD34 expression, whereas CD1a expression emerges and is transiently confined to cortical thymocytes [38] (Fig 1E,F). Within the CD34+ thymic population, ~50% is represented by pre-cortical T-cell progenitors (CD34highCD7+CD1a-, 1 E,F (grey cells)), allowing us to hypothesize that CD1a may be a feasible and safe target for immunotherapy in R/R cortical T-ALL, which have a fatal outcome [3,39-41].

Example 2: CD1a-redirected T-cells (CD1a CARTs) expand without T-cell fratricide

We designed a second-generation CD1a CAR consisting of anti-CD1a scFv, a CD8 TM spacer, and intracellular signaling domains from 4-1BB (CD137) and CD3ζ coupled in-frame with GFP through a T2A sequence (Fig 2A). The expression of the CD1a CAR was easily detected through coexpression of both scFv and GFP in 293T cells (Fig 2B) and in primary CD4+ and CD8+ T-cell subsets (Fig 2C). Importantly, activated (CD69+CD25+) CD1a CARTs (Fig 2D) continuously expanded 200-fold over a 12-day period, similar to MOCK T-cells (Fig 2E), demonstrating that redirecting CARTs against CD1a antigen does not induce T-cell fratricide.

Example 3: CD1a CARTs specifically eradicate T-ALL cell lines and primary blasts *in vitro*

CD1a CARTs were then tested *in vitro* using the CD1a+ T-ALL cell lines Jurkat and MOLT4, and the B-ALL cell line NALM6 as a negative control (Fig 2F). Compared with control CARTs (either MOCK T-cells or CD22 CARTs), CD1a CARTs specifically eliminated CD1a+ T-ALL cells in a manner dependent on the E:T ratio. A relatively low E:T ratio of 2:1 or 4:1 induced 50-80% specific cell lysis in 16h-assays (Fig 2H,I, 9). Importantly, barely any CD1a+ T-ALL cells survived exposure to CD1a CARTs in a 72 h-assay at a 1:1 E:T ratio (Fig 2I). CD1a CARTs produced high levels of the pro-inflammatory cytokines IL-2, TNFα and IFNγ on co-culture with CD1a+ T-ALL cells confirming their action (Fig 2K).

To further address their ability to eliminate primary tumors, CD1a CARTs were co-cultured with primary cortical T-ALL samples (either freshly harvested or PDX-derived), with a proportion of CD1a+ blasts ranging between 80% and 98% (Fig 3A). Compared with MOCK T-cells, CD1a CARTs specifically eliminated primary CD1a+ cortical T-ALL cells in 72h cytotoxicity assays at 4:1 E:T ratio

(Fig 3B,C). Normal hematopoietic cells (CD1a-) co-existing in BM with CD1a+ T-ALL blasts were not lysed by CD1a CARTs (Fig 3C). High-levels of IFNγ and TNFα were also secreted on co-culture with CD1a+ primary T-ALL cells (Fig 3D). Collectively, these results show that CD1a CARTs have a potent and specific anti-leukemic activity against T-ALL cell lines and primary blasts *in vitro*.

Example 4: CD1a CARTs demonstrate potent anti-leukemia activity *in vivo*

We next evaluated the activity of CD1a CARTs *in vivo* using both Luc-expressing Jurkat T-ALL cells (Fig 4, 10) and a primary cortical T-ALL xenograft model [34] (Fig 5). NSG mice were transplanted with $3x10^6$ Luc-expressing Jurkat cells three days prior to i.v. infusion of either $2x10^6$ or $5x10^6$ CD1a (or MOCK) CARTs, and leukemia establishment was followed-up weekly by BLI (Fig 4A, 10). In contrast to the mice receiving MOCK T-cells, which showed massive tumor burden by BLI, those mice receiving CD1a CARTs were practically leukemia-free by day 25 (Fig 4B,C, 10). The control of leukemia progression was CD1a CART cell dose-dependent (Fig 10B,C). Flow cytometry analysis of tumor burden in PB at sacrifice confirmed the BLI data (Fig 4D). Importantly, FACS analysis revealed T-cell persistence in all hematopoietic tissues analyzed (Fig 4E); however, we found a significantly increased biodistribution of CD1a CARTs in BM and spleen, as compared with T-cell biodistribution in mice receiving MOCK T-cells (Fig 4E), indicative of an active control of disseminated leukemia by CD1a CARTs.

In a clinically more relevant PDX model of cortical T-ALL, NSG mice were first transplanted with $1x10^6$ primary CD1a+ T-ALL blasts followed three days later by infusion of $1x10^6$ CD1a (or MOCK) CARTs, and leukemia engraftment was then followed-up bi-weekly by bleeding and endpoint BM analysis (Fig 5A). Engraftment of CD1a+ cortical T-ALL cells gradually increased over time both in BM (Fig 5B, 50% ± 13% and 55% ± 11% on week 6 and 9, respectively) and PB (Fig 5C, 4.4% ± 2% and 18% ± 6% on week 6 and 9, respectively) in MOCK T-cells-treated PDXs, and associated with a significantly lower 9-week OS (42% *vs* 100%, p=0.01; Fig 5D). In contrast, CD1a CARTs fully abolished T-ALL growth/engraftment (0.36% and 0% T-ALL blasts in BM and PB, respectively) and they persisted in BM and PB after 9 weeks (Fig 5B,C,E).

Example 5: *In vivo* persistent CD1a CARTs are functional in re-challenge assays

Because the persistence of CARTs in hematopoietic tissues is a major biological parameter for their clinical success, we next assessed whether CD1a CARTs persisting after 40-50 days remained functional and efficient in controlling T-ALL progression. To do this, T-ALL-transplanted mice in which the leukemia was abolished on treatment with CD1a CARTs were rechallenged with either Luc-Jurkat cells (Fig 6A-D) or primary T-ALLs from a primograft (Fig 6E-G). In contrast to controls in which the secondary leukemias rapidly (as soon as 2 weeks after) and massively engrafted, T-ALL engraftment

was barely detectable by either BLI or FACS in the Jurkat (Fig 6C) or primograft model after 6 weeks (Fig 6F).

Example 6: Patient-derived CD1a CARTs specifically target autologous CD1a+ blasts and retain antiviral activity

The proper choice of the target antigen and avoiding T-cell fratricide are crucial for the success of CARTs in the treatment of T-ALL. Accordingly, we examined whether PB-derived CD3+CD1a- T-cells from patients with cortical T-ALL can be isolated and genetically modified to express CD1a CAR (Fig 7). Thus, CD3+CD1a- T-cells from patients were isolated (>95% purity, data not shown), activated with CD3/CD28 and lentivirally transduced (31-70% transduction) with CD1a CAR or MOCK. Next, we investigated the cytolytic capacity of CD1a CARTs derived from primary T-ALLs against active T-ALL patient-matched PBMCs (Fig 7A). Total PBMCs were used as targets because it allows us to assess both the autologous cytotoxicity potential and the degree of fratricide. Within eFluor670-labelled target PBMCs, the great majority are CD1a+ blasts and ~15% are CD3+CD1a- normal T-cells (Fig 7B). As compared with MOCK T-cells, the CD1a CARTs showed a massive and specific cytolytic capacity against autologous CD1a+ blasts but not against CD1a- normal T-cells (Fig 6B), further demonstrating that CD1a CARTs are fratricide-resistant.

To further assess the potential thymic toxicity of CD1a CARTs, we next used human normal fetal thymus-derived CD7+ thymocytes as target cells. Only the CD1a+ cortical thymocytes (second and third grey box) were eliminated by the CD1a CARTs, whereas developmentally earlier and later CD1a- (first box) thymic T-lineage populations (CD7+CD34+ and CD7+CD34-) were not targeted (Fig 1E,F), limiting the on-target/off-tumor effects to a developmentally transient thymic population of cortical thymocytes. We finally sought to determine whether CD1a CARTs can protect, by themselves, the host by targeting the most common pathogens causing viremia in immunosuppressed patients. To do this, we tested the reactivity of CD1a CARTs to CMV, EBV and Flu antigens (CEF) and quantified the SCFs by IFNγ ELISpot. Both MOCK T-cells and CD1a CARTs responded very similarly to stimulation with viral peptides, suggesting that CD1a CARTs retain antiviral activity (Fig 7D).

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CLAIMS

1. A T cell comprising a nucleic acid encoding a chimeric antigen receptor (CAR) comprising:
(i) an extracellular domain comprising a CD1a targeting-moiety, wherein the CD1a targeting moiety is a scFV comprising a VL domain consisting of SEQ ID NO: 7 and a VH domain consisting of SEQ ID NO: 8;
(ii) a transmembrane domain; and
(iii) an intracellular signaling domain;

for use in a method of treating a CD1a-positive cancer.

2. The T cell for use according to claim 1, wherein the transmembrane domain comprises the transmembrane domain of CD28, CD3, CD45, CD4, CD8, CD9, CD16, CD22, CD33, CD37, CD64, CD80, CD86, CD134, CD137, or CD154.

3. The T cell for use according to claim 2, wherein the transmembrane domain comprises the transmembrane domain of CD8.

4. The T cell for use according to any one of claims 1-3, wherein the intracellular signaling domain comprises the intracellular domain of CD3ζ, FcRγ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b or CD66b.

5. The T cell for use according to claim 4, wherein the intracellular signaling domain comprises the intracellular domain of CD3ζ.

6. The T cell for use according to any one of claims 1-5, wherein the CAR further comprises a costimulatory signaling domain, preferably the costimulatory signaling domain comprises the intracellular domain of CD27, CD28, CD137, CD134, CD30, CD40, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, CD278 or CD276.

7. The T cell for use according to claim 6, wherein the costimulatory signaling domain comprises the intracellular domain of CD137.

8. A pharmaceutical composition comprising a plurality of cells as defined in any of the precedent claims and a pharmaceutically acceptable carrier or diluent, for use in a method of treating a CD1a-positive cancer.

9. The T cell for use according to any of claims 1 to 7, wherein the CD1a-positive cancer is cortical T-cell acute lymphoblastic leukemia, preferably relapsed/refractory cortical T-cell acute lymphoblastic leukemia.

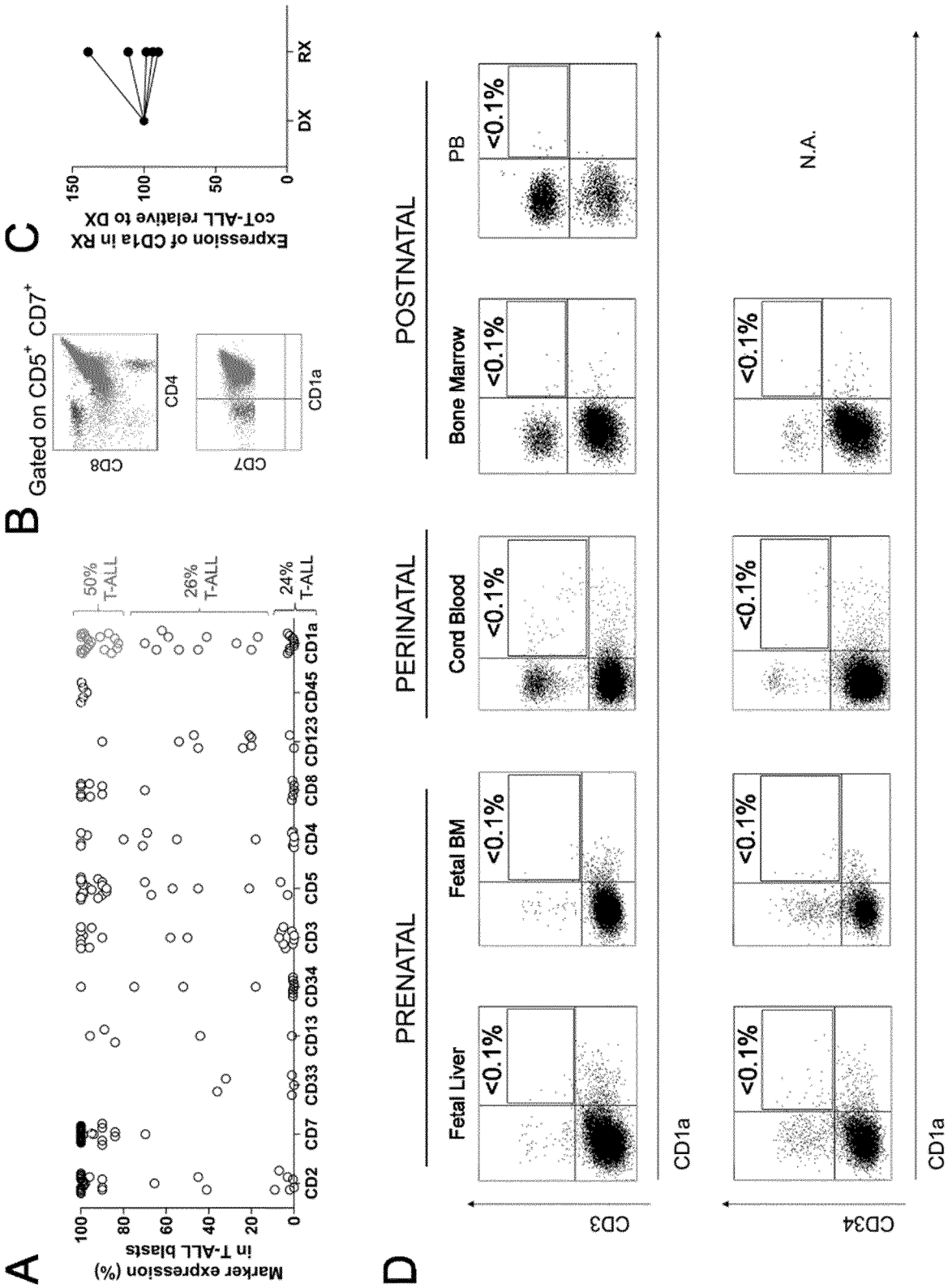
10. The pharmaceutical composition according to claim 8, wherein the CD1a-positive cancer is cortical T-cell acute lymphoblastic leukemia, preferably relapsed/refractory cortical T-cell acute lymphoblastic leukemia.

11. The T cell for use according to any of claims 1 to 7, wherein the CD1a-positive cancer is CD1a+ T-cell lymphoblastic lymphomas, preferably relapsed/refractory CD1a+ T-cell lymphoblastic lymphomas.

12. The pharmaceutical composition according to claim 8, wherein the CD1a-positive cancer is CD1a+ T-cell lymphoblastic lymphomas, preferably relapsed/refractory CD1a+ T-cell lymphoblastic lymphomas.

Figures

Figure 1



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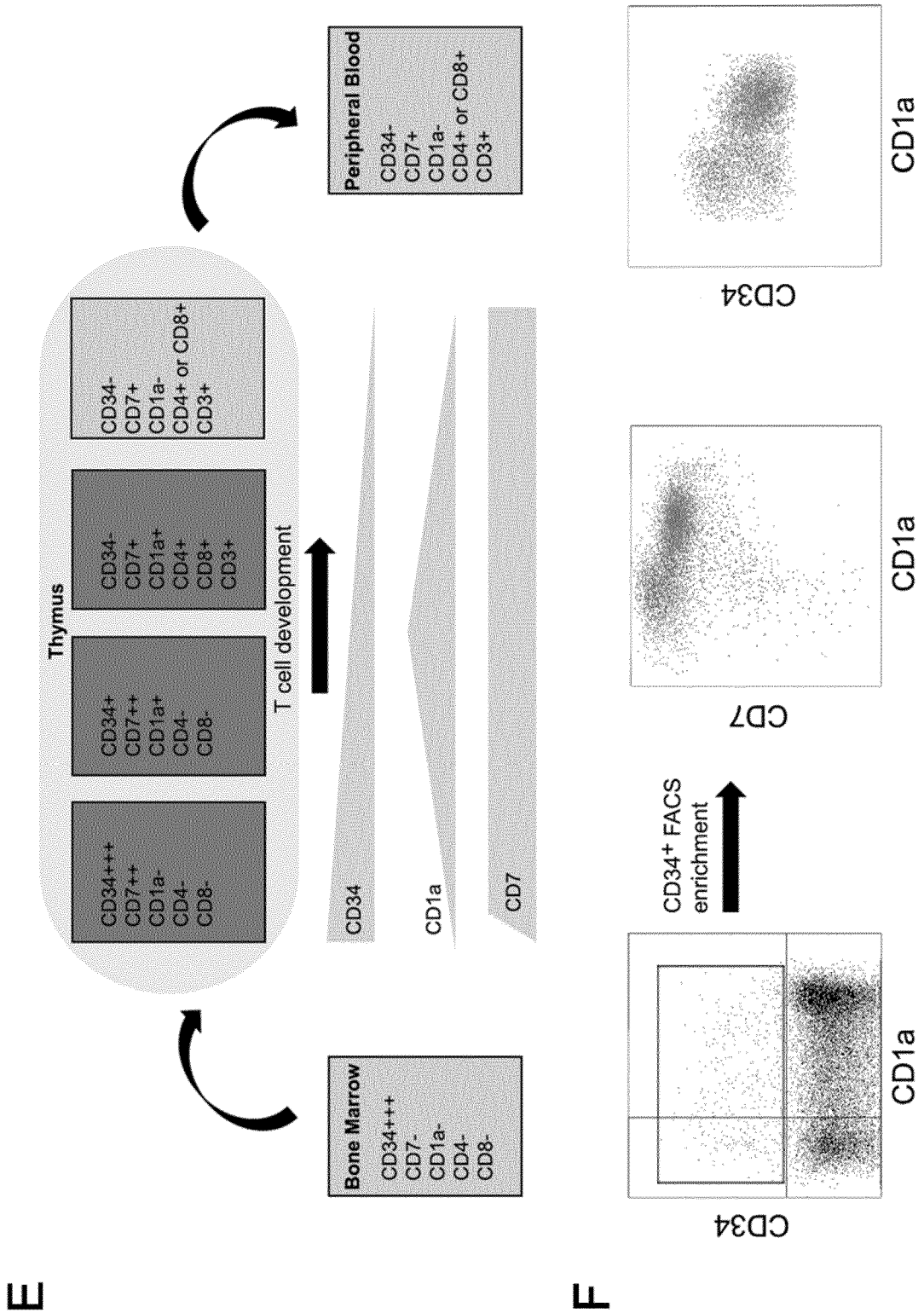


Figure 2

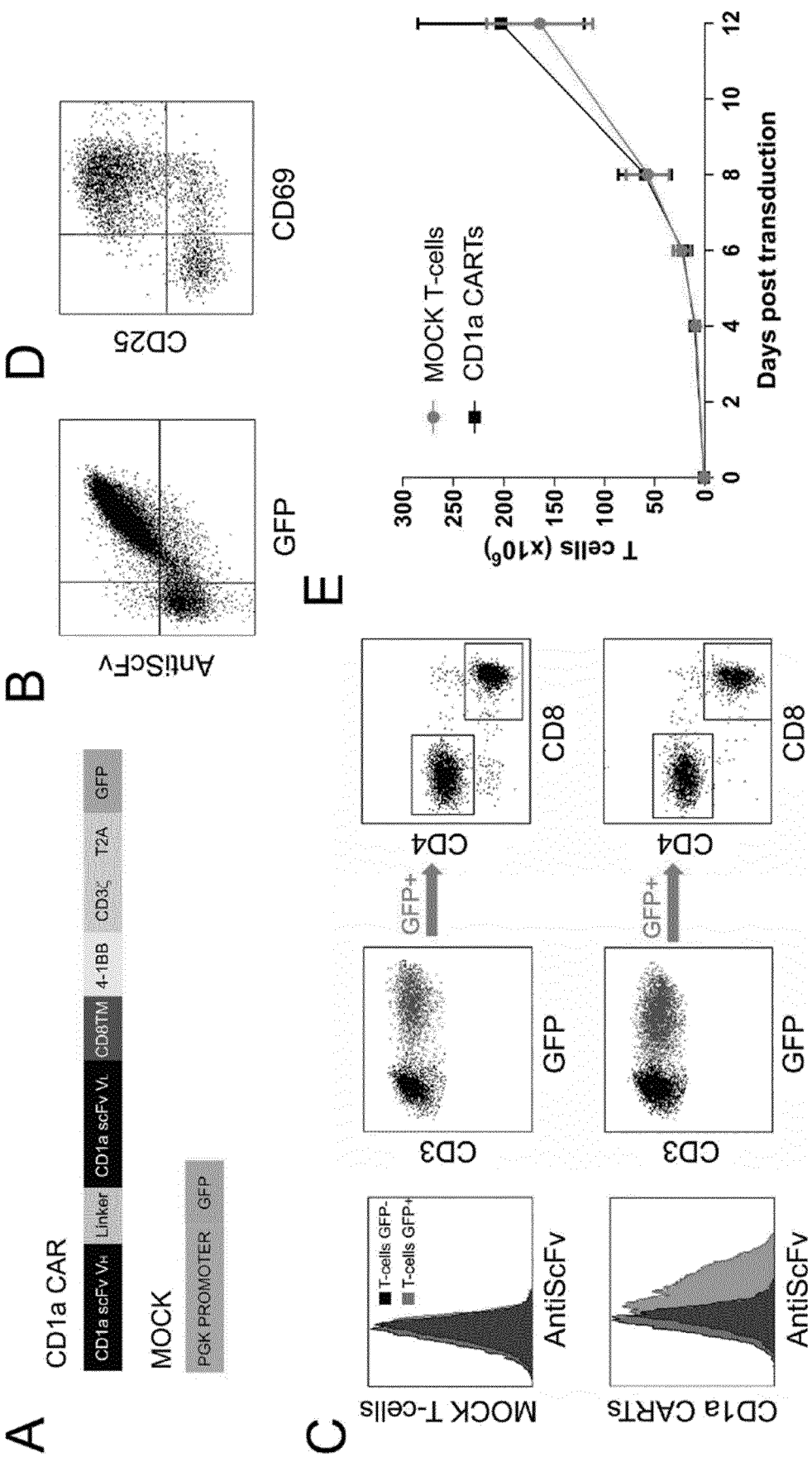

A
CD1a CAR
CD1a scFv VH
Linker
CD1a scFv VL
CD8TM
4-1BB
CD3ζ
T2A
GFP
MOCK
PGK PROMOTER
GFP
B
AntiScFv
GFP
D
CD25
CD69
E
MOCK T-cells
CD1a CARTs
T cells (x10⁶)
Days post transduction
C
MOCK T-cells
CD1a CARTs
T-cells GFP-
T-cells GFP+
AntiScFv
CD3
GFP
GFP+
CD4
CD8

Figure 2 (Cont.)

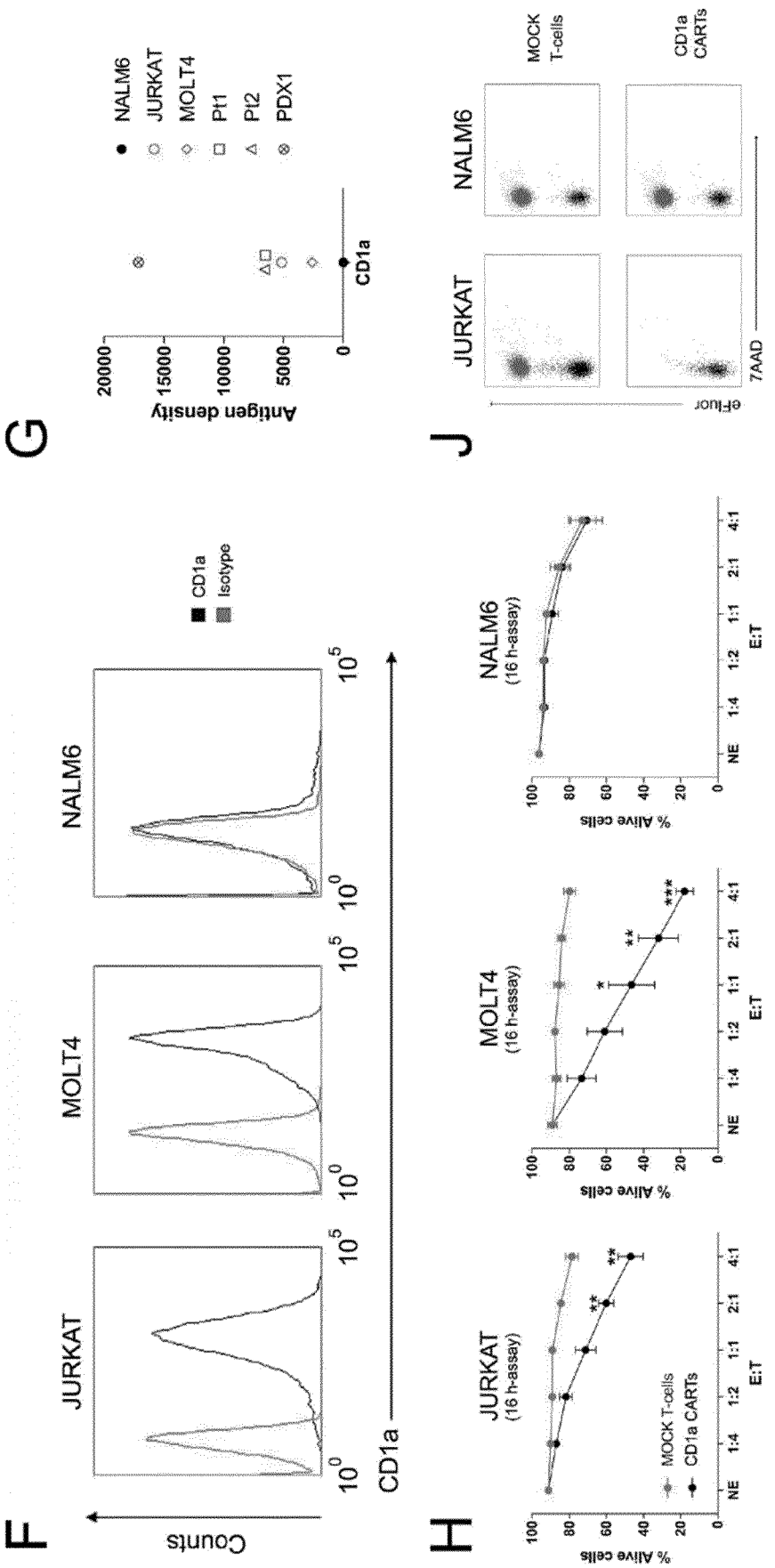


Figure 2 (Cont.)

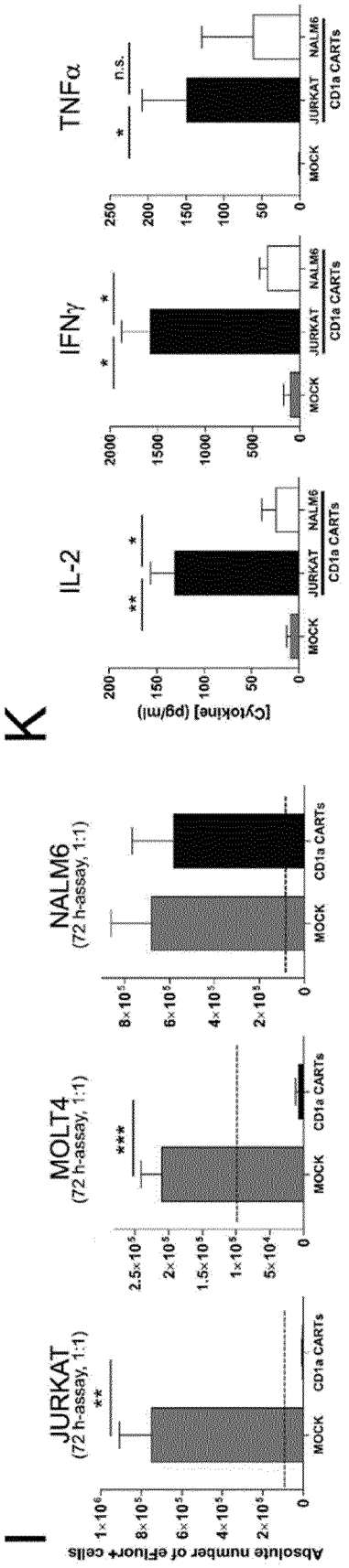


Figure 3

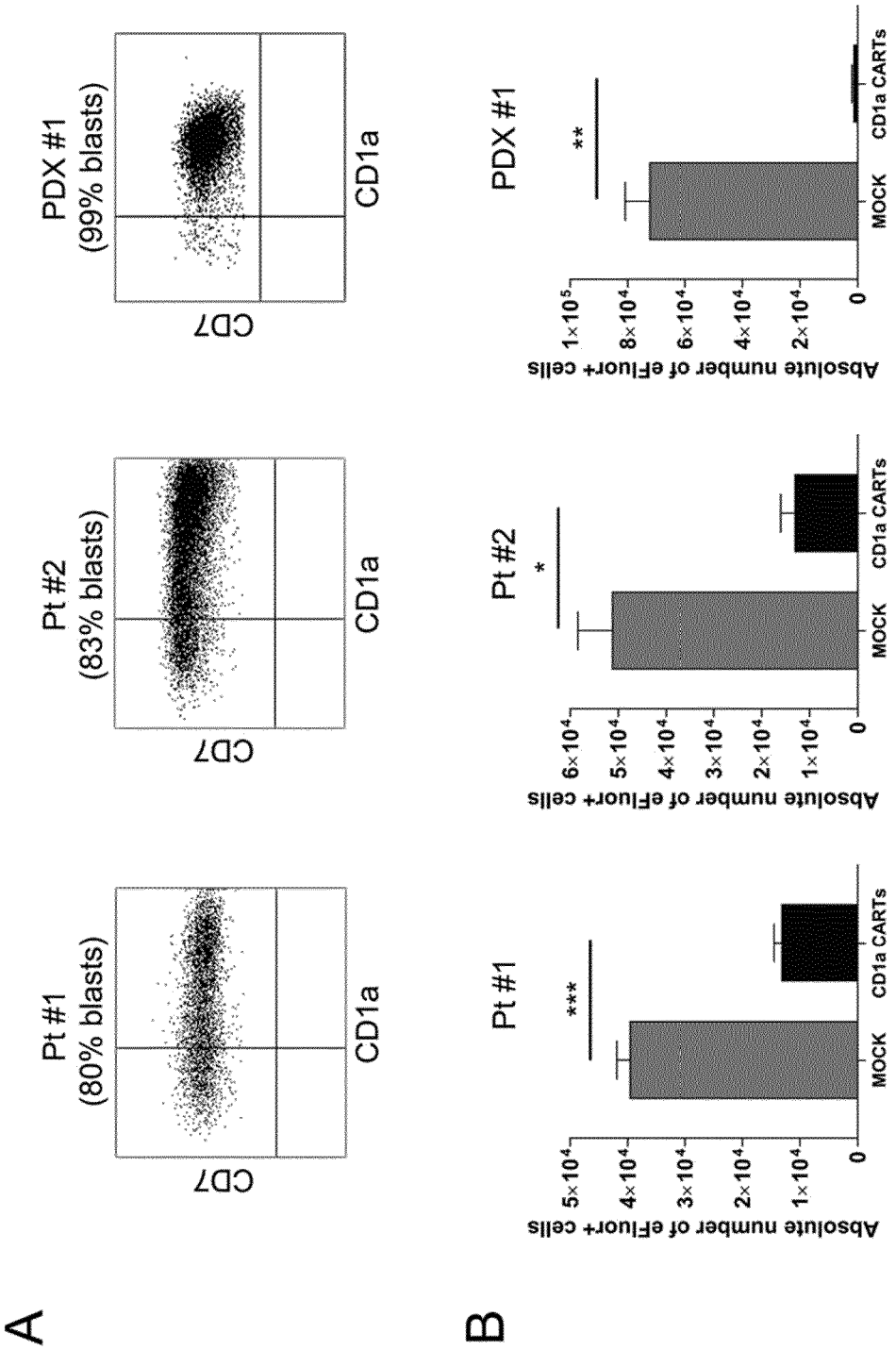

A
PDX #1
(99% blasts)
Pt #2
(83% blasts)
Pt #1
(80% blasts)
CD7
CD1a
B
PDX #1
Pt #2
Pt #1
**
*
***
MOCK
CD1a CARTs
Absolute number of eFluor+ cells
0
1×10^4
2×10^4
3×10^4
4×10^4
5×10^4
6×10^4
8×10^4
1×10^5

Figure 3(Cont.)

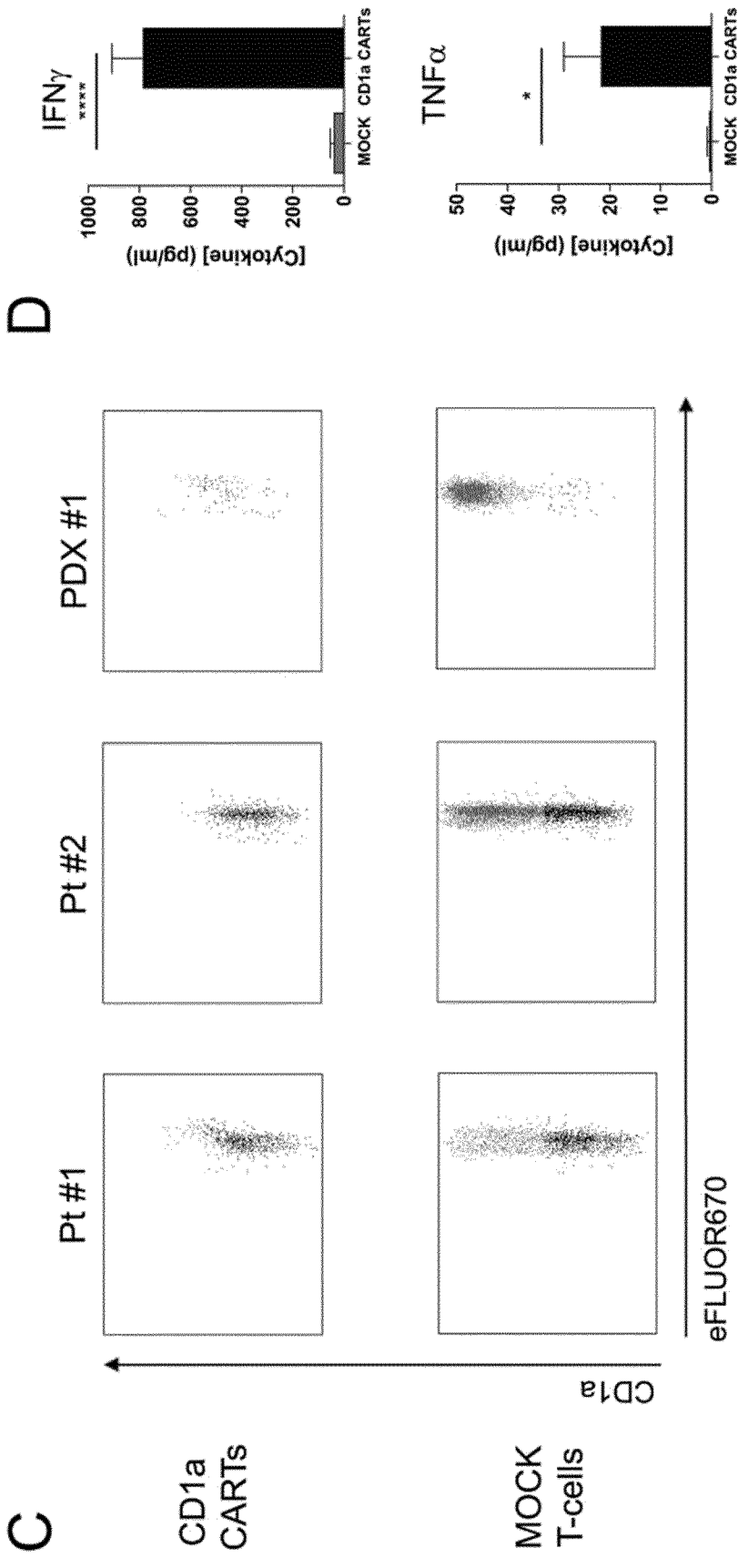


Figure 4

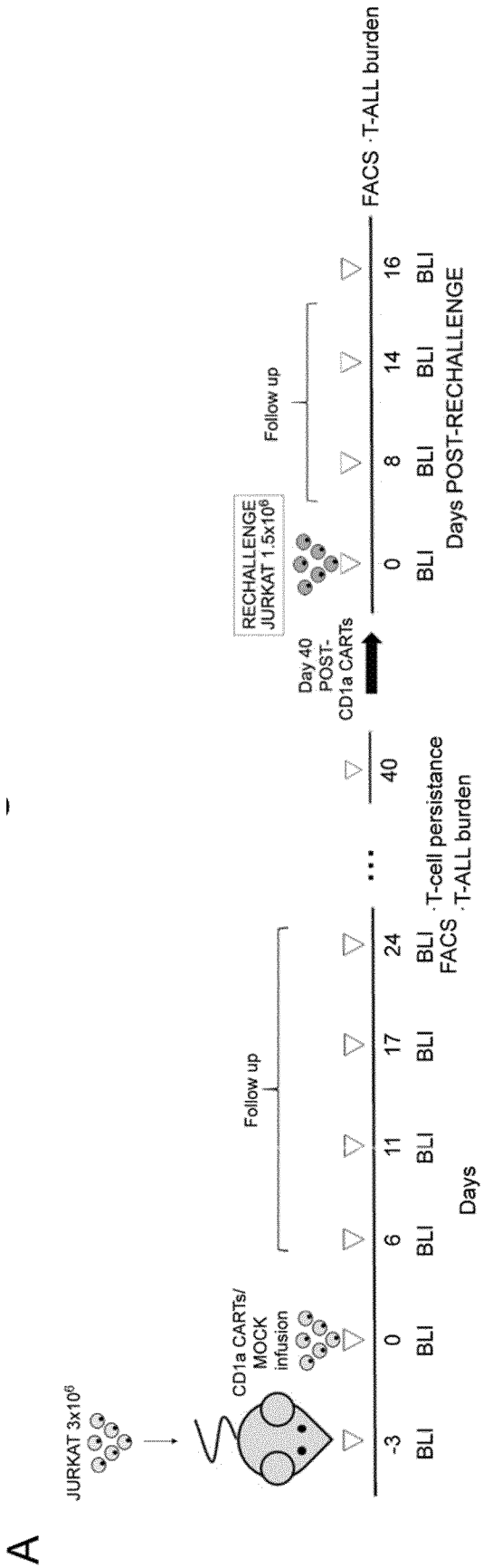
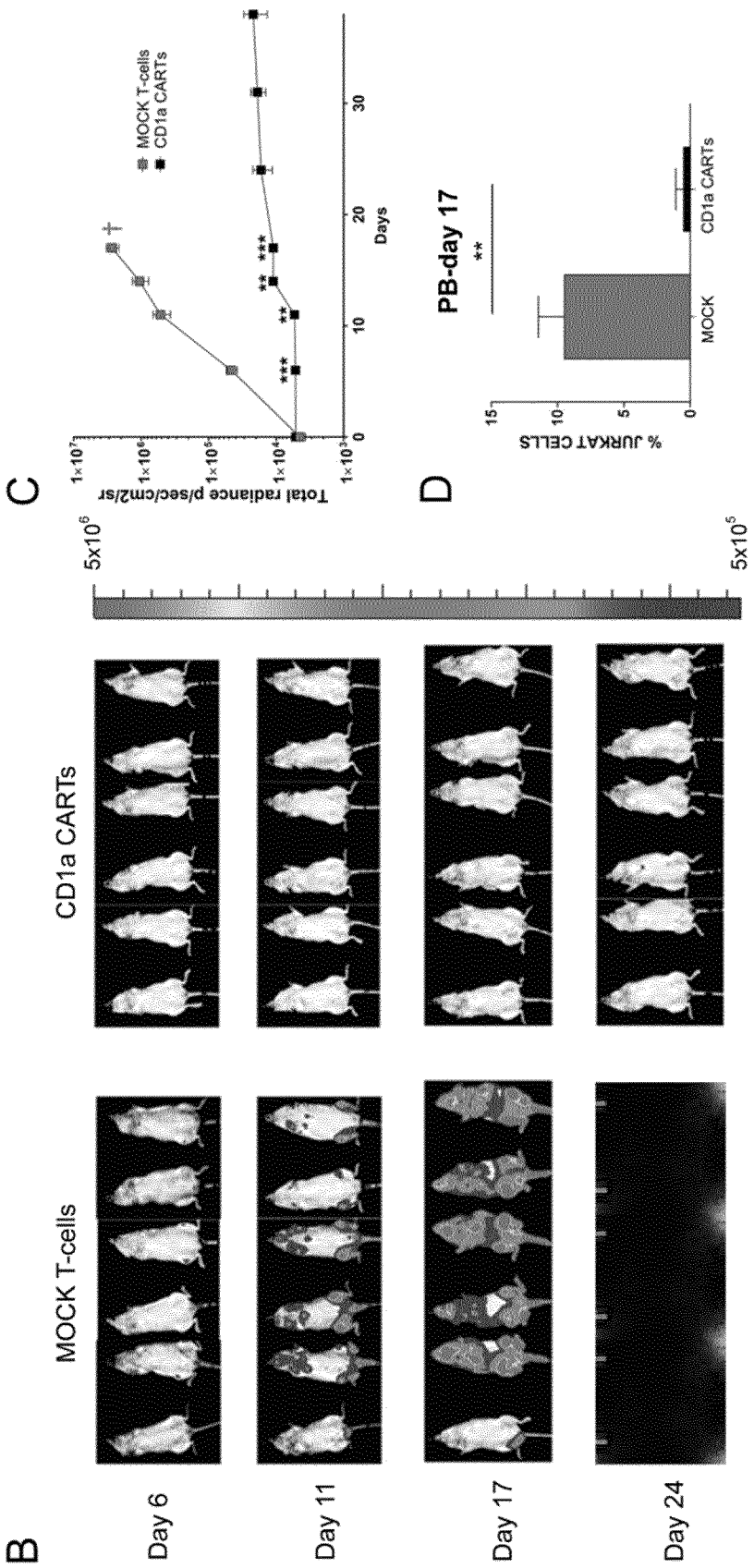

A
JURKAT 3x10^6
CD1a CARTs/ MOCK infusion
Follow up
-3 BLI
0 BLI
6 BLI
11 BLI
17 BLI
24 BLI FACS
T-cell persistance T-ALL burden
40
Days
Day 40 POST-CD1a CARTs
RECHALLENGE JURKAT 1.5x10^6
Follow up
0 BLI
8 BLI
14 BLI FACS
16 BLI
T-ALL burden
Days POST-RECHALLENGE

Figure 4 (Cont.)



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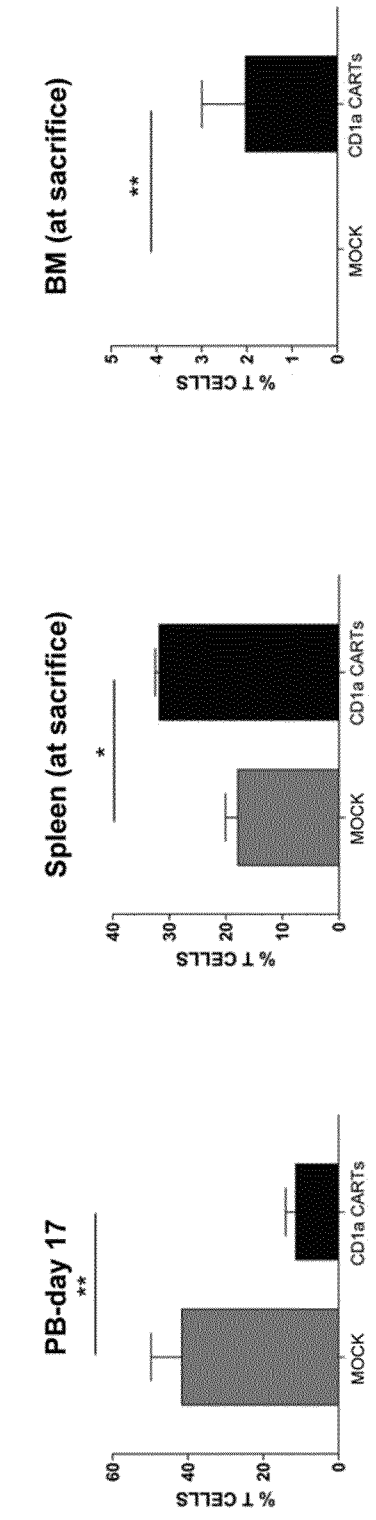


E

Figure 5

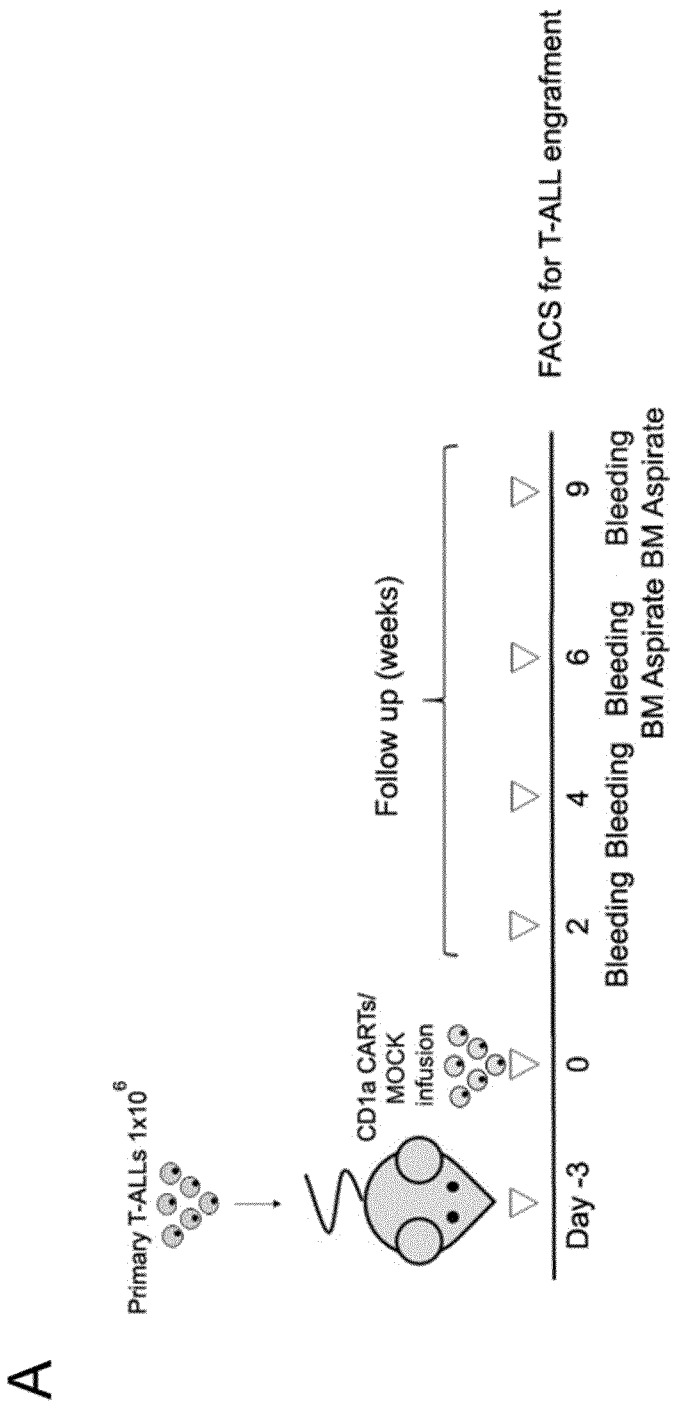
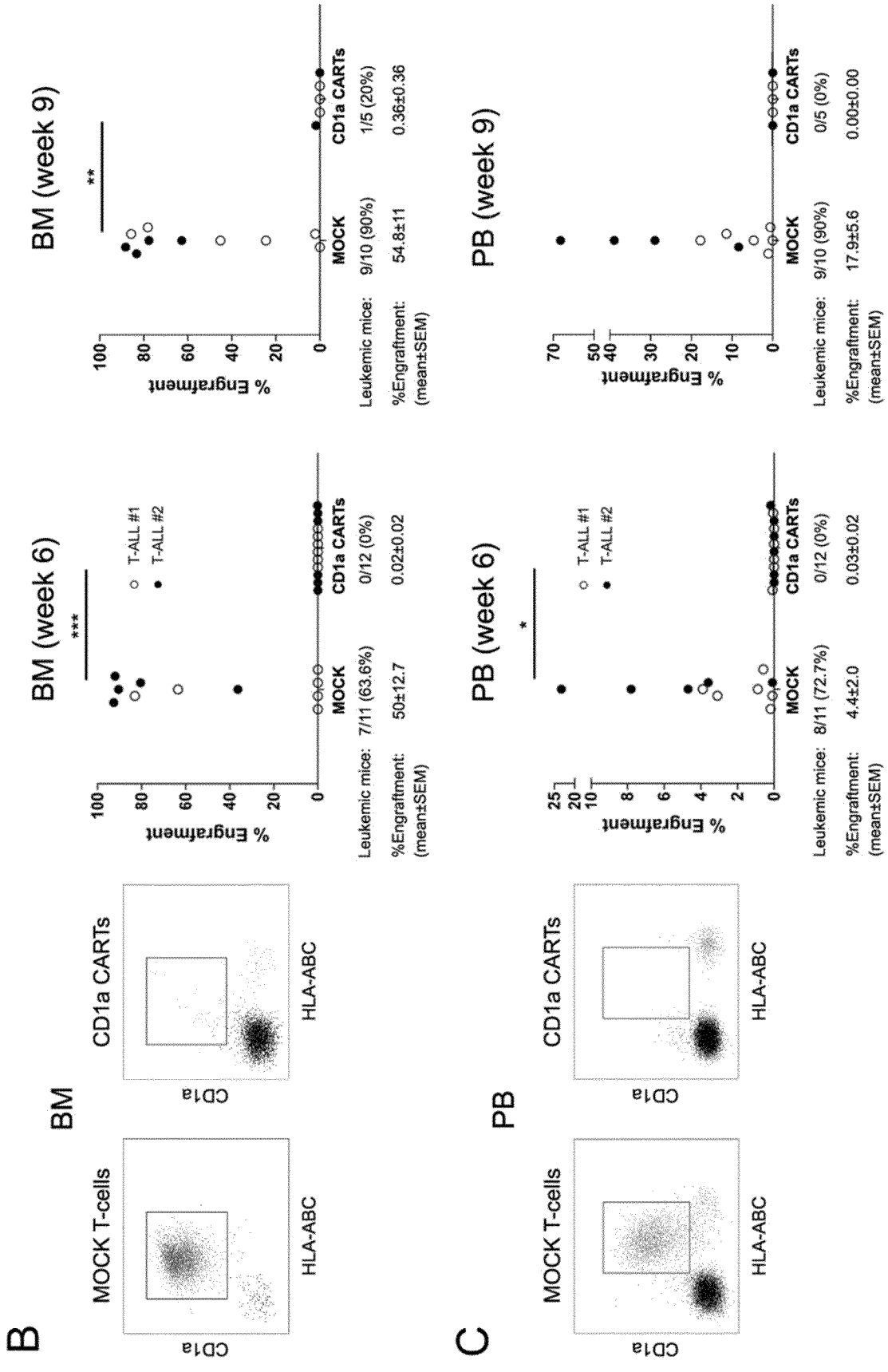

A
Primary T-ALLs 1x10^6
CD1a CARTs/
MOCK
infusion
Follow up (weeks)
FACS for T-ALL engrafment
Day -3
0
2
4
6
9
Bleeding
Bleeding
Bleeding
Bleeding
BM Aspirate
BM Aspirate

Figure 5 (Cont.)



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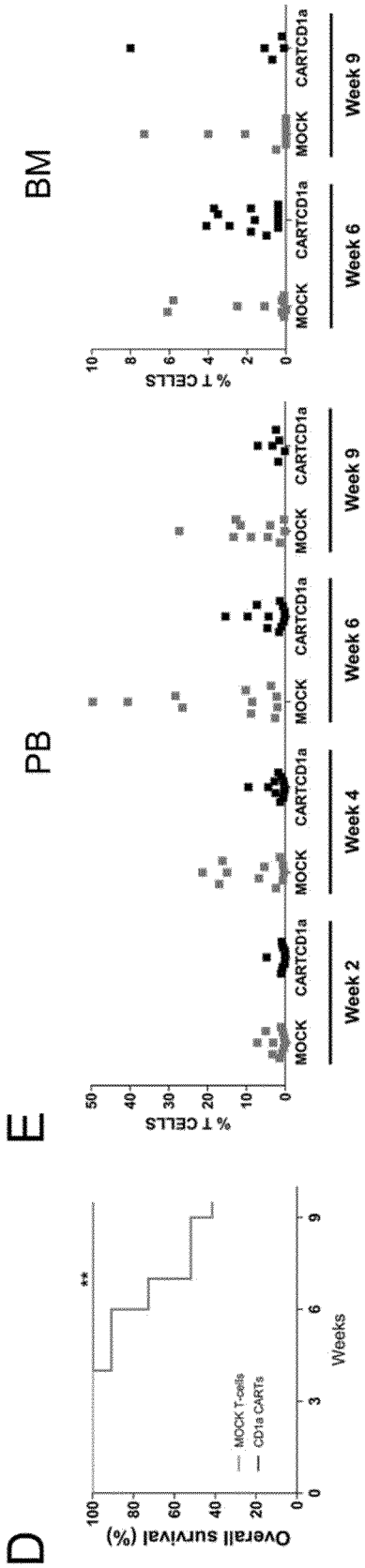


Figure 6

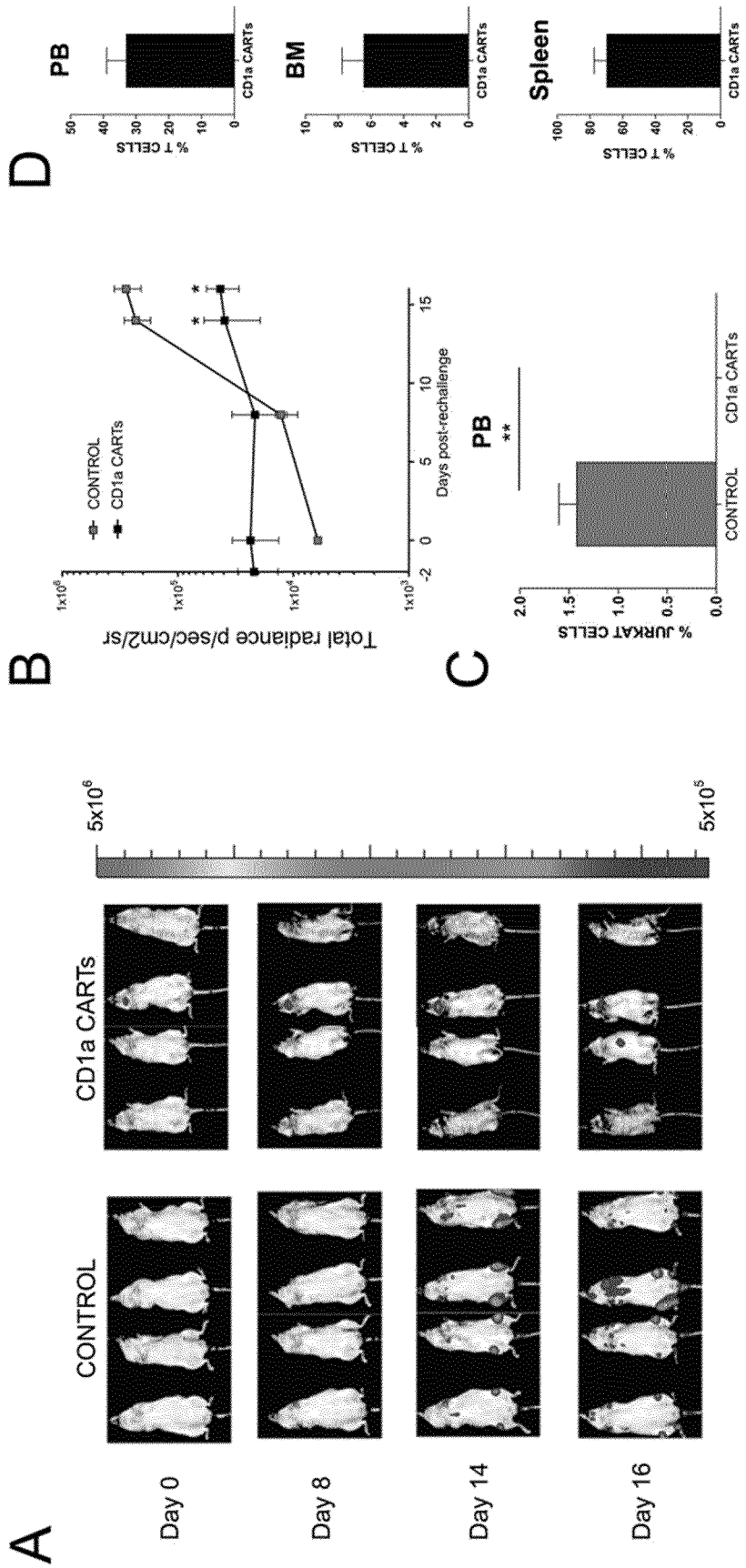

A
CONTROL
CD1a CARTs
5x10^6
5x10^5
Day 0
Day 8
Day 14
Day 16
B
Total radiance p/sec/cm2/sr
CONTROL
CD1a CARTs
Days post-rechallenge
C
PB
**
% JURKAT CELLS
CONTROL
CD1a CARTs
D
PB
BM
Spleen
% T CELLS
CD1a CARTs

Figure 6 (Cont.)

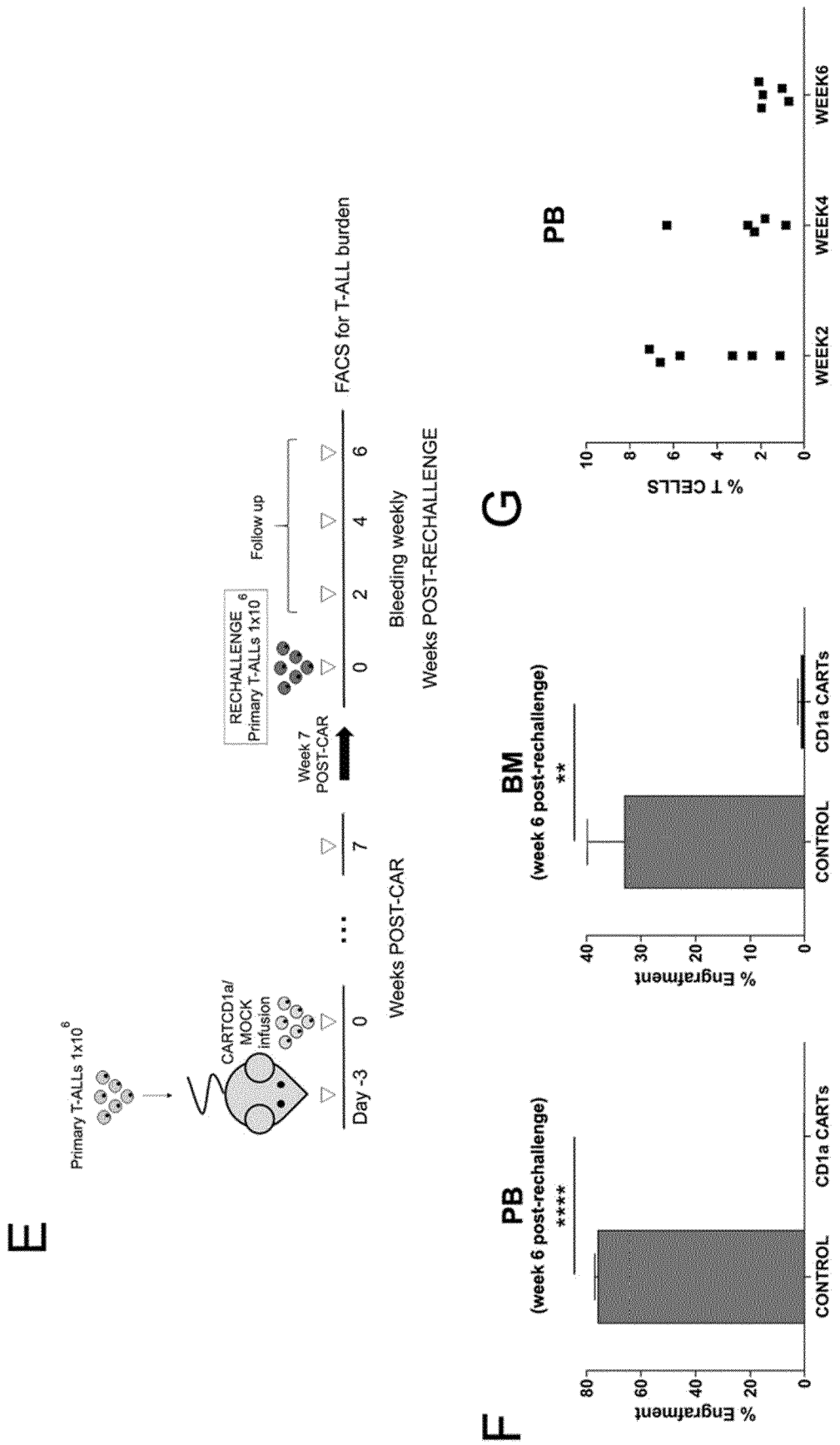


Figure 7

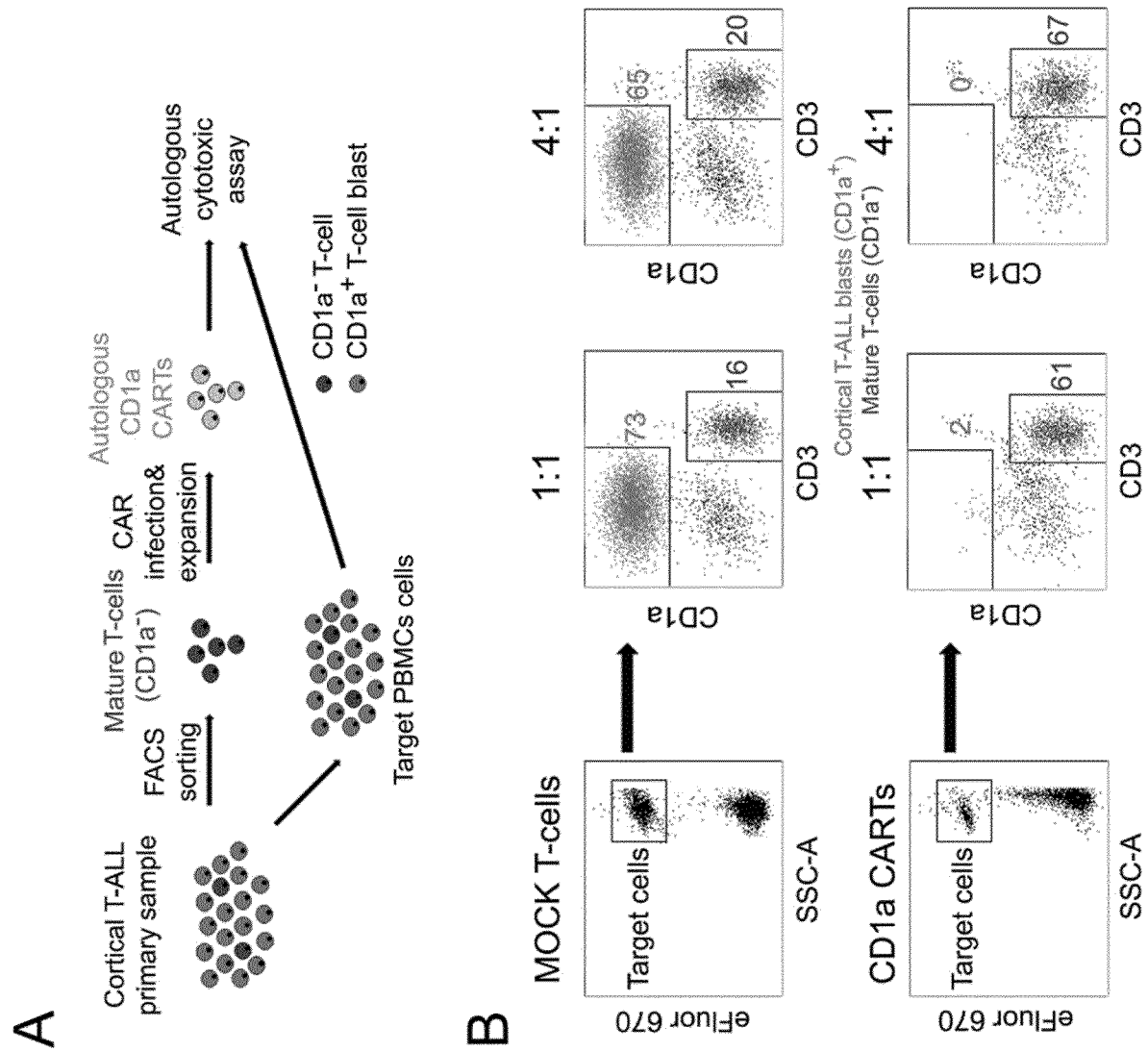

A
Cortical T-ALL primary sample
FACS sorting
Mature T-cells (CD1a⁻)
CAR infection& expansion
Autologous CD1a CARTs
Autologous cytotoxic assay
CD1a⁻ T-cell
CD1a⁺ T-cell blast
Target PBMCs cells
B
MOCK T-cells
CD1a CARTs
1:1
4:1
Target cells
SSC-A
eFluor 670
CD1a
CD3
73
16
65
20
2
61
0
67
Cortical T-ALL blasts (CD1a⁺)
Mature T-cells (CD1a⁻)

Figure 7 (Cont.)

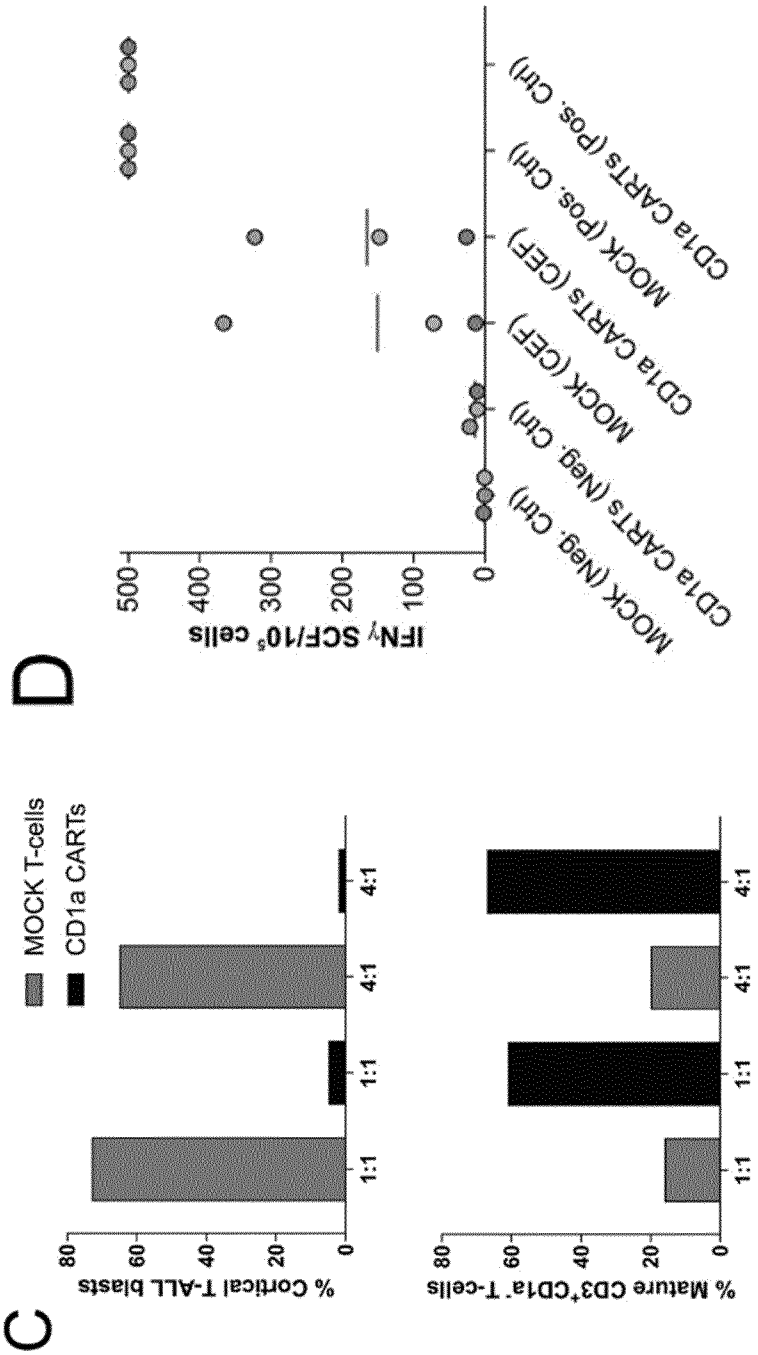


Figure 8

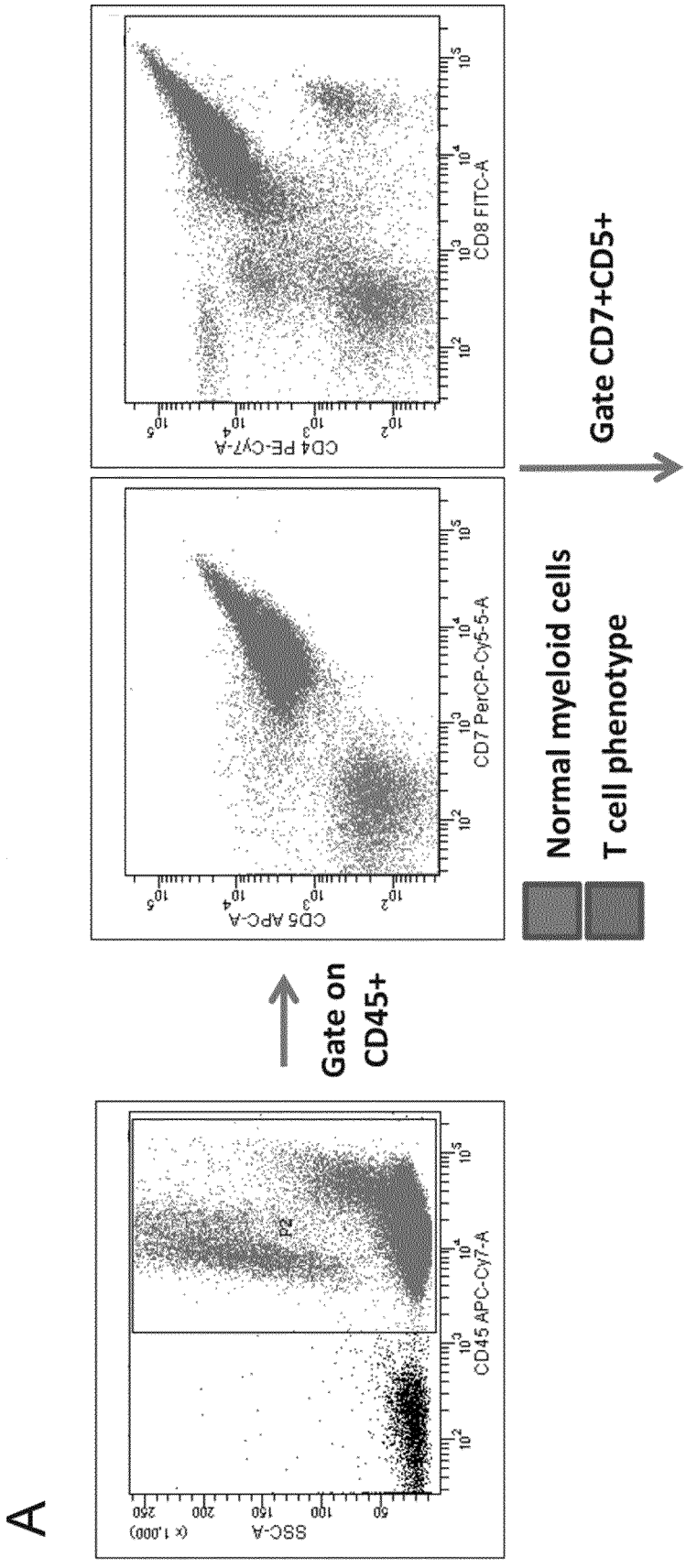
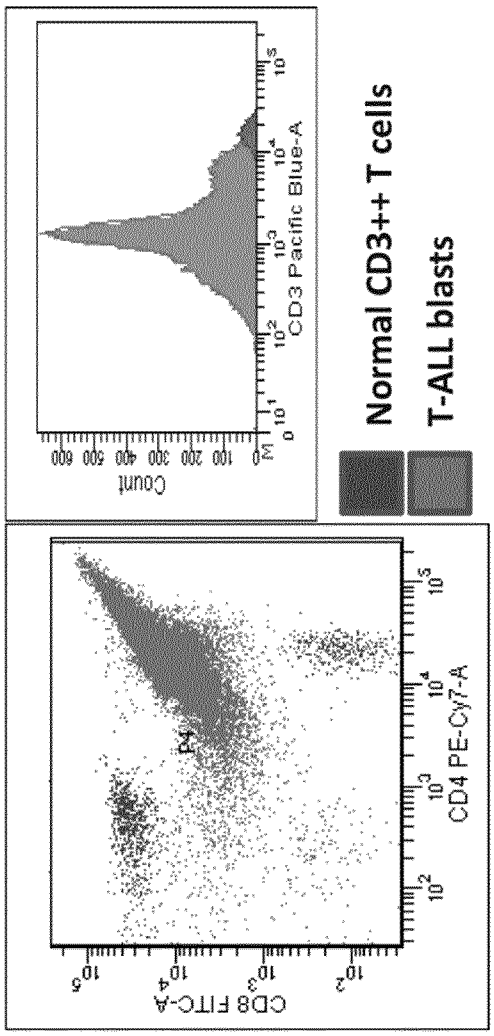

A
SSC-A
(x 1,000)
CD45 APC-Cy7-A
P2
Gate on CD45+
CD5 APC-A
CD7 PerCP-Cy5-5-A
CD4 PE-Cy7-A
CD8 FITC-A
Gate CD7+CD5+
Normal myeloid cells
T cell phenotype

Figure 8 (Cont.)



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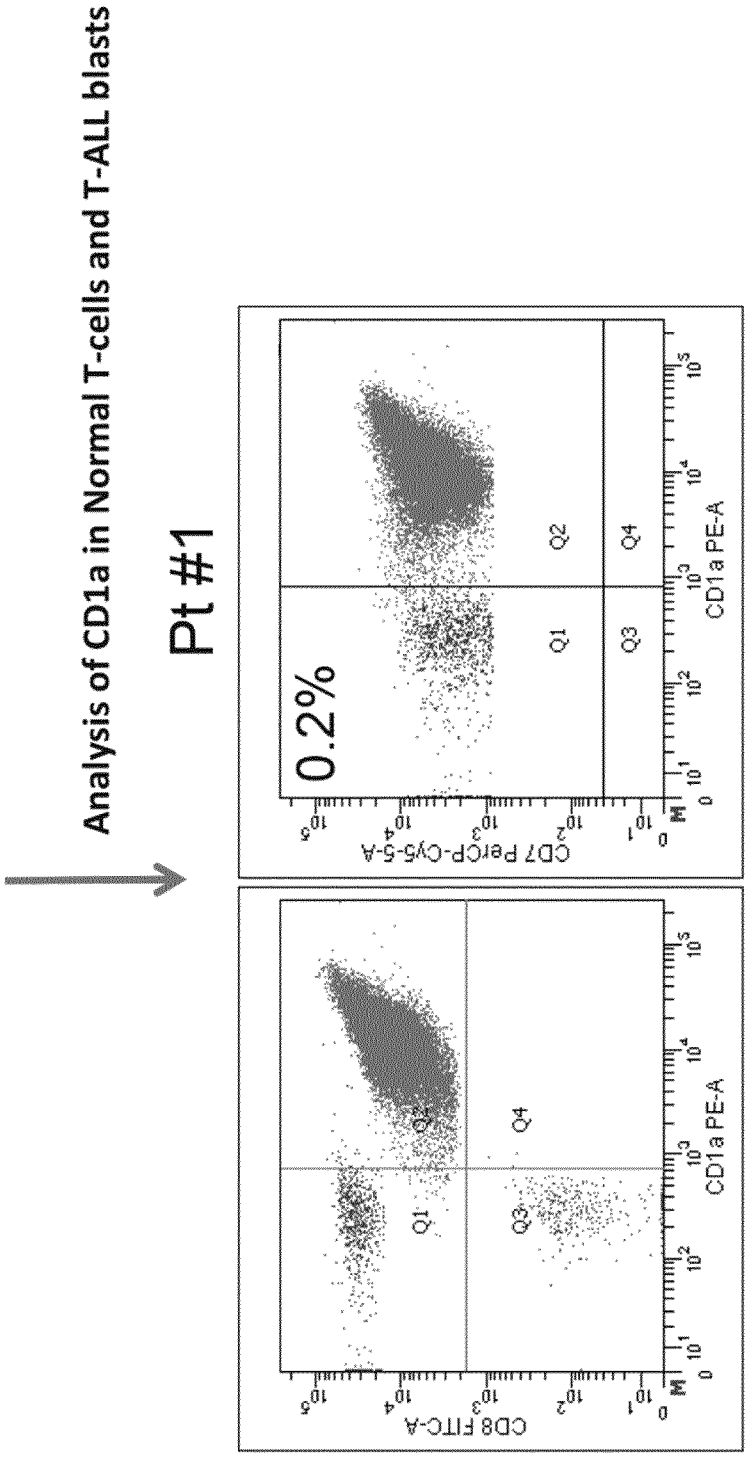

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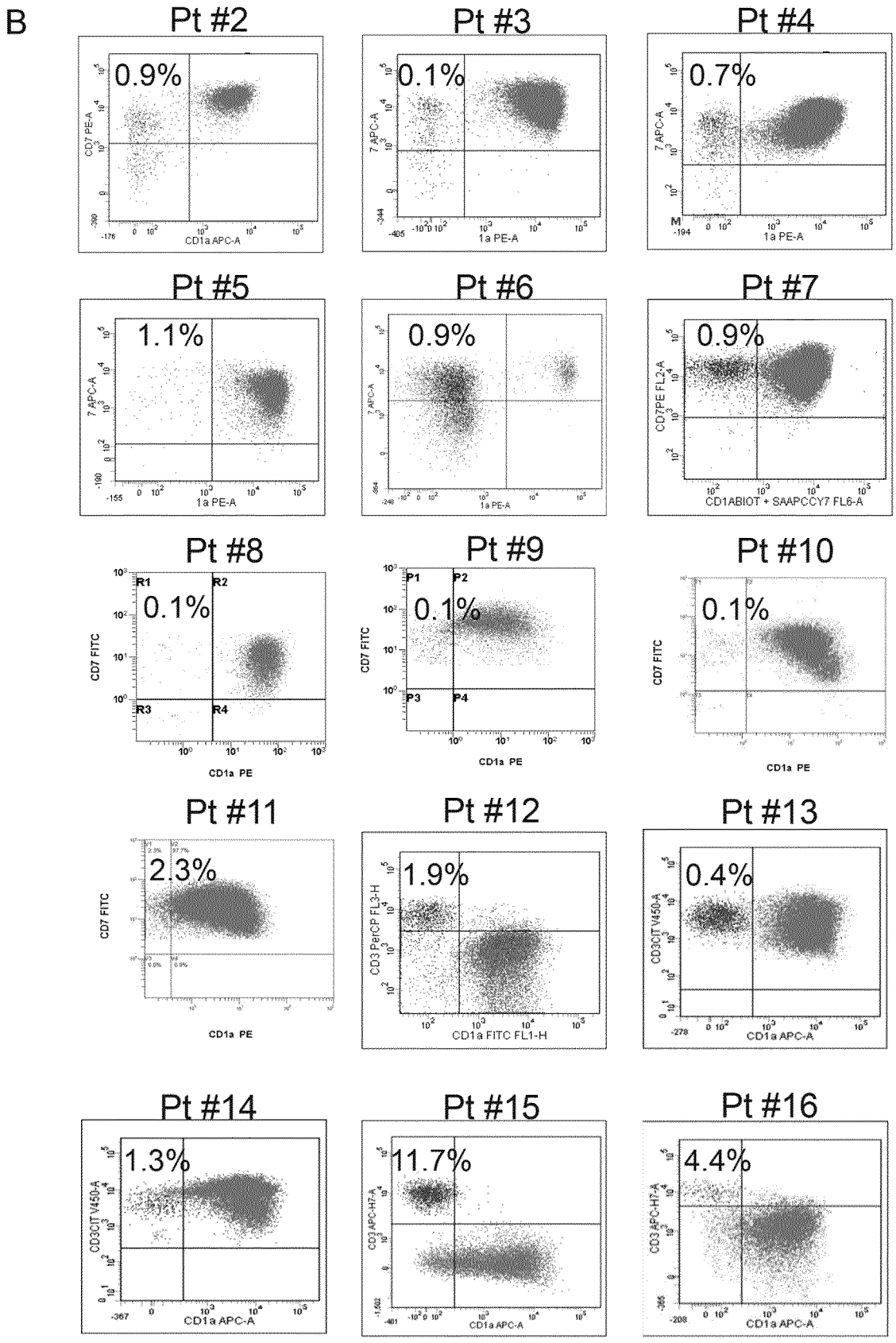


Figure 9

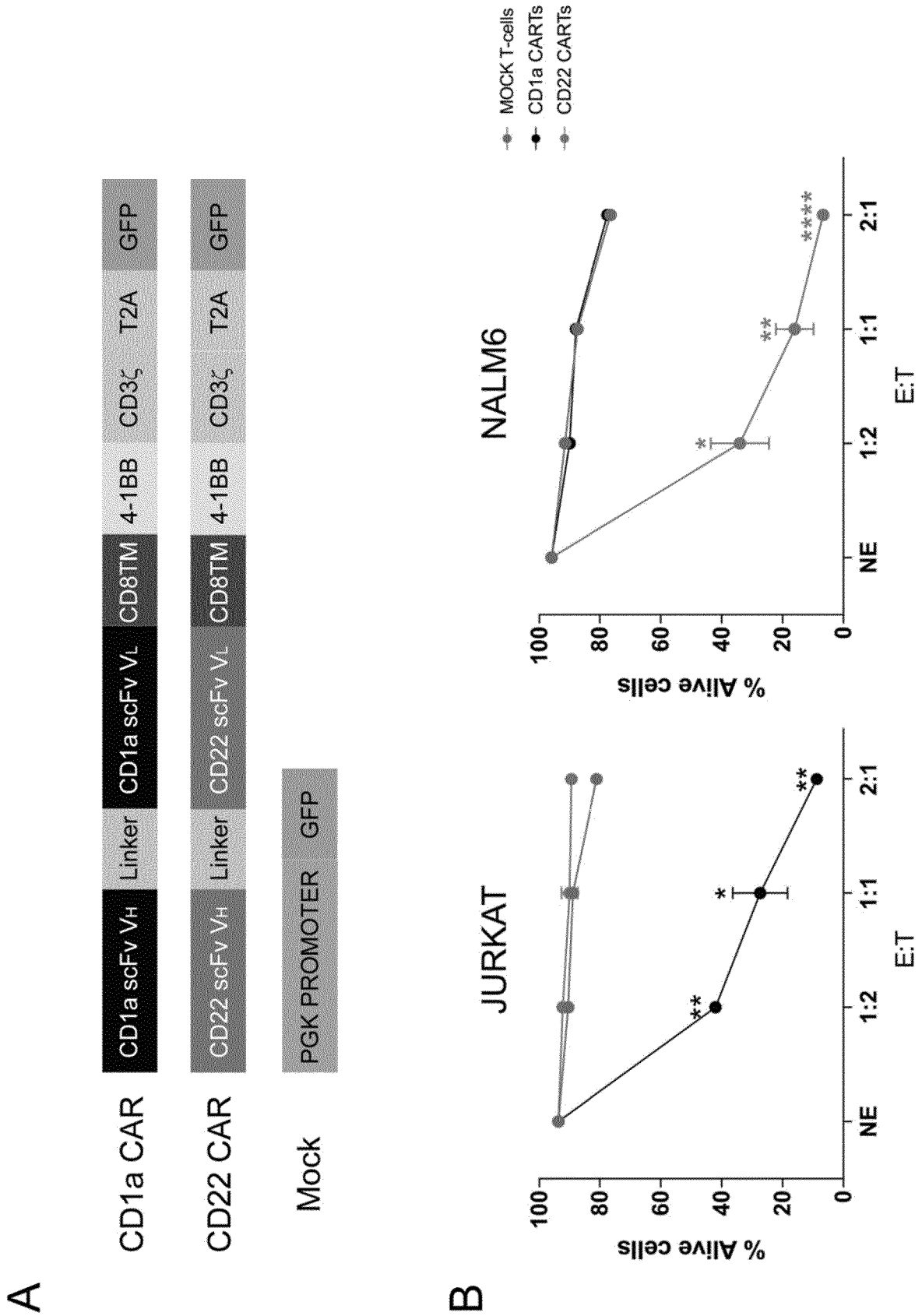

A
CD1a CAR
CD1a scFv VH
Linker
CD1a scFv VL
CD8TM
4-1BB
CD3ζ
T2A
GFP
CD22 CAR
CD22 scFv VH
Linker
CD22 scFv VL
CD8TM
4-1BB
CD3ζ
T2A
GFP
Mock
PGK PROMOTER
GFP
B
JURKAT
NALM6
MOCK T-cells
CD1a CARTs
CD22 CARTs
% Alive cells
0
20
40
60
80
100
NE
1:2
1:1
2:1
E:T
*
**
****

Figure 10

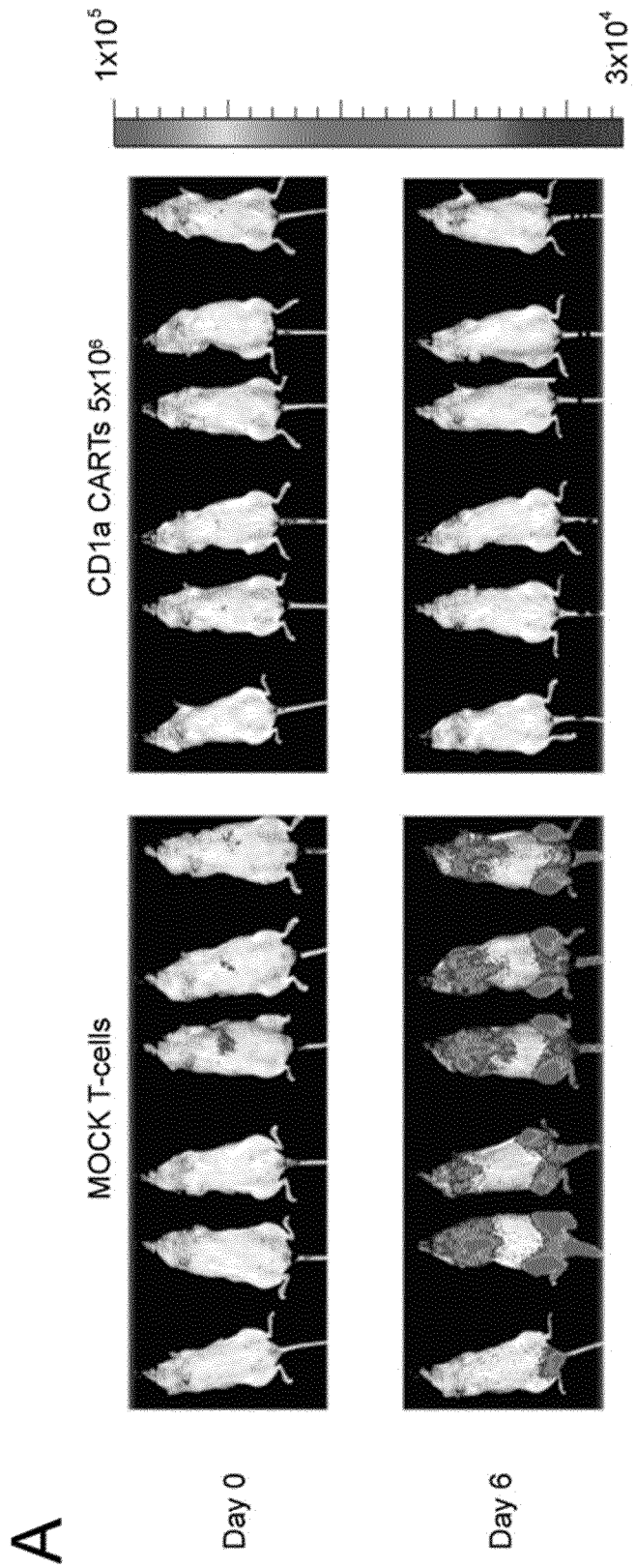


Figure 10 (Cont.)

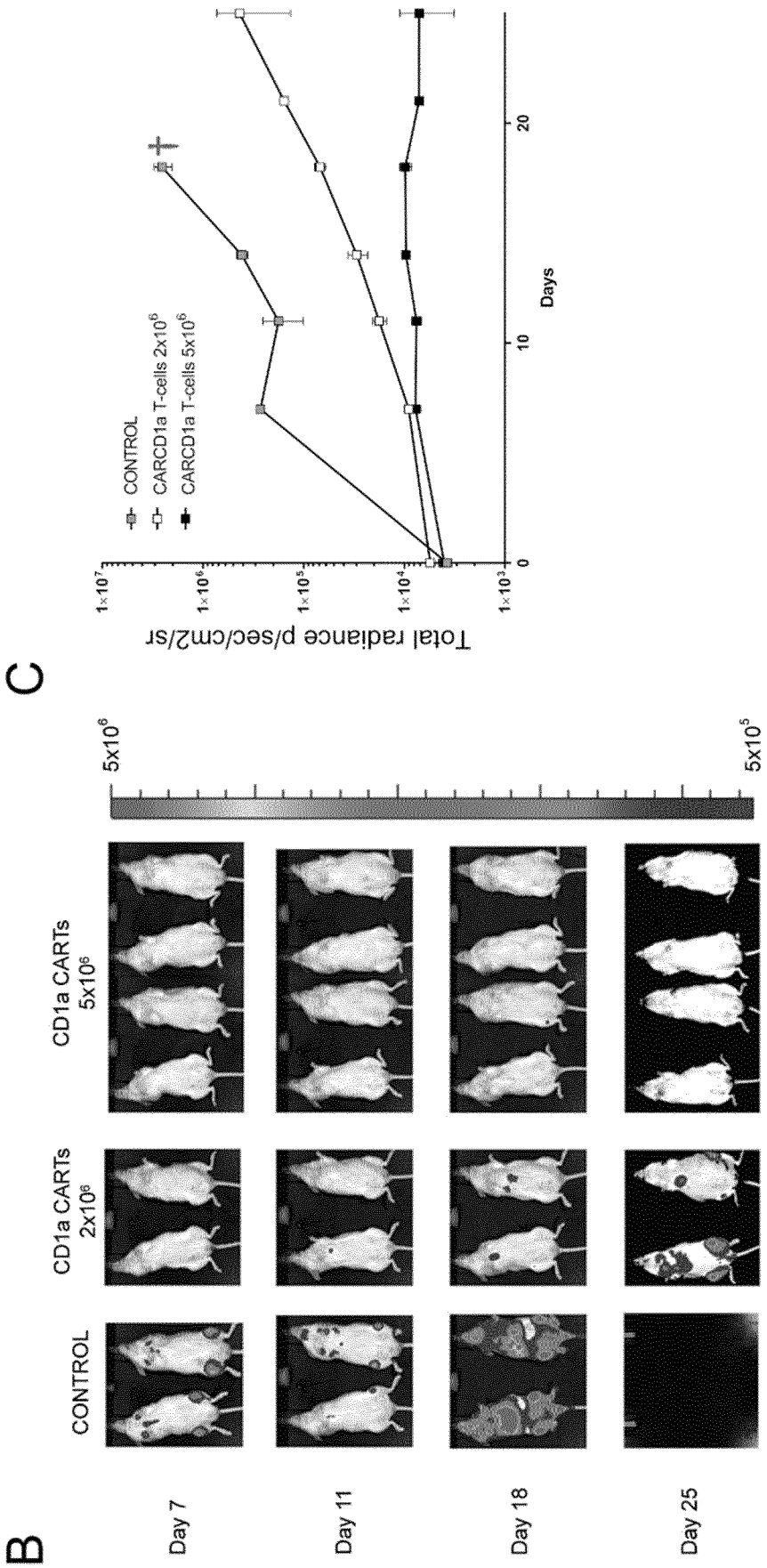


Figure 11

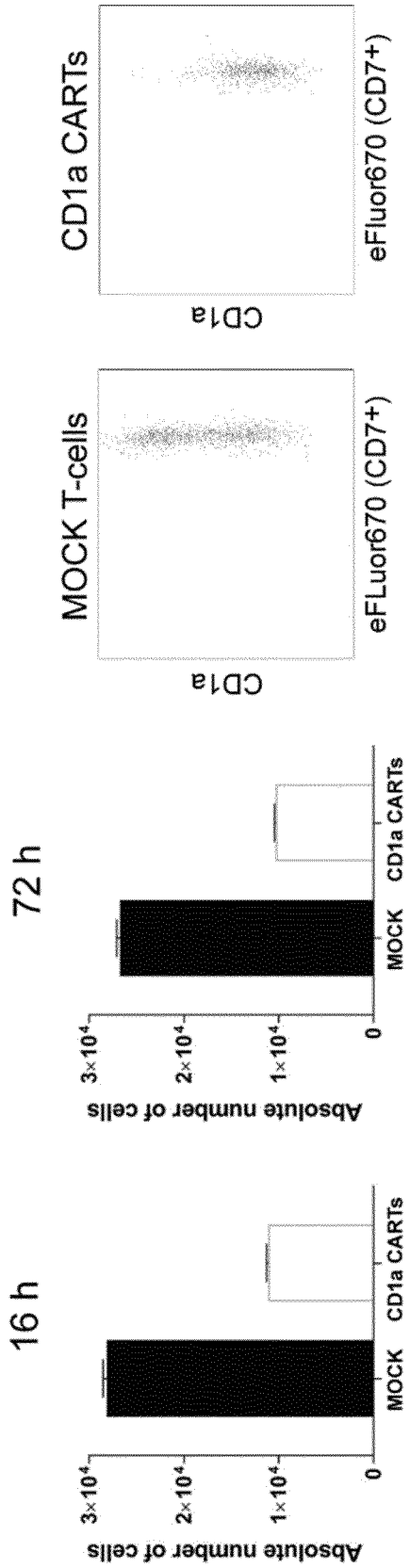
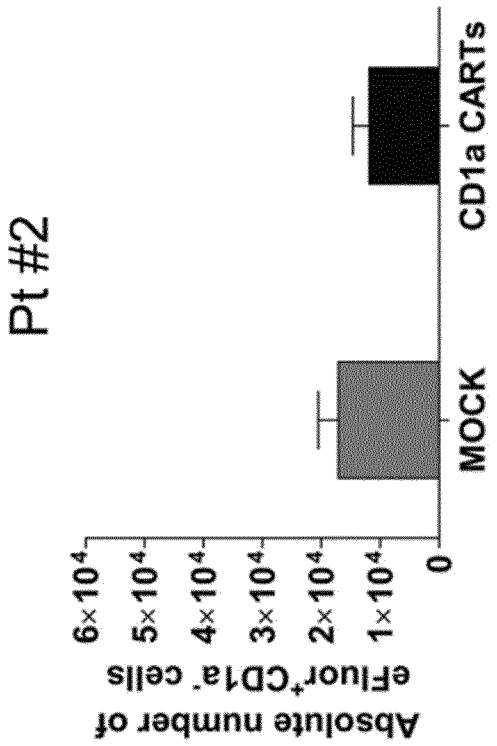
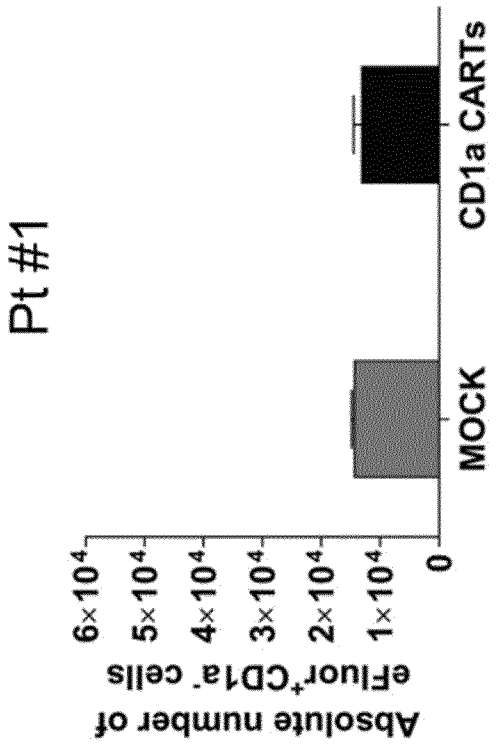

CD1a CARTs
CD1a
eFluor670 (CD7+)
MOCK T-cells
CD1a
eFLuor670 (CD7+)
72 h
Absolute number of cells
3×10⁴
2×10⁴
1×10⁴
0
MOCK
CD1a CARTs
16 h
Absolute number of cells
3×10⁴
2×10⁴
1×10⁴
0
MOCK
CD1a CARTs

Figure 12


Pt #2
Absolute number of eFluor+CD1a- cells
6×10^4
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4
0
MOCK
CD1a CARTs


Pt #1
Absolute number of eFluor+CD1a- cells
6×10^4
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4
0
MOCK
CD1a CARTs

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/053769

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/725 C07K16/28 C12N5/0783 A61P35/00
ADD. A61K39/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N A61K A61P

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MENENDEZ PABLO ET AL: "Adoptive Cellular Immunotherapy Using Cd1a CART-Cells for Cortical T-Cell Acute Lymphoblastic Leukemia", EXPERIMENTAL HEMATOLOGY, ELSEVIER INC, US, vol. 64, 22 August 2018 (2018-08-22), XP085449254, ISSN: 0301-472X, DOI: 10.1016/J.EXPHEM.2018.06.089	1,8-12
Y	abstract	2-7
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[X] 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

"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)

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"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
19 May 2020	29/05/2020

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MILIOTOU N. ANDROULLA ET AL: "CAR T-cell Therapy: A New Era in Cancer Immunotherapy", CURRENT PHARMACEUTICAL BIOTECHNOLOGY, vol. 19, no. 1, 31 May 2018 (2018-05-31), pages 5-18, XP055560547, NL ISSN: 1389-2010, DOI: 10.2174/1389201019666180418095526 figure 1	2-7
A	SAMUEL MURRAY ET AL: "Diagnostic and Therapeutic Evaluation of an Anti-Langerhans Cell Histiocytosis Monoclonal Antibody (NA1/34) in a New Xenograft Model", JOURNAL OF INVESTIGATIVE DERMATOLOGY, vol. 114, no. 1, 1 January 2000 (2000-01-01), pages 127-134, XP055606701, NL ISSN: 0022-202X, DOI: 10.1046/j.1523-1747.2000.00831.x abstract	1
A	N.N.: "CD1A (NA1/34): sc-53035", Santa Cruz Biotechnology: online catalogue, 25 April 2017 (2017-04-25), pages 1-1, XP055606820, Retrieved from the Internet: URL:https://datasheets.scbt.com/sc-53035.pdf [retrieved on 2019-07-18] the whole document	1
X	D SANCHEZ-MARTINEZ ET AL: "Adoptive Cellular Immunotherapy using CD1a CART-cells for Treatment of Cortical Pediatric T-Cell Acute Lymphoblastic Leukemia", KLINISCHE PAEDIATRIE, vol. 230, no. 3, 1 April 2018 (2018-04-01), page 174, XP055606754, DE ISSN: 1439-3824, DOI: 10.1055/s-0038-1645020	1,8-12
Y	the whole document	2-7

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	Diego Anchez-Martínez ET AL: "Fratricide-resistant CD1a-specific CAR T cells for the treatment of cortical T-cell acute lymphoblastic leukemia", , 23 May 2019 (2019-05-23), pages 2291-2304, XP055606731, Retrieved from the Internet: URL:http://www.bloodjournal.org/content/bloodjournal/133/21/2291.full.pdf [retrieved on 2019-07-18] the whole document -----	1-12