CCR9 TARGETING MOIETY FOR THE TREATMENT OF CCR9-POSITIVE CANCER

Technical Field

The present invention provides therapeutics for the treatment of CCR9-positive cancers such as T-cell acute lymphoblastic leukemia. In particular, the present invention provides a CCR9 targeting moiety. The present invention furthermore relates to a CCR9 targeting moiety comprising a further targeting moiety, preferably a CD1a targeting moiety, a dual CAR comprising a CCR9 and a CD1a targeting moiety, their use in the treatment of CCR9 and/or CD1a positive cancers, and the use of a CCR9 targeting moiety and a separate CD1a targeting moiety for such treatment.

Background art

T-cells are major effectors in cancer immunotherapy[1]. Infusion of unmodified donor- or third-party-derived T-cells can be used to control resistant disease in patients who have received allogeneic hematopoietic stem cell transplantation (alloHSCT)[2]. The effector functions of T-cells can be substantially boosted by redirecting them against tumor antigens *via* genetic engineering of T-cell receptors or CARs to generate tumor-reactive T-cells for adoptive transfer[3]. CARs are fusion molecules typically containing an extracellular single chain variable fragment (scFv) of a monoclonal antibody specific to a surface molecule on the tumor cell, a spacer domain that provides flexibility and optimizes effector-target cell engagement, a transmembrane domain, and the T-cell signaling domains 4-1BB (or CD28) and CD3ζ, which drive survival/proliferation and cytotoxicity, respectively[4,5]. CAR T-cell (CART) therapy has revolutionized cancer treatment, especially for relapse/refractory (R/R) B-cell acute lymphoblastic leukemia (B-ALL) and B-cell lymphomas, for which a specific and relatively safe target antigen exists (CD19)[6]. Indeed, impressive rates of clinical complete response (>85%) have been independently reported in patients with R/R B-ALL receiving CD19-directed CARTs[7].

However, CART therapies are still in their infancy, and our current understanding of the mechanisms underlying successful adoptive immunotherapy and the clinic-biological parameters predicting response to immunotherapy remain elusive. Of note, CART therapies for non-B-cell malignancies are lagging owing to the absence of safe and specific targets, and/or CART delivery-associated challenges.

T-cell lineage acute lymphoblastic leukemia (T-ALL) is a phenotypically and genetically heterogeneous malignant disorder that results from leukemic transformation of thymic T-cell precursors[8]. It comprises 10–15% of all acute leukemias diagnosed in children and adults[9,10]. Despite improved survival rates thanks to intensive chemotherapy regimens, event-free (EFS) and overall (OS) survival remains <70%, and R/R T-ALL has a particularly poor outcome. There

are currently no potential curative options for R/R T-ALL beyond HSCT and conventional chemotherapy, which is linked to large trade-offs in toxicities[9,11], bolstering the need for novel targeted therapies. Strategies targeting T-cell malignancies using any kind of immunotherapy (including CARTs) remain challenging because of the shared expression of target antigens between normal and malignant T-cells, ultimately leading to life-threatening T-cell aplasia and fratricide of CARTs, which limits their therapeutic efficacy[12–18]. Several clinical trials in the last few years have explored the therapeutic use of CD5- and CD7-directed CARTs for T-ALL with encouraging results[19–21]. However, CD7-directed CART therapy will likely induce both immunodeficiency and CAR T-cell fratricide because of the shared expression of CD7 in normal and tumoral T-cells, and it may only be useful as a bridge to allo-HSCT. Although allogenic CART therapies using gene-edited T cells depleted of CD7, TRAC, CD52 or HLA-II represent innovative strategies[22–25], improved efficacious autologous CARTs with a safe profile are in high demand for T-ALL.

Cortical T-ALL (coT-ALL) is a major subgroup of T-ALL characterized by the cell surface expression of CD1a, a lipid-presenting molecule whose expression is essentially restricted to coT-ALL and Langerhans cell histiocytosis. CD1a is not expressed in any human tissue (completely absent in normal mature T-cells) with the exception of cortical thymocytes and Langerhans cells during development[26,27]. The feasibility of fratricide-resistant CD1a-specific CARTs as an *unprecedented* adoptive immunotherapy strategy for coT-ALL, which showed robust cytotoxicity against CD1a+ T-ALL cell lines and primary coT-ALL blasts both *in vitro* and *in vivo* has been reported. CAR-CD1a T-cells are fratricide-resistant and remain functional *in vivo* after 8 weeks, as demonstrated in leukemia re-challenge experiments[28].

CCR9 is a 7-transmembrane protein chemokine receptor whose unique ligand is CCL25[29]. High expression of CCR9 is associated with poor prognosis or metastasis in different types of solid tumors[30–33]. Antibodies against CCR9 have recently demonstrated antileukemic effects in preclinical models of T-cell leukemia[34]. Importantly, a CCR9-directed CAR has very recently been proposed for R/R T-ALL[35].

The present invention aims to provide a novel therapy for treating T-ALL.

Figures

Figure 1. CCR9 expression in healthy tissues and T-ALL samples. A) Single-cell RNA seq analysis of CCR9 expression in healthy tissues (Tabula Sapiens Consortium, Science (2022)). Two-dimensional clustering analysis on Uniform Manifold Approximation and Projection (UMAP) to visualize CCR9 expression on Thymocytes and T cells; B) CCR9 expression in human neonatal thymus subpopulations determined by FACS (n=4). C) & D) CCR9 expression in human peripheral blood (PB, n=18) and bone marrow (BM, n=13) respectively, from adult and pediatric

healthy donors. E) Gating strategy, representative histogram, and total CCR9 expression in blasts and normal CD4 (nCD4) and CD8 (nCD8) T cells from T-ALL patients (n=170). F) CCR9 expression in blasts from T-ALL patients stratified across immunophenotypes/subtypes (n=170). ETP, Early T-cell precursor (ETP) acute lymphoblastic leukemia. G) CCR9 expression in T-ALL blasts at diagnosis (Dx) and relapse (Rel), two patient-matched samples are shown.

Figure 2. CCR9 antibody generation strategy. Anti-CCR9 antibody-secreting hybridomas were generated by mouse immunisation with human CCR9 peptides. Extracellular sequences/regions of CCR9 that were chosen for mouse immunisation. From extracellular sequence 1 two different 21-aminoacid long peptides were used (#1 and #2). From extracellular sequence 2 one peptide was used (#3). PNMADD peptide in extracellular sequence 1 denotes the epitope that is recognized by the prior art anti-CCR9 antibody clones 92R and 91R (SunRock, Somovilla-Crespo et al, Front Immunol (2018) and PCT/EP2014/075578). Lower panel, CCR9 transmembrane domains and location of extracellular sequences 1 and 2 and peptides #1, #2 and #3.

Figure 3. Screening of Hybridoma clones via flow cytometry. A total of 82 hybridoma (hyb) candidates were tested by FACS analysis (MADDY peptide: 40 clones; MEDY peptide: 6 clones; YSQIK peptide: 36 clones) as further described in Example 2. A) The figure shows histogram analysis of exemplary results of 18 representative tests in WT and knock-out (KO) for CCR9 MOLT4 cells. Only one hybridoma clone of the 82 tested positive by flow cytometry in WT cells but not in CCR9 KO cells (Clone 115, dashed square). B) Clone 115 was further tested using the CCR9-negative 300.19 cell line (WT) and its CCR9-overexpressing counterpart. C) Clone 115 titer determined by ELISA (upper panel) and SDS-PAGE followed by Coomassie staining quality control analysis (lower panel) under reducing (line 1) and non-reducing conditions (line 2). MW, molecular weight marker. Antibody dilutions used for ELISA titer determination (upper) and sample amount loaded in SDS-PAGE (lower) are indicated.

Figure 4. Structure of one chimeric antigen receptor (CAR) of CCR9 of present invention

Figure 5. CCR9 scFv humanization strategy. Summary scheme of humanization strategies followed to obtain the different humanization constructs h1CAR-CCR9 (HUM1) and h2CAR-CCR9 (HUM2).

Figure 6. Sequence-based humanization. A) Alignment between the murine heavy chain and the germline IGHV1-3*01 sequence that shares the highest sequence identity to the heavy chain (60.2%). The number of different residues (with different levels of conservation) without considering CDRs is 32. B) Comparison between the murine and IGHV1-3*01 sequence. Letters and circles indicate aminoacid changes introduced (see Examples section for a detailed explanation). CDRs and stems are indicated for the heavy chain (H1 to H3).

Figure 7. Sequence-based humanization. A) Alignment between the murine light chain and the germline IGKV2D-29*02 that shares the highest sequence identity to the light chain (82%). The number of different residues without considering CDRs is 13. B) Comparison between the murine and IGKV2D-29*02 sequence. Letters and circles indicate aminoacid changes introduced (see Examples section for a detailed explanation). CDRs and stems are indicated for the heavy light chains (L1 to L3).

Figure 8. Sequence-based humanization. Alignment of murine, germline and proposed humanized sequences, showing the conservation degree for both heavy (upper panel) and light (lower panel) chains.

Figure 9. Sequence-based humanization. Heavy and light chain conservation among the different human sequences.

Figure 10. Sequence-based humanization. Sequence comparison between murine, human germline, and humanized sequence-based "strict" (minimum changes were introduced) and sequence-based "relaxed" (more changes were allowed) candidates, for the heavy chain (upper panel) and for the light chain (lower panel). CDRs and stems are indicated for the heavy (H1 to H3) and light chains (L1 to L3).

Figure 11. Sequence-based humanization. Sequences of the VH and VL chains of the scFvs of murine clone 115 ("murine scFv") as well as the two humanized scFvs ("HUM1 scFv" and HUM2 scFv") anti-CCR9. The CDRs are highlighted.

Figure 12. CCR9 detection in T cells. A) Representative flow cytometry plots showing CAR construct detection in transduced T cells (GFP+); (mur: murine CAR; HUM1 CAR-CCR9 and HUM2 CAR-CCR9) and with anti-murine scFv antibodies. B) Median fluorescence intensity (MFI) of GFP in the transduced populations (each point represents an independent donor).

Figure 13. CAR-CCR9 in vitro cytotoxicity. A) CCR9 expression in indicated cells lines analyzed by flow cytometry with high expression (MOLT4, T-cell acute lymphoblastic leukemia), median expression (SupT1, T cell lymphoma) and no expression (MV4;11, B Myelomonocytic Leukemia). B) Effector T cells were co-cultured with the selected target cell lines at different effector target ratios (E:T) for 24h (top) and 48h (bottom), and residual non-apoptotic target cells were measured by flow cytometry (n=5 donors). UT, untransduced cells.

Figure 14. CAR-CCR9 functionality in vivo. A) Schematic showing in vivo testing. NSG mice (n=5) were injected with $1.5x10^6$ (1.5 M) MOLT4Luc cells intravein (iv), and three days later $4x10^6$ (4 M) effector T cells (UT, untransduced; mur, murine CAR-CCR9; humanized 1 CAR-CCR9, HUM1; humanized 2 CAR-CCR9, HUM2) were administered. Disease progression was monitored by

bioluminescence bi-weekly, and mice were culled and organs collected and analyzed at day 13 after T cell administration. B) Bioluminescence images monitoring disease progression at indicated timepoints. C) Total radiance quantification at the indicated timepoints (AvgRad, log10 scale). D) Flow cytometry analysis of peripheral blood (PB), bone marrow (BM) and spleen at sacrifice. Percentage of MOLT4 cells (top) and effector T cells (bottom) in the different treatment groups.

Figure 15. CAR-CCR9 functionality in vivo in a T-ALL PDX model. A) CCR9 expression levels. NSG mice (n=4-5) were injected with $1.0x10^6$ PDX843 cells intravein (iv), and three days later $4x10^6$ effector T cells (UT, untransduced; humanized 2 CAR CCR9) were administered as indicated in Figure 14. Disease progression was monitored by bioluminescence weekly, and mice were culled and organs collected and analyzed after two weeks after T cell administration. B) Bioluminescence images monitoring disease progression at indicated timepoints. C) Total radiance quantification at the indicated timepoints (AvgRad, log10 scale). D) Flow cytometry analysis of peripheral blood (PB), bone marrow (BM) and spleen at sacrifice (w, week). Percentage of blast cells (top) and effector T cells (bottom) in the different treatment groups.

Figure 16. SDS-PAGE analysis of recombinant scFvs. A) "CCR9_HUM2", B) "Comp-1"; C) "Comp-2" (243LO326); D) "Comp-3" (SR_92R). Lane M1: Protein Markers. R: Reducing condition. NR: Non-reducing condition.

Figure 17. Kinetics of scFv protein (His tag) immobilized onto NTA biosensor interacting with CCR9 N-terminal 1-48 peptide determined by Bio-Layer Interferometry (BLI), Association and dissociation of peptide with scFv protein (His tag) was monitored by OctetRED 384. A) "CCR9_HUM2", B) "Comp-1", C) "Comp-2" (243LO326), D) "Comp-3" (SR_92R). Each line represents the binding kinetics obtained at different peptide concentrations applied from 6.25 nM to 200 nM.

Figure 18. CD1a and CCR9 co-expression in T-ALL. a) Gating strategy followed for the quantification of CCR9 and CD1a expression in blasts by flow cytometry. Example of a patient with high CD1a expression (75%). B to F) CCR9 and CD1a co-expression in 15 patients arbitrary grouped as follows: high CD1a (B), high CCR9 (C), co-expression of both high CD1a and CCR9 (D), mix of blast cell populations co-expressing CD1a and CCR9 or with single antigen expression (E and F). G) Quantification in a cohort of 170 T-ALL patients. An arbitrary cut-off was stablished as 20% and patients were classified in three groups according with the % of expression of CCR9 and CD1a calculated as indicated in panel A. Absolute numbers of patients in each group are also indicated related to the total number of patients analyzed.

Figure 19. Generation of MOLT4 cells knock-out for CCR9 and CD1a. A) Representative FACS plots showing the expression of CD1a and CCR9 in wild-type cells (wt), cells knock-out for CD1a (CD1aKO), knock-out for CCR9 (CCR9KO) and double knock-out for both CD1a and CCR9 (dKO). B) Expansion profile of cells. C) Cytotoxicity of CAR-Ts (experiment performed as described in Figure 13) as a single therapy against CCR9 or CD1a and as a dual therapy targeting both CCR9 and CD1a by the co-transduction of single CARs (Co CCR9_CD1a CAR). In all cases, the % of transduced cells was quantified using GFP expression and normalized to the lowest using UT cells. For a typical experiment, transductions were >40%. NE, non-effector; UT, untransduced. The percentage of viable MOLT4 target cells are shown for three independent PBMC donors.

Figure 20. Setting-up of a model for dual CAR therapy studies. A) Representative FACS plot of a patient with a complex population of blast cells expressing CD1a or CCR9 or co-expressing both antigens at the same time (left panel). Mix of MOLT 4 cells wt, CCR9 KO and CD1a KO at proportion 1:1:1 to mimic the population of cells found in T-ALL patients. B) Comparative cytotoxicity analysis as done in Figure 19, by incubating single effector of CAR-Ts against CCR9 or CD1a or as a dual therapy of CAR-Ts co-transduced for both CCR9 and CD1a CAR (Co CCR9_CD1a) with a mix 1:1:1 of MOLT4 cells wt and KO for CD1a and CCR9. Left panel, cytotoxicity assay at different E:T ratios for 24 h. Middle and right panels, cytotoxicity assays at 1:3 E:T ratio and at different time-points expressed as % of live target cells (middle) or total cells (right). 2-way ANOVA for multiple comparisons (***, p<0,0001, Co CCR9_CD1a vs CCR9 or CD1a CAR). C) Quantification of pro-inflammatory cytokines IFNγ, IL-2 and TNFα of the cytotoxicity assay indicated in panel B, at 24 h determined by ELISA. ELISA, enzyme-linked immunosorbent assay; NE, non-effector; UT, untransduced PBMCs; CCR9 CAR and CD1a CAR, infection of single CARs at a MOI of 10; Co CCR9_CD1a CAR, co-transduction of single CCR9 and CD1a CARs at a MOI of 20. Efficiency of transduction was normalized by GFP expression and adjusted to the minimum by using UT cells.

Figure 21. Dual CAR-CCR9/CD1a functionality in vivo. In vivo testing as done in Figure 14. NSG mice (n=6) were injected with a mix of 0.8 x10^6 Luc-MOLT4 cells/mouse: mixture of 30% wt + 30% CD1aKO + 40% CCR9KO. Mice were randomized 3 days later by groups having similar initial luciferase activity and injected with 3 x10^6 CAR T cells/mouse (after MOLT4 injection) intravein (iv). Effector T cells: UT, untransduced; CCR9 and CD1a CAR-Ts; Mix-CD1a/CCR9 CAR-Ts dual therapy is a 50% mix of CD1a + 50% CCR9 CAR T cells. Disease progression was monitored by bioluminescence at days (d) 7, 14, 21 and 26 after T cell administration, and mice were culled and organs collected and analyzed by flow cytometry at day 26. A) Total radiance quantification at the indicated timepoints (AvgRad, log10 scale). B) Flow cytometry analysis of bone marrow (BM) at sacrifice. Percentage of MOLT4 cells in the different treatment groups. C)

Bioluminescence images monitoring disease progression at indicated timepoints. D-E) Example of dual therapy similarly performed to panels A-B, but co-transducing T cells as indicated in Figures 19 and 20 (Co-CCR9/CD1a CAR-Ts dual therapy is an infection of CD1a and CCR9 CARs T cells at a MOI of 20. D) Total radiance quantification at the indicated timepoints (AvgRad, log10 scale). E) Flow cytometry analysis of bone marrow (BM) at sacrifice. Percentage of MOLT4 cells in the different treatment groups. Indicated statistical analysis in panels A and D at day 21 and 18, respectively, comparing dual therapy (Mix or co-transduction) vs single treatments (Two-way ANOVA for multiple comparisons. * p<0,05; **<0,0001; ***<0,00001).

Figure 22. Dual CAR-CCR9/CD1a functionality in vivo in a PDX model. In vivo testing as done in Figure 15 with a PDX co-expressing CCR9 and CD1a. A) CCR9 and CD1a expression levels. NSG mice (n=4-5) were injected with $1.0x10^6$ PDX cells intravein (iv), and three days later $2x10^6$ effector T cells (UT, untransduced; CCR9 and CD1a CAR T-cells or a CAR-Ts resulting from the co-transduction of both CARs Co-CCR9_CD1a). Because this PDX has no luciferase activity, disease progression was monitored weekly by bleeding and blast quantification in peripheral blood (PB) (B). Mice were culled and organs collected and analyzed after eight weeks (w) after T cell administration. C-E) Flow cytometry analysis of peripheral blood (PB) at week 8 (data shown in panel B), spleen (SP) and bone marrow (BM) at sacrifice showing the percentage of blast cells in the different treatment groups.

Figure 23. Bicistronic vectors co-expressing CCR9 and CD1a CARs. A) Structure of different bicistronic versions, from N-t to C-t having first the CCR9 CAR and second the CD1a CAR (Bicis 1) or first the CD1a CAR and second the CCR9 CAR (Bicis 2) separated by a F2A self-cleaving peptide sequence. Both CARs are expressed under the same EF1-a promoter and also co-express a GFP separated from the second CAR by a T2A self-cleaving peptide sequence. CCR9 and CD1a scFvs are the humanized versions as described herein with the corresponding heavy, VH, and light, VL, chains. Constructs may have different signal peptide sequences (S): S1 is a modified signal peptide from human CD8a used in previous single CCR9 and CD1a CARs; S2 is the signal peptide from hIgG1 (human immunoglobulin light chain variable region); S3, is the signal peptide from mIgG (murine immunoglobulin heavy chain). Depending on the combinations of signal peptides S1 to S3, we have Bicis 1-2, 1-3, 2-2 and 2-3, according with the corresponding pictures. TM is the transmembrane from human CD8a (hinge and transmembrane region), L is the peptide linker region and 4-1BB and CD3ζ are the co-stimulatory and cytotoxic domains, respectively. TM*, 4-1BB*, L*, 4-1BB* and CD3ζ*, correspond to sequences encoding the same protein but with different DNA codon usage resulting in different DNA sequences to avoid homologous recombination. B) Expression of the indicated constructs by CAR T-cells determined by GFP co-expression: single CCR9 and CD1a CARs, co-transduced CCR9 and CD1a CARs (Co) and the four bicistronic CAR vectors, compared to control untransduced T cells (UT). C to

F) Cytotoxicity experiments performed at 1:1 E:T ratio and 24h. Target (T) cells are MOLT4 wt (C), CCR9^{KO} (D), CD1a^{KO} (E) or CCR9_CD1a^{dKO} (F). The percentage of viable cells are shown after co-culture of UT or CAR T-cells from three independent PBMC donors.

Summary of the invention

In one aspect the present invention relates to a CCR9 targeting moiety that specifically binds to the amino acid sequence of SEQ ID NO:1 (SMEDYVNFN).

In a second aspect the present invention relates to a CCR9 targeting moiety comprising an antibody, F(ab')2, Fab, scFab or scFv, said antibody, F(ab')2, Fab, scFab or scFv comprising

a) a light chain (VL) comprising at least one complementarity determining region (CDR) selected from:

(i) a CDR comprising the amino acid sequence shown in SEQ ID NO:2 [CDR-L1], or a variant thereof;

(ii) a CDR comprising the amino acid sequence shown in SEQ ID NO:3 [CDR-L2], or a variant thereof; and

(iii) a CDR comprising the amino acid sequence shown in SEQ ID NO:4 [CDR-L3], or a variant thereof; and

b) a heavy chain (VH) comprising at least one complementarity determining region (CDR) selected from:

(i) a CDR comprising the amino acid sequence shown in SEQ ID NO:5 [CDR-H1], or a variant thereof;

(ii) a CDR comprising the amino acid sequence shown in SEQ ID NO:6 [CDR-H2], or a variant thereof; and

(iii) a CDR comprising the amino acid sequence shown in SEQ ID NO:7 [CDR-H3], or a variant thereof.

In one embodiment of the first and second aspect of the present invention the CCR9 targeting moiety comprises

a. a VL domain consisting of SEQ ID NO:8 and a VH domain consisting of SEQ ID NO:9; or
b. a VL domain consisting of SEQ ID NO:10 and a VH domain consisting of SEQ ID NO:11; or
c. a VL domain consisting of SEQ ID NO:12 and a VH domain consisting of SEQ ID NO:13.

In one embodiment of the first and second aspect of the present invention the CCR9 targeting moiety is a scFv comprising

a. a VL domain consisting of SEQ ID NO:8 and a VH domain consisting of SEQ ID NO:9; or
b. a VL domain consisting of SEQ ID NO:10 and a VH domain consisting of SEQ ID NO:11; or
c. a VL domain consisting of SEQ ID NO:12 and a VH domain consisting of SEQ ID NO:13.

In one embodiment of present invention the CCR9 targeting moiety is a murine CCR9 targeting moiety.

In one embodiment of present invention the CCR9 targeting moiety is a humanized CCR9 targeting moiety.

In one preferred embodiment of present invention the CCR9 targeting moiety is a humanized targeting moiety comprising the amino acid sequence of SEQ ID NO:16.

In one preferred embodiment of present invention the CCR9 targeting moiety is a humanized targeting moiety comprising the amino acid sequence of SEQ ID NO:17.

In one preferred embodiment of present invention the CCR9 targeting moiety is a murine targeting moiety comprising the amino acid sequence of SEQ ID NO:18.

In a preferred embodiment of present invention the CCR9 targeting moiety consists of the amino acid sequence of SEQ ID NO:16, SEQ ID NO:17 or SEQ ID NO:18.

In a third aspect the present invention relates to a chimeric antigen receptor (CAR) comprising:

a) an extracellular domain comprising a CCR9 targeting moiety according to the first and/or second aspect of present invention;
b) a transmembrane domain; and
c) an intracellular signaling domain.

In one embodiment of the CAR of present invention 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 a preferred embodiment the transmembrane domain comprises the transmembrane domain of CD8.

In one embodiment of the CAR of present invention the intracellular signaling domain comprises the intracellular domain of CD3ζ, FcRγ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b or CD66b, preferably of CD3ζ.

In a further embodiment of the CAR of present invention, 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, or CD276.

In one preferred embodiment the costimulatory signaling domain comprises the intracellular domain of CD137.

In a preferred embodiment of the CAR of present invention, the CAR comprises, preferably consists of the amino acid sequence according to SEQ ID NO:22, SEQ ID NO:23 or SEQ ID NO:24.

In a further embodiment of present invention, the CCR9 targeting moiety, or the CAR further comprise a second targeting-moiety.

In one embodiment said second targeting moiety is selected from a CD3, CD4, CD5, CD7, CD37, CD30, CD33, CD99, CCR7, CDR3, TRBC1/2, or CD1a targeting moiety, preferably wherein said second targeting moiety is a CD1a targeting moiety.

In a preferred embodiment said second targeting moiety is a CD1a targeting moiety. In an even more preferred embodiment, the CD1a targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:31 or SEQ ID NO:33 and a VH domain consisting of SEQ ID NO:32 or SEQ ID NO:34.

In a fourth aspect the present invention relates to a dual CAR comprising a first and a second CAR, wherein the first CAR is a CAR according to the third aspect of present invention and the second CAR is a CAR comprising a CD1a targeting moiety, preferably the CD1a targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:31 or SEQ ID NO:33 and a VH domain consisting of SEQ ID NO:32 or SEQ ID NO:34.

In a fifth aspect the present invention relates to a nucleic acid encoding the CAR of present invention.

In a sixth aspect the present invention relates to a cell comprising said nucleic acid and/or the CAR of present invention. In a preferred embodiment the cell is a T-cell.

In a seventh aspect the present invention relates to a pharmaceutical composition comprising a plurality of cells of present invention and a pharmaceutically acceptable carrier or diluent.

In an eighth aspect the present invention relates to the cell or the pharmaceutical composition of the invention for use as a medicament.

In a ninth aspect the present invention relates to the cell or the pharmaceutical composition of the invention for use in a method of treating a CCR9-positive cancer, wherein the method comprises administering the cell or composition to a patient in need thereof.

In one embodiment the CCR9-positive cancer is T-cell acute lymphoblastic leukemia or T-cell lymphoma, preferably relapsed/refractory T-cell acute lymphoblastic leukemia.

In a tenth aspect the present invention relates to the cell or the pharmaceutical composition of the invention for use in a method of treating a CD1a-positive cancer, preferably a CD1a and CCR9-positive cancer, wherein the method comprises administering the cell or composition to a patient in need thereof.

In one embodiment the CD1a-positive cancer is cortical T-cell acute lymphoblastic leukemia, preferably, relapsed/refractory cortical T-cell acute lymphoblastic leukemia.

In an eleventh aspect the present invention relates to the cell or the pharmaceutical composition of the invention for use in the method for treating a CD1a-positive and/or a CCR9-positive cancer, preferably a CD1a and CCR9-positive cancer as described above, the method further comprising administering simultaneously or subsequently a CD1a CAR comprising a CD1a targeting moiety, a transmembrane domain and an intracellular signaling domain.

In one embodiment the CD1a targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:31 or SEQ ID NO:33 and a VH domain consisting of SEQ ID NO:32 or SEQ ID NO:34.

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 constant 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 "CCR9" refers to C-C chemokine receptor (CCR) type 9/CDw199 and is a 7-transmembrane protein chemokine receptor whose unique ligand is CCL25. High expression of CCR9 is associated with poor prognosis or metastasis in different types of solid tumors. Antibodies against CCR9 have recently demonstrated antileukemic effects in preclinical models of T-cell leukemia.

The term "CCR9-targeting moiety" refers to a substance that is able to bind CCR9. Within the context of a CAR, a CCR9-targeting moiety targets T cells to a CCR9-positive cell, preferably a cancer cell. Within the context of a CAR, it is to be understood that the CCR9-targeting moiety is

genetically encodable. Exemplary sequence and data related to human CCR9 has been deposited in the UniProtKB database under ID number P51686.

"CCR9-positive" cancer, including a "CCR9-positive" cancerous disease, is one comprising cells, which have CCR9 present at their cell surface. The term "CCR9-positive" also refers to a cancer that produces sufficient levels of CCR9 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 CCR9. In some embodiments, the CCR9-positive cancer is T-cell acute lymphoblastic leukemia. In some embodiments the CCR9-positive cancer is T-cell lymphoma.

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 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 or other effector cells, such as for example NK cells, gamma delta T cells, or others, 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 caretaker 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 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 and/or CCR9-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 II 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 naïve 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 glutathione, thioctic acid, sodium thioglycolate, thioglycerol, [alpha]-monothioglycerol, and sodium thiosulfate; 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. 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/jdispatcher/psa/emboss_needle.

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.

Detailed description of the invention

The inventors have shown that CCR9 expression varies in healthy tissues and T-ALL samples (Example 1). Specifically, and as can be seen from Figure 1, CCR9 expression is detected in thymocytes and T cells from the small intestine and in human neonatal thymus subpopulations. Low CCR9 expression was found in human peripheral blood and bone marrow from healthy donors with only a slight increase in B-cells (Figure 1B to 1D). In contrast, CCR9 expression is detected in blast cells from T-ALL patients stratified across immunophenotypes/subtypes (Figure 1E & F). In addition, CCR9 expression in T-ALL blasts is similarly detected at diagnosis (Dx) and relapse (Rel) (Figure 1G). CCR9 is therefore a promising target for T-ALL therapy and the inventors have thus embarked on the generation of a CCR9 antibody that can be used in such T-ALL therapy targeting T-ALL cells via CCR9.

Firstly, a murine CCR9 antibody was generated by mouse immunisation with human CCR9 peptides as shown in Figure 2 and Example 2. The immune response was verified by ELISA against the peptides used for the immunisation and screenings at polyclonal stage were conducted and positive clones further tested by FACS (see Figure 3A for a representative analysis). Hybridoma clones were tested by incubating their conditioned media containing the secreted antibodies ("supernatant") with the T-ALL CCR9+ cell line MOLT-4 and the $CCR9^{KO}$ MOLT-4. Only one hybridoma clone tested positive (Clone 115) (Figure 3A) and was thus further tested using a CCR9-negative 300.19 cell line (WT) and its CCR9-overexpressing counterpart (Figure 3B). The isotype of the antibody is IgG1 and ELISA titer and SDS-PAGE quality control analysis are shown in Figure 3C. Finally, the hybridoma #115 was sequenced and a second-generation 4-1BB-based CAR-CCR9 was cloned into a clinically validated pCCL lentivector (Figure 4).

To avoid immunogenic response in humans and to make the CAR T-cells more suitable for use in the clinic, as a next step humanization of the murine scFv was performed using two strategies as summarized in Figure 5 and described in more detail in Example 3. The CAR-CCR9 generated following a sequence-based "strict" method is called hereinafter h1CAR-CCR9 or HUM1. The conservation among the different human sequences for the heavy and light chains is indicated in Figures 8, 9 and 10 and is used to assess the level of conservation among human sequences in non-conserved positions (that are not the CRDs and stem regions) between murine and human sequences. The CAR-CCR9 generated following a sequence-based "relaxed" method is called hereinafter h2CAR-CCR9 or HUM2.

The inventors could show that PBMCs transduced with mCAR-CCR9 and h1CAR-CCR9 and h2CAR-CCR9 could be detected by FACS (Example 4 and Figure12).

They then assessed the capacity of CCR9-CAR T-cells to eliminate target (T) CCR9+ MOLT4 and SupT1 cells (Example 5 and Figure 13). As shown in Figure 13B, murine and HUM2 CCR9 CAR-Ts are equally effective in killing MOLT4 or SupT1 cells but not MV4;11 cells. Killing activity of HUM1 was slightly lower. These results clearly show that humanized CCR9-CAR T-cells are as effective as murine CAR T-cells to promote cell death *in vitro*.

The activity of murine and humanized CCCR9-CAR-Ts *in vivo* using Luc/GFP-expressing MOLT4 T-ALL cells was also evaluated (Example 6, Figure 14). In contrast to the mice receiving UT cells, which showed massive tumor burden by bioluminescence (BLI), those mice given CCR9-CAR-Ts had a lower disease progression until day 13 (Figure 14B). Quantification of BLI by IVIS showed no differences among the murine CAR-CCR9 and the humanized versions (Figure 14C). For the analysis of tumour circulating cells and persistence of the transferred CARTs, the mice were sacrificed at the end of the experiment (day 13). The results show a significant reduction of tumor burden in PB, BM and spleen in mice receiving humanized or murine CAR-Ts related to UT (Figure 14D). Overall, the inventors could show the efficacy of the murine and humanized CCR9-CAR T-cell products, showing a potent and specific antileukemic activity against T-ALL cell lines *in vitro* and potent antileukemic activity *in vivo*.

To assess to which epitope in CCR9 the murine and humanized CCR9-CAR T-cells bind, epitope mapping was performed as further explained in Example 7. A strong binding was observed against the complete antigen peptide MADDYGSESTSSMEDYVNFNF used to generate the 115 antibody. In addition, the purified antibody 115 detected Peptide 3 (SSMEDYVNFN) and Peptide 4 (SMEDYVNFNF) in ELISA with strong signal but no signal was detected with Peptide 1 (MADDYGSEST) and Peptide 2 (GSESTSSMED). As a result, the 9 AA epitope sequence recognized by antibody 115 on the MADDYGSESTSSMEDYVNFNF peptide is SMEDYVNFN.

A further epitope mapping experiment was performed with the humanized CCR9 ("CCR9_HUM") scFv as described in Example 9 in which the epitope could be confirmed. At the same time the epitopes of three other CCR9 antibodies disclosed in the prior art were determined and the inventors could show that the epitope sequence recognized by the prior art antibodies was different from the epitope sequence recognized by "CCR9_HUM".

CCR9 TARGETING MOIETY

In view of the above results, in one aspect the present invention relates to a CCR9 targeting moiety that specifically binds to the amino acid sequence of SEQ ID NO:1 (SMEDYVNFN).

In a second aspect the present invention relates to a CCR9 targeting moiety comprising an antibody, F(ab')2, Fab, scFab or scFv, said antibody, F(ab')2, Fab, scFab or scFv comprising

a) a light chain domain (VL) comprising at least one complementarity determining region (CDR) selected from:

(i) a CDR comprising the amino acid sequence shown in SEQ ID NO:2 [LCDR-1], or a variant thereof;

(ii) a CDR comprising the amino acid sequence shown in SEQ ID NO:3 [LCDR-2], or a variant thereof; and

(iii) a CDR comprising the amino acid sequence shown in SEQ ID NO:4 [LCDR-3], or a variant thereof; and

b) a heavy chain domain (VH) comprising at least one complementarity determining region (CDR) selected from:

(i) a CDR comprising the amino acid sequence shown in SEQ ID NO:5 [HCDR-1], or a variant thereof;

(ii) a CDR comprising the amino acid sequence shown in SEQ ID NO:6 [HCDR-2], or a variant thereof; and

(iii) a CDR comprising the amino acid sequence shown in SEQ ID NO:7 [HCDR-3], or a variant thereof.

Table 1 below depicts the amino acid sequences SEQ ID NO:2 to SEQ ID NO:7 corresponding to the six CDRs LCDR1-3 and HCDR1-3.

Table 1: Sequences depicting the six CDRs HCDR1-3 and LCDR1-3

LCDR-1	SEQ ID NO:2	QSLVHSNGKTY
LCDR-2	SEQ ID NO:3	KVS
LCDR-3	SEQ ID NO:4	AQSTHVWT
HCDR-1	SEQ ID NO:5	GYSFTDYI
HCDR-2	SEQ ID NO:6	IDPNNYNT
HCDR-3	SEQ ID NO:7	ARDVY

In one embodiment of this second aspect said VL domain comprises the CDRs LCDR1, LCDR2 and LCDR3 and said VH domain comprises the CDRs HCDR1, HCDR2 and HCDR3, wherein:

- LCDR1 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:2, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:2;
- LCDR2 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:3, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:3;

- LCDR3 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:4, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:4;
- HCDR1 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:5, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:5;
- HCDR2 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:6, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:6; and
- HCDR3 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:7, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:7.

In one embodiment the CCR9 targeting moiety is a murine CCR9 targeting moiety.

In one embodiment the CCR9 targeting moiety is a humanized CCR9 targeting moiety.

In some embodiments of present invention, the CCR9-targeting moiety is an antibody, anticalin, repebody, monobody, scFv, Fab, scFab, affibody, fynomer, DARPin, nanobody, or peptide aptamer that specifically binds to CCR9.

In one embodiment of the first and second aspect of the present invention the CCR9 targeting moiety comprises a VL domain consisting of SEQ ID NO:8 and a VH domain consisting of SEQ ID NO:9 (see table 2 below). In a preferred embodiment the CCR9 targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:8 and a VH domain consisting of SEQ ID NO:9.

In one embodiment of the first and second aspect of the present invention the CCR9 targeting moiety comprises a VL domain consisting of SEQ ID NO:10 and a VH domain consisting of SEQ ID NO:11 (see table 2 below). In a preferred embodiment the CCR9 targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:10 and a VH domain consisting of SEQ ID NO:11.

In one embodiment of the first and second aspect of the present invention the CCR9 targeting moiety comprises a VL domain consisting of SEQ ID NO:12 and a VH domain consisting of SEQ ID NO:13 (see table 2 below). In a preferred embodiment the CCR9 targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:12 and a VH domain consisting of SEQ ID NO:13.

Table 2: Sequences depicting the VL and VH domain of the two humanized CCR9 scFvs (HUM1 and HUM2) and the murine CCR9 scFvs (MUR). The CDRs are highlighted.

VL (HUM1)	SEQ ID NO:8	DVVMTQTPLSLSVTPGQPASISCRSSQSLVHSNGKTYLQWYLQ KPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAED VGVYFCAQSTHVWTFGGGTKVEIKRA
VH (HUM1)	SEQ ID NO:9	QVQLKQSGPEVVKPGTSVKVSCTASGYSFTDYIIYWVKQAHGR SLEWMGYIDPNNYNTRYSQKFQGKVTITVDKSSTSAYMELNSL TSDDTAVYYCARDVYWGQGTTVTVSS
VL (HUM2)	SEQ ID NO:10	DVVMTQTPLSLSVTPGQPASISCRSSQSLVHSNGKTYLQWYLQ KPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAED VGVYFCAQSTHVWTFGGGTKVEIKRA
VH (HUM2)	SEQ ID NO:11	QVQLVQSGAEVKKPGASVKVSCTASGYSFTDYIIYWVKQAHGQ SLEWMGYIDPNNYNTRYSQKFQGKVTITVDKSSTSAYMELNSL TSDDTAVYYCARDVYWGQGTTVTVSS
VL (MUR)	SEQ ID NO:12	DVVMTQTPLSLTVSLGDQASISCRSSQSLVHSNGKTYLQWYLQ KPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLKISRVEAED LGVYFCAQSTHVWTFGGGTKLEIKRA
VH (MUR)	SEQ ID NO:13	QVQLKQSGPELVKPGTSVRVSCTASGYSFTDYIIYWVKQSHGR SLEWIGYIDPNNYNTRYSQKFKGKATLTVDKSSTSAFMHLNSLT SDDSAVYYCARDVYWGQGTTLTVSS

In some embodiments, the CCR9-targeting moiety is an antibody, F(ab')2, Fab, preferably scFv, 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 SEQ ID NO:2, LCDR2 consists of SEQ ID NO:3, LCDR3 consists of SEQ ID NO:4, HCDR1 consists of SEQ ID NO:5, HCDR2 consists of SEQ ID NO: 6, and HCDR3 consists of SEQ ID NO:7.

In a preferred embodiment the humanized CCR9 targeting moiety is a scFv comprising a VL domain and a VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and wherein:

- LCDR1 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:2, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:2;
- LCDR2 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:3, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:3;

- LCDR3 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:4, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:4;
- HCDR1 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:5, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:5;
- HCDR2 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:6, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:6; and
- HCDR3 comprises, consists, or consists essentially of an amino acid sequence as shown in SEQ ID NO:7, or a sequence which is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% identical to SEQ ID NO:7.

In an even more preferred embodiment, the humanized CCR9 targeting moiety is a scFv comprising a VL domain and a VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and wherein:

- LCDR1 consists of an amino acid sequence as shown in SEQ ID NO:2;
- LCDR2 consists of an amino acid sequence as shown in SEQ ID NO:3;
- LCDR3 consists of an amino acid sequence as shown in SEQ ID NO:4;
- HCDR1 consists of an amino acid sequence as shown in SEQ ID NO:5;
- HCDR2 consists of an amino acid sequence as shown in SEQ ID NO:6;
- HCDR3 consists of an amino acid sequence as shown in SEQ ID NO:7.

In a preferred embodiment the CCR9 targeting moiety is an antibody, F(ab')2, Fab, preferably scFv, or scFab comprising a VL domain and a VH domain, wherein the VL domain comprises, consists, or consists essentially of SEQ ID NO:8, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:8; and wherein the VH domain comprises, consists, or consists essentially of SEQ ID NO:9, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:9.

In a preferred embodiment the CCR9 targeting moiety is an scFv comprising a VL domain and a VH domain, wherein the VL domain comprises, consists, or consists essentially of SEQ ID NO:8, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:8; and wherein the VH domain comprises, consists, or consists essentially of SEQ ID NO:9, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:9.

In another preferred embodiment the CCR9 targeting moiety is an antibody, F(ab')2, Fab, preferably scFv, or scFab comprising a VL domain and a VH domain, wherein the VL domain comprises, consists, or consists essentially of SEQ ID NO:10, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:10; and wherein the VH domain comprises, consists, or consists essentially of SEQ ID NO:11, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:11.

In a preferred embodiment the CCR9 targeting moiety is an scFv comprising a VL domain and a VH domain, wherein the VL domain comprises, consists, or consists essentially of SEQ ID NO:10, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:10; and wherein the VH domain comprises, consists, or consists essentially of SEQ ID NO:11, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:11.

In another preferred embodiment the CCR9 targeting moiety is an antibody, F(ab')2, Fab, preferably scFv, or scFab comprising a VL domain and a VH domain, wherein the VL domain comprises, consists, or consists essentially of SEQ ID NO:12, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:12; and wherein the VH domain comprises, consists, or consists essentially of SEQ ID NO:13, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:13.

In a preferred embodiment the CCR9 targeting moiety is an scFv comprising a VL domain and a VH domain, wherein the VL domain comprises, consists, or consists essentially of SEQ ID NO:12, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:12; and wherein the VH domain comprises, consists, or consists essentially of SEQ ID NO:13, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:13.

In one embodiment, the CCR9-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:8 and the VH domain consists of SEQ ID NO:9.

In one embodiment, the CCR9-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:10 and the VH domain consists of SEQ ID NO:11.

In one embodiment, the CCR9-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:12 and the VH domain consists of SEQ ID NO:13.

In a preferred embodiment, the VL and the VH domains comprised in the CCR9-targeting moiety are linked by a peptide linker. In some embodiments, the linker comprises at least 5 amino acids, preferably between 5 and 25, preferably between 10-20 amino acids, most preferably 20 amino acids. Preferably, at least 10, at least 15 of the amino acids are G. Preferably, the peptide linker comprises, consists or consists essentially of SEQ ID NO:14, or a sequence that has 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:14 (see table 3 below).

In a preferred embodiment, the CCR9-targeting moiety further comprises a signal peptide that is placed preferably at the N-terminal region of the CCR9-targeting moiety. Preferably, the signal peptide comprises, consists or consists essentially of SEQ ID NO:15, or a sequence that has 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:15 (see table 3 below).

Table 3: Sequences depicting the peptide linker and signal peptide according to one embodiment

Peptide linker	SEQ ID NO:14	GGGGSGGGGSGGGGSGGGGS
Signal Peptide	SEQ ID NO:15	MALPVTGLLLSLGLLLHAARPTG

Preferably, the CCR9 targeting moiety comprises, consists, or consists essentially of SEQ ID NO: 16, SEQ ID NO: 17 or SEQ ID NO:18, or a sequence that has 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:16, SEQ ID NO:17, or SEQ ID NO:18. Most preferably, the CCR9 targeting moiety consists of SEQ ID NO:16, SEQ ID NO: 17 or SEQ ID NO:18.

Table 4: Sequences depicting humanized and murine CCR9 targeting moieties of present invention

Humanized CCR9 targeting moiety (HUM1)	SEQ ID NO:16	QVQLKQSGPEVVKPGTSVKVSCTASGYSFTDYIIYWVK QAHGRSLEWMGYIDPNNYNTRYSQKFQGKVTITVDKSS TSAYMELNSLTSDDTAVYYCARDVYWGQGTTVTVSSG GGGSGGGGSGGGGSGGGGSDVVMTQTPLSLSVTPGQ PASISCRSSQSLVHSNGKTYLQWYLQKPGQSPKLLIYKV SNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYFCA QSTHVWTFGGGTKVEIKRA

Humanized CCR9 targeting moiety (HUM2)	SEQ ID NO:17	QVQLVQSGAEVKKPGASVKVSCTASGYSFTDYIIYWVK QAHGQSLEWMGYIDPNNYNTRYSQKFQGKVTITVDKSS TSAYMELNSLTSDDTAVYYCARDVYWGQGTTVTVSSG GGGSGGGGSGGGGSGGGGSDVVMTQTPLSLSVTPGQ PASISCRSSQSLVHSNGKTYLQWYLQKPGQSPKLLIYKV SNRFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYFCA QSTHVWTFGGGTKVEIKRA
Murine CCR9 targeting moiety (MUR)	SEQ ID NO:18	QVQLKQSGPELVKPGTSVRVSCTASGYSFTDYIIYWVKQ SHGRSLEWIGYIDPNNYNTRYSQKFKGKATLTVDKSSTS AFMHLNSLTSDDSAVYYCARDVYWGQGTTLTVSSGGG GSGGGGSGGGGSGGGGSDVVMTQTPLSLTVSLGDQA SISCRSSQSLVHSNGKTYLQWYLQKPGQSPKLLIYKVSN RFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCAQS THVWTFGGGTKLEIKRA

Preferably, the CCR9 targeting moiety is a scFv that comprises, consists, or consists essentially of SEQ ID NO:16, or a sequence that has 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:16. Most preferably, the CCR9 targeting moiety is a scFv consisting of SEQ ID NO:16.

Preferably, the CCR9 targeting moiety is a scFv that comprises, consists, or consists essentially of SEQ ID NO:17, or a sequence that has 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:17. Most preferably, the CCR9 targeting moiety is a scFv consisting of SEQ ID NO:17.

Preferably, the CCR9 targeting moiety is a scFv that comprises, consists, or consists essentially of SEQ ID NO:18, or a sequence that has 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:18. Most preferably, the CCR9 targeting moiety is a scFv consisting of SEQ ID NO:18.

CHIMERIC ANTIGEN RECEPTOR (CAR)

The CCR9 targeting moieties as defined above can be part of a chimeric antigen receptor. Thus, in a third aspect the present invention relates to a chimeric antigen receptor (CAR) comprising:

a) an extracellular domain comprising a CCR9 targeting moiety according to the first and/or second aspect of present invention;

b) a transmembrane domain; and

c) an intracellular signaling 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 someone embodiment of present invention, 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 at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity on an amino acid level to the respective transmembrane domain.

In one embodiment of present invention, 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 a preferred embodiment the transmembrane domain comprises the transmembrane domain of CD8.

In a preferred embodiment the transmembrane domain comprises the transmembrane domain of CD8, or a variant thereof, wherein the variant thereof has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity on an amino acid level to the hinge and transmembrane domain of CD8 (SEQ ID NO.19).

In some embodiments, the transmembrane domain comprises the hinge and transmembrane domain of CD8 (SEQ ID NO.19).

The CARs according to present invention further comprise an 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 one embodiment, 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 at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity on an amino acid level to respective intracellular domain.

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

In a preferred embodiment, the intracellular signaling domain comprises the intracellular domain of CD3ζ or a variant thereof, wherein the variant thereof has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity on an amino acid level to the intracellular domain of CD3ζ (SEQ ID NO.20).

In a more preferred embodiment, the intracellular signaling domain comprises or consists of the intracellular domain of CD3ζ (SEQ ID NO.20).

Table 5: Sequences of Transmembrane and signaling domains

Transmembrane domain CD8	SEQ ID NO:19	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFA CDIYIWAPLAGTCGVLLLSLVITLYC
Intracellular signaling domain CD3ζ	SEQ ID NO:20	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERR RGKGHDGLYQGLSTATKDTYDALHMQALPPR
Costimulatory signaling domain CD137	SEQ ID NO:21	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

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 at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity on an amino acid level to sequence of the respective intracellular domain.

In a preferred embodiment, the costimulatory signaling domain comprises the intracellular domain of CD137 or a variant thereof, wherein the variant thereof has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity on an amino acid level to the intracellular domain of CD137 (SEQ ID NO:21).

In a more preferred embodiment, the costimulatory signaling domain consists of SEQ ID NO:21.

The following full sequence CARs are specifically envisaged:

A CAR comprising

(i) a scFv comprising a VL domain and VH domain, wherein said VL domain comprises LCDR1 (SEQ ID NO:2), LCDR2 (SEQ ID NO:3) and LCDR3 (SEQ ID NO:4) polypeptides and said VH domain comprises HCDR1 (SEQ ID NO:5), HCDR2 (SEQ ID NO:6) and HCDR3 (SEQ ID NO:7) polypeptides;

(ii) a transmembrane domain comprising SEQ ID NO:19 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:19;

(iii) an intracellular signaling domain comprising SEQ ID NO:20 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:20; and

(iv) a costimulatory signaling domain comprising SEQ ID NO:21 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:21.

A CAR comprising:

(i) a scFv comprising a VL domain and VH domain, wherein said VL domain comprises LCDR1 (SEQ ID NO:2), LCDR2 (SEQ ID NO:3) and LCDR3 (SEQ ID NO:4) polypeptides and said VH domain comprises HCDR1 (SEQ ID NO:5), HCDR2 (SEQ ID NO:6) and HCDR3 (SEQ ID NO:7) polypeptides;

(ii) a transmembrane domain consisting of SEQ ID NO:19;

(iii) an intracellular signaling domain consisting of SEQ ID NO:20; and

(iv) a costimulatory signaling domain consisting of SEQ ID NO:21.

A CAR comprising:

(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO:8 and the VH domain consists of SEQ ID NO:9;

(ii) a transmembrane domain comprising SEQ ID NO:19 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:19;

(iii) an intracellular signaling domain comprising SEQ ID NO:20 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:20; and

(iv) a costimulatory signaling domain comprising SEQ ID NO:21 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:21.

A CAR comprising:

(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO:10 and the VH domain consists of SEQ ID NO:11;

(ii) a transmembrane domain comprising SEQ ID NO:19 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:19;

(iii) an intracellular signaling domain comprising SEQ ID NO:20 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:20; and

(iv) a costimulatory signaling domain comprising SEQ ID NO:21 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:21.

A CAR comprising:

(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO:12 and the VH domain consists of SEQ ID NO:13;

(ii) a transmembrane domain comprising SEQ ID NO:19 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:19;

(iii) an intracellular signaling domain comprising SEQ ID NO:20 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:20; and

(iv) a costimulatory signaling domain comprising SEQ ID NO:21 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:21.

A CAR comprising:

(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO:8 and the VH domain consists of SEQ ID NO:9;

(ii) a transmembrane domain consisting of SEQ ID NO:19;

(iii) an intracellular signaling domain consisting of SEQ ID NO:20; and

(iv) a costimulatory signaling domain consisting of SEQ ID NO:21.

A CAR comprising:

(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO:10 and the VH domain consists of SEQ ID NO:11;

(ii) a transmembrane domain consisting of SEQ ID NO:19;

(iii) an intracellular signaling domain consisting of SEQ ID NO:20; and

(iv) a costimulatory signaling domain consisting of SEQ ID NO:21.

A CAR comprising:

(i) a scFv comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO:12 and the VH domain consists of SEQ ID NO:13;

(ii) a transmembrane domain consisting of SEQ ID NO:19;

(iii) an intracellular signaling domain consisting of SEQ ID NO:20; and

(iv) a costimulatory signaling domain consisting of SEQ ID NO:21.

A CAR comprising

(i) a scFv consisting of SEQ ID NO:16;

(ii) a transmembrane domain comprising SEQ ID NO:19 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:19;

(iii) an intracellular signaling domain comprising SEQ ID NO:20 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:20; and

(iv) a costimulatory signaling domain comprising SEQ ID NO:21 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:21.

A CAR comprising

(i) a scFv consisting of SEQ ID NO:17;

(ii) a transmembrane domain comprising SEQ ID NO:19 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:19;

(iii) an intracellular signaling domain comprising SEQ ID NO:20 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:20; and

(iv) a costimulatory signaling domain comprising SEQ ID NO:21 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:21.

A CAR comprising

(i) a scFv consisting of SEQ ID NO:18;

(ii) a transmembrane domain comprising SEQ ID NO:19 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:19;

(iii) an intracellular signaling domain comprising SEQ ID NO:20 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:20; and

(iv) a costimulatory signaling domain comprising SEQ ID NO:21 or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity to SEQ ID NO:21.

A CAR comprising:

(i) a scFv consisting of SEQ ID NO:16;

(ii) a transmembrane domain consisting of SEQ ID NO:19;

(iii) an intracellular signaling domain consisting of SEQ ID NO:20; and

(iv) a costimulatory signaling domain consisting of SEQ ID NO:21.

A CAR comprising:

(i) a scFv consisting of SEQ ID NO:17;

(ii) a transmembrane domain consisting of SEQ ID NO:19;

(iii) an intracellular signaling domain consisting of SEQ ID NO:20; and

(iv) a costimulatory signaling domain consisting of SEQ ID NO:21.

A CAR comprising:

(i) a scFv consisting of SEQ ID NO:18;

(ii) a transmembrane domain consisting of SEQ ID NO:19;

(iii) an intracellular signaling domain consisting of SEQ ID NO:20; and

a costimulatory signaling domain consisting of SEQ ID NO:21.

A CAR comprising or consisting of SEQ ID NO:22, or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:22.

A CAR comprising or consisting of SEQ ID NO:23, or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:23.

A CAR comprising or consisting of SEQ ID NO:24, or a sequence that has at least 95%, at least 96%, at least 97%, at least 98%, at least 99% sequence identity with SEQ ID NO:24.

Table 6: Sequences depicting the CAR-CCR9s (h1CAR-CCR9 (HUM1); h2CAR-CCR9 (HUM2); mCAR-CCR9 (MUR)

mCAR-CCR9 (MUR)	SEQ ID NO:22	MALPVTGLLLSLGLLLHAARPTGQVQLKQSGPELVKPGTSVRV SCTASGYSFTDYIIYWVKQSHGRSLEWIGYIDPNNYNTRYSQKF KGKATLTVDKSSTSAFMHLNSLTSDDSAVYYCARDVYWGQGT TLTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQTPLSLTVSL GDQASISCRSSQSLVHSNGKTYLQWYLQKPGQSPKLLIYKVSN RFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCAQSTHVWT FGGGTKLEIKRATTTPAPRPPTPAPTIASQPLSLRPEACRPAAG GAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLL YIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSAD APAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQ RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPR
H1CAR-CCR9 (HUM1)	SEQ ID NO:23	MALPVTGLLLSLGLLLHAARPTGQVQLKQSGPEVVKPGTSVKV SCTASGYSFTDYIIYWVKQAHGRSLEWMGYIDPNNYNTRYSQK FQGKVTITVDKSSTSAYMELNSLTSDDTAVYYCARDVYWGQGT TVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQTPLSLSVTP GQPASISCRSSQSLVHSNGKTYLQWYLQKPGQSPKLLIYKVSN RFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYFCAQSTHVWT FGGGTKVEIKRATTTPAPRPPTPAPTIASQPLSLRPEACRPAAG GAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLL YIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSAD APAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQ RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPR
H2CAR-CCR9 (HUM2)	SEQ ID NO:24	MALPVTGLLLSLGLLLHAARPTGQVQLVQSGAEVKKPGASVKV SCTASGYSFTDYIIYWVKQAHGQSLEWMGYIDPNNYNTRYSQK FQGKVTITVDKSSTSAYMELNSLTSDDTAVYYCARDVYWGQGT TVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQTPLSLSVTP GQPASISCRSSQSLVHSNGKTYLQWYLQKPGQSPKLLIYKVSN RFSGVPDRFSGSGSGTDFTLKISRVEAEDVGVYFCAQSTHVWT FGGGTKVEIKRATTTPAPRPPTPAPTIASQPLSLRPEACRPAAG GAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLL YIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSAD

		APAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQ RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPR

In a preferred embodiment of the CAR of present invention the CAR consists of SEQ ID NO:22.

In a preferred embodiment of the CAR of present invention the CAR consists of SEQ ID NO:23.

In a preferred embodiment of the CAR of present invention the CAR consists of SEQ ID NO:24.

SECOND TARGETING MOIETY

In a further embodiment of present invention, the humanized CCR9 targeting moiety, or the CAR as described herein above further comprise a second targeting-moiety.

Said second targeting moiety can be a targeting moiety that targets proteins that are overexpressed in T-ALL, such as for example CD3, CD4, CD5, CD7, CD37, CD30, CD33, CD99, CCR7, CDR3, TRBC1/2, or CD1a.[44]

In one embodiment the said second targeting moiety is CD3, CD4, CD5, CD7, CD37, CD30, CD33, CD99, CCR7, CDR3, TRBC1/2, or CD1a targeting moiety.

In a preferred embodiment said second targeting moiety is a humanized CD1a targeting moiety.

In one embodiment the humanized CD1a targeting moiety comprises a VL domain and a VH domain, wherein said VL domain comprises LCDR1, LCDR2 and LCDR3 polypeptides and said VH domain comprises HCDR1, HCDR2 and HCDR3 polypeptides, and wherein:

- LCDR1 comprises, consists, or consists essentially of SEQ ID NO:25, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:25;
- LCDR2 comprises, consists, or consists essentially of SEQ ID NO:26, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:26;
- LCDR3 comprises, consists, or consists essentially of SEQ ID NO:27, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:27;
- HCDR1 comprises, consists, or consists essentially of SEQ ID NO:28, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:28;

- HCDR2 comprises, consists, or consists essentially of SEQ ID NO:29, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:29; and
- HCDR3 comprises, consists, or consists essentially of SEQ ID NO:30, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:30.

Table 7: Sequences depicting the six CDRs HCDR1-3 and LCDR1-3 of the CD1a targeting moiety

LCDR-1	SEQ ID NO:25	QASQDINKYIA
LCDR-2	SEQ ID NO:26	IHYTSTL
LCDR-3	SEQ ID NO:27	LHYDNLPWT
HCDR-1	SEQ ID NO:28	SGYAFSTYTMH
HCDR-2	SEQ ID NO:29	YINPNSASTS
HCDR-3	SEQ ID NO:30	ARGFYTMDY

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.

In some embodiments, the CD1a-targeting moiety is an antibody, F(ab')2, Fab, preferably scFv, 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 as described herein.

In a preferred embodiment the CD1a-targeting moiety is an scFv.

In one embodiment the humanized CD1a targeting moiety is an antibody, F(ab')2, Fab, preferably scFv, or scFab comprising a VL domain and a VH domain, wherein the VL domain comprises, consists, or consists essentially of SEQ ID NO:31, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:31; and wherein the VH domain comprises, consists, or consists essentially of SEQ ID NO:32, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:32.

In another embodiment the humanized CD1a targeting moiety is an antibody, F(ab')2, Fab, preferably scFv, or scFab comprising a VL domain and a VH domain, wherein the VL domain comprises, consists, or consists essentially of SEQ ID NO:33, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO: 33; and

wherein the VH domain comprises, consists, or consists essentially of SEQ ID NO: 34, or a sequence with at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:34.

Table 8: Sequences depicting the different VL and VH domains of the CD1a targeting moieties

VL	SEQ ID NO:31	RDIQMTQSPSSLSASVGDRVTITCQASQDINKYIAWYQFKPGKGP RLLIHYTSTLEPAIPSRFSGSGSGREYTFTISSLQPEDIATYYCLHYD NLPWTFGGGTKVEIKRA
VH	SEQ ID NO:32	QVQLQQSGAEVKKPGASVKVSCKASGYAFSTYTMHWVRQAPGQ GLEWMGYINPNSASTSYAQKFQGRVTMTADKSTNTAYMELSSLR SEDTAVYYCARGFYTMDYWGQGTLVTVSS
VL	SEQ ID NO:33	DIQMTQSPSSVSASVGDRVTISCQASQDINKYIAWYQFKPGKGPR LLIHYTSTLQPAIPSRFSGSGSGREYTLTISSLQPEDFATYYCLHYD NLPWTFGGGTKVEVRRA
VH	SEQ ID NO:34	QVQLVQSGPEVAKPGASVRLSCKASGYAFSTYTMHWVRQAPGQ GLEWMGYINPNSASTSYNQNFKARVTLTADKSTNTAYMHLSSLTS DDTAVYYCGRGFYTMDYWGQGTLVTVSSA

In a preferred embodiment, the CD1a-targeting moiety is an antibody, F(ab')2, Fab, preferably scFv an antibody, scFv, Fab, or scFab comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO:31 and the VH domain consists of SEQ ID NO:32. Preferably the CD1a-targeting moiety is an scFv.

In another preferred embodiment the CD1a-targeting moiety is an antibody, F(ab')2, Fab, preferably scFv an antibody, scFv, Fab, or scFab comprising a VL domain and VH domain, wherein the VL domain consists of SEQ ID NO:33 and the VH domain consists of SEQ ID NO:34. Preferably the CD1a-targeting moiety is an scFv.

In a preferred embodiment, the VL and the VH domains comprised in the CD1a-targeting moiety are linked by a peptide linker. In some embodiments, the linker comprises at least 5 amino acids, preferably between 5 and 25, preferably between 10-20 amino acids, most preferably 20 amino acids. Preferably, at least 10, at least 15 of the amino acids are G. Preferably, the peptide linker comprises, consists or consists essentially of SEQ ID NO:14, or a sequence that has 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:14.

In a preferred embodiment, the CD1a-targeting moiety further comprises a signal peptide that is placed preferably at the N-terminal region of the CD1a-targeting moiety. Preferably, the signal peptide comprises, consists or consists essentially of SEQ ID NO:15, or a sequence that has

90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO: 15.

Preferably, the CD1a targeting moiety comprises, consists, or consists essentially of SEQ ID NO: 35, or a sequence that has 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO: 35. Most preferably, the CD1a targeting moiety consists of SEQ ID NO:35.

In another preferred embodiment, the CD1a targeting moiety comprises, consists, or consists essentially of SEQ ID NO:36, or a sequence that has 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 100% sequence identity to SEQ ID NO:36. Most preferably, the CD1a targeting moiety consists of SEQ ID NO:36.

Table 9: Sequences depicting humanized CD1a targeting moieties

Humanized CD1a targeting moiety - 1	SEQ ID NO:35	QVQLQQSGAEVKKPGASVKVSCKASGYAFSTYTMHWV RQAPGQGLEWMGYINPNSASTSYAQKFQGRVTMTADK STNTAYMELSSLRSEDTAVYYCARGFYTMDYWGQGTL VTVSSGGGGSGGGGSGGGGSGGGGSRDIQMTQSPSS LSASVGDRVTITCQASQDINKYIAWYQFKPGKGPRLLIHY TSTLEPAIPSRFSGSGSGREYTFTISSLQPEDIATYYCLH YDNLPWTFGGGTKVEIKRA
Humanized CD1a targeting moiety - 2	SEQ ID NO:36	QVQLVQSGPEVAKPGASVRLSCKASGYAFSTYTMHWV RQAPGQGLEWMGYINPNSASTSYNQNFKARVTLTADKS TNTAYMHLSSLTSDDTAVYYCGRGFYTMDYWGQGTLV TVSSAGGGGSGGGGSGGGGSGGGGSDIQMTQSPSSV SASVGDRVTISCQASQDINKYIAWYQFKPGKGPRLLIHY TSTLQPAIPSRFSGSGSGREYTLTISSLQPEDFATYYCLH YDNLPWTFGGGTKVEVRRA

In one preferred embodiment of present invention the CAR is therefore a CAR comprising a CCR9 targeting moiety as well as a CD1a targeting moiety as described herein. It is to be understood that such CAR encompasses all possible modes of combination of the two targeting moieties in the CAR. For example, a dual CAR is envisaged, preferably with the CCR9 targeting moiety in distal position. Further dual CAR options are described in the section here below.

In one embodiment the present invention also relates to a CD1a CAR comprising a CD1a targeting moiety as described herein.

In a further preferred embodiment, the CD1a CAR comprises a transmembrane domain and/or an intracellular signaling domain as described herein above for the CCR9 CAR.

DUAL CARs

Dual-targeting CAR T-cell therapy utilizes dual CAR strategies to identify two tumor-associated antigens in cancer. This can be achieved using two single CAR T-cell products with different antigen-binding specificities or a single CAR T-cell product capable of targeting two different antigens.

The inventors assessed the expression of CCR9 and CD1a in 170 primary T-ALL samples and found that 70% of the T-ALL samples were CCR9 positive and 59% were CD1a positive (Example 10). Dual staining of CCR9 and CD1a revealed the presence of phenotypically different T-ALL clones including double negative T-ALLs, double positive T-ALLs and single (CCR9 or CD1a) T-ALLs analyzed in human samples (Figure 18). The variable proportions of CD1a- and CCR9-expressing blasts within each patient (Figure 18) indicate the clinical advantage of a dual targeting of both CD1a and CCR9 that will cover 85% of patients that represents a significantly higher proportion of T-ALL blasts. Moreover, dual CD1a and CCR9 therapy will also contribute to reduce immune escape of those patients co-expressing both antigens (44%).

Efficacy and safety of a dual CAR-T targeting strategy against CCR9 and CD1a were tested on double positive cells for CCR9 and CD1a (wt), knock-out cells for CD1a (CD1aKO), knock-out for CCR9 (CCR9KO) and double knock-out for both CD1a and CCR9 (dKO) (Figure 19A and Example 11). The efficacy of single CAR-Ts against CD1a and CCR9, and the dual therapy, was similar as expected because cells co-express both antigens. This is demonstrated after incubation of target wt cells (T) with effector CAR-Ts cells (E) at different E:T ratios, compared to only untransduced cells (UT) (Figure 19C). Crossed experiments showed that single CAR-Ts against CCR9 eliminate MOLT4 CD1a KO but not CCR9 KO cells, and the opposite occurs with single CAR-Ts against CD1a. The dual combination of both CARs, after co-transduction, eliminates both CCR9 or CD1a KO cells with minimal potential loss compared to the single treatments. Moreover, the dual therapy did not show a significant increase of toxicity by using single Knock-out cells for CCR9 or CD1a or double-knock out cells.

In order to test the efficacy of a dual CAR-T targeting strategy against CCR9 and CD1a versus the single therapy, a population of MOLT4 cells co-expressing both antigens (wt cells) or expressing only CCR9 (CD1a KO cells) or only CD1a (CCR9 KO cells) were artificially mixed as further explained in Example 12. This complex population of tumor cells faithfully represents the % of blast cells found in a patient, as shown in Figure 20A. The inventors found that dual therapy using co-transduction of single CARs is superior in specific cytotoxicity compared with the use of

single CARs, as shown in Figure 20B at different E:T ratios or a fixed E:T ratio and at different time-points. Surprisingly, dual therapy was able to eliminate 100% of cells at 48 h compared to single CAR-T treatments. Importantly, as shown in Figure 20C, secretion of IL-2, TNFα, and IFNγ cytokines after 24h was similar in all the conditions and not a significant increase was observed comparing dual vs single therapy. This fact of killing a double population of cells positive for both CCR9 and CD1a without increasing the release of proinflammatory cytokines is very important to not increase the toxicity of dual therapy that may cause cytokine release syndrome (CRS), as a undesirable side-effect.

Furthermore, the inventors assessed the *in vivo* activity of single CCR9 and CD1a and dual CAR-Ts in vivo (Example 13 and Figure 21). Surprisingly, as occurred with UT cells, those mice given single CCR9-CARTs or CD1a-CARTs had massive tumor burden, suggesting that single CARs are unable to control the disease progression when a mix of blast cells expressing both antigen targets are present. However, the group of mice receiving de dual therapy (Mix-CD1a/CCR9 CAR-Ts) had minimal disease progression until day 26 (Figure 21). Quantification of BLI by IVIS showed no differences among the UT and CAR-CCR9 or CAR-CD1a, whose levels were significantly higher than those of mice receiving the dual therapy (Figure 21A and C). As expected, the results also show a significant reduction of tumor burden in BM in mice receiving the dual CAR-T therapy related to the single or UT (Figure 21B). Overall, the inventors could show the efficacy of the dual CCR9-CD1a-CAR T-cell therapy, showing a potent, synergistic and specific antileukemic activity against T-ALL expressing both CD1a and CCR9 in vivo. Interestingly, the synergistic effect in vivo is surprising compared with the additive effect observed in vitro in Figure 20B and C and may not be anticipated by the skilled person. Similar results were obtained when the dual therapy was co-transduction of T cells with CCR9 and CD1a CARs (Example 13 and Figure 21D and E).

The activity of single CCR9 or CD1a CAR-T therapy was also tested in vivo in a pre-clinical model of patient-derived xenografts (PDX) as described in Example 14. In this case, PDX2 T-ALL keep high levels of CCR9 and CD1a, similarly to those found in patients co-expressing both targets (see Figure 22). After tumor implantation, mice received a lower dose of CAR-Ts in comparison to the experiment shown in Example 13 in order to demonstrate long-term efficacy of treatments. In contrast to the mice receiving UT cells, which showed massive tumor burden by blast quantitation in PB, those mice given single CCR9 or CD1a CARTs or dual CCR9-CD1a CAR-T (co-transduction of single CARs) had a much lower disease progression until week 8 (Figure 22B). Importantly, after long-term observation at week 8, the dual combination of CARs showed a considerable reduction of tumor burden in PB, BM and spleen, compared with mice receiving single CCR9 or CD1a CAR-Ts related to UT, despite the co-expression of both antigens (Figure 22A). These results clearly show the advantage of dual therapy vs monotherapy in particular

when long-term progression is monitored. At the end of the experiment, tumor burden reduction in PB and SP was quite evident in those mice receiving the dual therapy (Figure 22, C and D). Overall, the inventors could show the efficacy of the dual CCR9/CD1a-CAR-T therapy product, as a mix of single CAR-Ts (Figure 21) or after co-transduction of single CARs (Figure 21 and 22), showing a potent and specific antileukemic activity of dual therapy in vivo that is stronger compared to monotherapy.

Finally, the activity of single CAR-Ts against CD1a and CCR9 (monotherapy) was compared with a dual-therapy approach by using the co-transduction of single CARs (Co) or the use of different bicistronic CAR versions co-expressing CCR9 and CD1a (Example 15 and Figure 23). Four bicistronic versions that differ in having different signal peptides, order of scFvs, and CAR DNA sequences were tested and compared between them. To determine the functionality of bicistronic in comparison with co-transduction constructs, cytotoxicity assays with MOLT4 cells expressing both CCR9 and CD1a or with the different knock-out versions, as a control of specificity were performed. In Figure 23 (panels C to F) it can be clearly observed that the different bicistronic CAR-Ts are as potent as the co-transduction, resulting in similar killing efficiency. The cytotoxic activity of bicistronic CAR-Ts against single positive cells for CD1a or CCR9 is as effective as the monotherapy and the co-transduction approach, as shown by using the corresponding CCR9 or CD1a KO cells, respectively.

In a fourth aspect the present invention therefore relates to a dual CAR comprising a first and a second CAR, wherein the first CAR can be a CAR comprising a CCR9 binding moiety as described herein above under “CHIMERIC ANTIGEN RECEPTOR (CAR)” and the second CAR is a CAR comprising a CD1a targeting moiety as described herein above under “SECOND TARGETING MOIETY”.

In a preferred embodiment the CD1a targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:31 or SEQ ID NO:33 and a VH domain consisting of SEQ ID NO:32 or SEQ ID NO:34.

In a preferred embodiment the CD1a targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:31 and a VH domain consisting of SEQ ID NO:32.

In one embodiment of this aspect the CAR is a bispecific CAR T-cell capable of targeting two antigens, specifically CCR9 and CD1a. The bispecific CAR can be generated via a bivalent vector or a bicistronic vector.

The potential advantage of bivalent CARs includes the relative ease and cost of manufacturing a single CAR T-cell product using one vector that uniformly expresses both scFvs.

In one embodiment the bispecific CAR is a bivalent CAR, also referred to as “tandem” CAR.

In a preferred embodiment the bispecific CAR is a bicistronic CAR.

In one preferred embodiment of this fourth aspect of present invention the CAR is a bicistronic CAR construct comprising:

(a) a first CAR comprising a CCR9 targeting moiety as described herein, a first signal peptide, a first transmembrane domain, and a first intracellular T cell signaling domain; and

(b) a second CAR comprising a CD1a targeting moiety as described herein, a second signal peptide, a second transmembrane domain, and a second intracellular T cell signaling domain; and

(c) a cleavable domain; wherein the cleavable domain is positioned between the first and second CARs.

In one preferred embodiment of this fourth aspect of present invention the CAR is a bicistronic CAR construct comprising:

(a) a first CAR comprising a CD1a targeting moiety as described herein, a first signal peptide, a first transmembrane domain, and a first intracellular T cell signaling domain; and

(b) a second CAR comprising a CCR9 targeting moiety as described herein, a second signal peptide, a second transmembrane domain, and a second intracellular T cell signaling domain; and

(c) a cleavable domain; wherein the cleavable domain is positioned between the first and second CARs.

In a preferred embodiment, the first and second signal peptides are different.

In a more preferred embodiment, the first signal peptide is SEQ NO:15.

In a preferred embodiment the second signal peptide is a different signal peptide than the first one and is able to translocate the second CAR to the target cellular membrane. Preferred are the signal peptides from human IgGI or murine IgG.

The cleavable domain can release the first and second CARs from the CAR construct.

The first CAR and the second CAR of the inventive CAR constructs can be joined to each other through 1, 2, 3, 4 or more cleavable domains. The cleavable domain(s) may comprise one or

more of any suitable cleavable domains, including domains recognized by cleavage enzymes or domains that are self-cleaving. Suitable domains include, for example, the 2A peptide. In certain embodiments, the 2A peptide is selected from the group consisting of porcine teschovirus-1 2A (P2A), Thosea asigna virus 2 A (T2A), equine rhinitis A virus 2 A (E2A), and foot-and-mouth disease virus 2 A (F2A), which sequences are

P2A sequence is ATNFSLLKQAGDVEENPGP (SEQ ID NO: 79)
T2A sequence is EGRGSLLTCGDVEENPGP (SEQ ID NO: 80)
E2A sequence is QCTNYALLKLAGDVESNPGP (SEQ ID NO: 81)
F2A sequence is VKQTLNFDLLKLAGDVESNPGP (SEQ ID NO: 82)

In a preferred embodiment the cleavable domain is foot-and-mouth disease virus 2 A (F2A).

In one preferred embodiment of the bicistronic CAR, the CCR9 targeting moiety is SEQ NO: 24.

Alternative dual antigen targeting models include co-infusion or co-transduction of two CARs also leading to bispecific targeting. The present invention therefore also relates in a further aspect to a single CCR9 CAR as described herein and a single CD1a CAR and their use for co-infusion or co-transduction.

In a further preferred embodiment, the CAR-CCR9 of present invention can thus be combined with a CAR-CD1a as described herein during treatment.

NUCLEIC ACIDS

In a fifth aspect the present invention relates to a nucleic acid encoding any one of the targeting moieties 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 the target, specifically CCR9 and/or 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.

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. 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.

The nucleic acid of present invention may be a bicistronic vector or a bivalent vector. The nucleic sequence of the two CARs in the bicistronic vector may be different to avoid homologous recombination between the first and the second CAR.

In a preferred embodiment, the DNA sequence of the second CAR is modified by using alternative codon usage in mammalian cells. Modification can involve the linker region between scFv, the transmembrane domain and the intracellular domains CD3ζ and 4-1BB.

CELLS

In a sixth aspect the present invention relates to a cell comprising said nucleic acid and/or the CAR of present invention. In a preferred embodiment 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.

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. 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 effector T cell is another allogeneic T cell, such as a gamma-delta T cell (referred to as a CAR gamma-delta T cell) prepared from numerous sources, including peripheral blood mononuclear cells (PBMCs), cord blood, and pluripotent stem cells (iPSCs), or it may be an invariant natural killer T (iNKT) cell (referred to as CAR iNKT cell).

In some embodiments the effector cell may be a natural killer (NK) cell (referred to as CAR NK cell) prepared form numerous sources, including peripheral blood mononuclear cells (PBMCs), cord blood, and pluripotent stem cells (iPSCs).

In some embodiments the effector cell may be a macrophage (referred to as CAR macrophage).

In some embodiments, the T cell is a CD3-positive and CCR9 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.

In some embodiments the cell can contain one CAR or two different CARs, specifically a CD1a CAR and a CCR9 CAR as described herein, or a bi-valent (tandem) CAR.

PHARMACEUTICAL COMPOSITION

In a seventh aspect the present invention relates to a pharmaceutical composition comprising a plurality of cells of 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 an eigth aspect the present invention relates to the cell or the pharmaceutical composition of the invention for use as a medicament.

METHODS OF TREATMENT

In a ninth aspect the present invention relates to the cell or the pharmaceutical composition of the invention for use in a method of treating a CCR9-positive cancer, wherein the method comprises administering the cell or composition to a patient in need thereof.

In one embodiment the CCR9-positive cancer is T-cell acute lymphoblastic leukemia, preferably, relapsed/refractory T-cell acute lymphoblastic leukemia or T-cell lymphoma.

In a tenth aspect the present invention relates to the cell or the pharmaceutical composition of the invention for use in a method of treating a CD1a-positive cancer, preferably a CD1a and CCR9-positive cancer, wherein the method comprises administering the cell or composition to a patient in need thereof.

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

In an eleventh aspect the present invention relates to the cell or the pharmaceutical composition of the invention for use in the method for treating a CD1a-positive and/or a CCR9-positive cancer, preferably a CD1a and CCR9-positive cancer, the method further comprising administering simultaneously or subsequently a CD1a CAR comprising a CD1a targeting moiety, a transmembrane domain and an intracellular signaling domain.

It is to be understood that the CD1a targeting moiety can be any of the targeting moieties as described herein above under the item “second targeting moiety”.

In a preferred embodiment the CD1a targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:31 or SEQ ID NO:33 and a VH domain consisting of SEQ ID NO:32 or SEQ ID NO:34.

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. 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 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 (2013)); 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)).

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

Example 1: CCR9 expression in healthy tissues and T-ALL samples

CCR9 expression was assessed in healthy tissues and T-ALL samples. For this a Single-cell RNA seq analysis of CCR9 expression was performed in healthy tissues as described in Tabula Sapiens Consortium, Science (2022) (Figure 1A). CCR9 expression is detected in thymocytes and T cells from the small intestine. Furthermore, CCR9 expression was determined in human neonatal thymus subpopulations by FACS (n=4). Low CCR9 expression was found in human peripheral blood (PB, n=18) and bone marrow (BM, n=13) respectively, from adult and pediatric healthy donors, with the exception of B-cells where is slightly increased (Figure 1B to 1D). By contrast, CCR9 is expressed in blast cells from T-ALL patients (n=170) (Figure 1E). CCR9 expression is detected in blasts from T-ALL patients stratified across immunophenotypes/subtypes (n=170) (Figure 1F). In addition, CCR9 expression in T-ALL blasts is similarly detected at diagnosis (Dx) and relapse (Rel) (Figure 1G).

Example 2: CCR9 antibody generation strategy

Anti-CCR9 antibody-secreting hybridomas were generated by mouse immunisation with human CCR9 peptides with an extra Cys added at C-term fused with KLH to improve immune response and to BSA for ELISA screenings (ProteoGenix) (Figure 2). Given the membrane-bound nature of CCR9, two extracellular sequences/regions were chosen for mouse immunisation. From the extracellular sequence 1, the longest, two different 21-aminoacid long peptides were used (#1 and #2). From the extracellular sequence 2, one 21-aminoacid long peptide was used (#3). Immunization of 5 mice with peptides was achieved by 4-6 injections until obtain an optimal immune response. Immune response was tested after bleeding and titer test by ELISA against the peptides and fusion of spleen cells from best mice was conducted with mouse myeloma cell lines by following standard methods. Screenings at polyclonal stage were conducted by ELISA against the peptides and positive ones were further tested by FACS (Figure 3A shows a representative analysis). Hybridoma clones were tested by incubating their conditioned media containing the secreted antibodies (“supernatant”) with the T-ALL CCR9+ cell line MOLT-4 and

the CCR9^{KO} MOLT-4. A secondary anti-mouse IgG (H+L) antibody conjugated with Alexa Fluor (A) 647 was used to detect labelled cells. Only one hybridoma clone tested positive by flow cytometry (Clone 115) (Figure 3A). As a summary, a total of 82 hybridoma candidates were tested (Figure 3):

- From MADDYGSESTSSMEDYVNFNF peptide #1: 40 clones were positive by ELISA screening but only clone 115 were tested positive by FACS (Figure 3).
- From MEDYVNFNFTDFYCEKNNVRQ peptide #2: 6 clones were positive by ELISA screening but none of them were positive by FACS.
- From YSQIKEESGIAICTMVYPSDE peptide #3: 36 clones were positive by ELISA but none of them were positive by FACS.

Clone 115 was further tested using the CCR9-negative 300.19 cell line (WT) and its CCR9-overexpressing counterpart (Figure 3B). After selection, hybridoma sub-cloning, isotype determination, high-scale antibody production and purification by ProteinA/G was conducted by standard methods. Isotype is IgG1 and 10 mg of antibody at 2,8 mg/mL was obtained after concentration, dialysis (PBS pH 7,4) and sterilization by membrane filtration. ELISA titer and SDS-PAGE quality control analysis is shown in Figure 3C. The hybridoma #115 was sequenced to obtain the scFv (SEQ number 12 and 13 and Figure 11), using a mouse IgG library primer set (Sánchez-Martínez et al, *Blood* (2019)), and a second-generation 4-1BB-based CAR-CCR9 was cloned into a clinically validated pCCL lentivector (Figure 4).

Example 3: Humanization of clone 115

As CAR-CCR9 was of murine origin (mCAR-CCR9), humanization of the murine scFv was performed to avoid immunogenic response in humans and to make the CAR T-cell product more suitable for using in the clinics. Humanization of mCAR-CCR9 has followed 2 strategies as summarized in Figure 5:

1) Sequence-based “Strict” mode

3) Sequence-based “Relaxed” mode

1. Sequence-based “Strict” mode:

This approach follows the subsequent protocol:

- Use the sequence of the murine scFv to query the database of IgG (http://www.imgt.org/).
- The search returns a list of human Ig genes ranked by E-value.
- Choose the one with the highest sequence identity to both heavy and light chains (Figures 6A and 7A).
- Besides the CDRs and stems regions look at other changes (non-conserved) and assess whether to change it or not given the conservation among the different human sequences (Figures 8, 9 and 10) and the potential impact at structural/functional level. The latter is assisted by looking

at the position and interactions that non-conserved positions make to its environment using the structural model of scFv (for example, close to CDRs or stems regions; residues important for packing; etc).

- Two different strategies to decide how many changes to perform on the non-conserved position were devised: strict and relaxed. The sequence-based "strict mode" tries to make the minimum changes to achieve humanization. In contrast, the "relaxed mode" allows more changes to the human sequence.

The CAR-CCR9 generated following this sequence-based "strict" method is named hereinafter as h1CAR-CCR9 or HUM1. In this case, regarding the heavy chain the query to the IgG database gives the Germline IGHV1-3*01 that shares the highest sequence identity to the heavy chain (60.2%). For the heavy chain, the number of different residues (with different levels of conservation) without considering CDRs is 32 (Figure 6A). Regarding the light chain, we found the Germline IGKV2D-29*02 shares the highest sequence identity to the light chain (82%). For the light chain, the number of different residues without considering CDRs is 13.

The conservation among the different human sequences for the heavy and light chains is indicated in Figures 8, 9 and 10 and is used to assess the level of conservation among human sequences in non-conserved positions (that are not the CRDs and stem regions, indicated in Figures 6, 7 and 10) between murine and human sequences.

According with the above instructions the decision-make process is summarized below. For the heavy chain (see Figure 6B for the indicated positions):

- Position a: K5 murine (V human) which is facing T23 at the Nt stem of H1, probably important for packing function (murine: K-T and human V-K). We decide to keep the murine (K) residue.
- Position b: P9 murine (A human), L11 murine (V human) and V12 murine (K human).
  P9 is located at the start of a beta-sheet with a potential structural role. We decide to keep the murine residue (P).
  L11 is fine to change to V (human) as is fully exposed. It pairs with T109 in the Ct stem of H3 and T109 is both conserved in human/murine. We decide to keep the human (V).
  V12 is buried in the structure and might be important for packing. The corresponding in human is a K, which is clearly a not conserved substation, hence we decide to keep the murine (V).
- Position c: T16 murine (A human) is fully exposed in a loop but close to V12 (packing loop). We decide to keep the murine (T).
- Position d: R19 murine (K human) is fully exposed in the middle of a beta-sheet. Conservative substitution. We decide to keep the human (K).

- Position e: A stretch with a number of changes: 38-KQSHGRS-44 murine (40-RQAPGQR-44 human) located on a loop between H1 and H2 as well as K38 at interaction distance to D89 D90 at the base of H3 Nt beta.

  K38: contacts with Nt stems of H3: D89 (D murine->E human) and D90 (which is conserved in both). We decide to keep the murine (K).

  S40: Conservative substitution. We decide to keep the human (A).

  H41: Human has a P, which is a very unique amino-acid structure-wise. H might be important for loop conformation in murine (as P in human). We decide to keep the murine (H).

  R43: It may be important for loop conformation. We decide to keep the murine (R)

  S44: Located at VL-VH packing and Nt stem of L3. It faces the 103-GGG. We decide to keep the murine (S).
- Position f: I48 murine (M human): Buried in the core, so might be important in human scaffold: We decide to keep the human (M).
- Position g: R59 murine (K human). It's aligned to important positions at Nt stem of H2: Y50. We decide to keep the murine (R)
- Position h: composed of stretch 65-KGKA-68 murine (QGRV human)

  K67 interacts with D90 (conserved), which is related to position K38 in position e (murine preserved). We decide to keep the murine (K67). For the rest amino-acids, we decide to keep the human (Q65 which is fully exposed and V68 which is at starting of a beta sheet.
- Position i: composed of stretch 70-LTVDK-74 (ITRDT human)

  L70 murine (I human) which is fully buried. We decide to keep the human (I) as might be important for the fold of the human scaffold.

  V72 and K74 are both facing H2 and K74 is facing H1. We decide to keep both murine residues.
- Position j: stretch 76-STS-79 murine (AST human). Part or a loop that stacks against H1. Just after K74 in (i). We decide to keep all murine residues in the region.
- Position k: stretch 80-FMHLN-85 murine (YMELS in human). F80 and H82 are exposed and related to R19 (where human was kept). We decide to keep both human (Y80 and E82). N84 faces a loop containing K67 (important position). We decide to keep the murine (N84).
- Position l: stretch 87-TSDDS91 murine (RSEDT in human). All residues are located in a loop. T87 neighbors D89 which relates to (e) and (h). We decide to keep the murine (T87). D89 seems to be important in murine and given the conserved nature of the substitution to human E, we decide to keep the murine. S91 (murine) is a conserved substitution (T). We decide to keep the human (T91).
- Position m: L108 murine (V human). Buried residues and conserved substitution. We decide to keep the human (V).

And for the light chain (Figure 7B):

- Position a: V2 murine (I human) at Nt is sandwiched between L1 and L3. We decide to keep the murine (V).

- Position b: composed of stretch 12-TVSLGDQ-18 murine (SVTPGQP human) are all in a exposed loop just before the Nt beta of L1.
  T12 is S in human is a conservative substitution. Keep the human.
  S14 is T in human is a conservative substitution. Keep the human.
  L15, Q18 are both P in human and might play a structural in human scaffold without obvious implication to function of murine. Keep the human.
  D17 is Q in human is a conservative substitution. Keep the human
- Position c: K50 murine (Q human). It is s located at the base of Nt beta of L2 and close to the interface with H3. We decide to keep the murine (K).
- Position d: L88 murine (V human). It is partially exposed with conserved residues around V in human. A conservative substitution. We decide to keep the human (V).
- Position e: F92 is a Y in human. Might be important for interface packing and it is close to position h in heavy chain. We decide to keep the murine (F).
- Position f: L108 murine (V human) is a fully buried aminoacid and could be important for structure of the human scaffold. We decide to keep the human (V).

Figure 8 summarizes the above indicted changes and the proposed humanized sequence. According with this methodology, the final selected sequence in the 'strict mode' is the next:

Heavy chain
QVQLKQSGPEVVKPGTSVKVSCTASGYSFTDYIIYWVKQAHGRSLEWMGYIDPNNYNTRYSQ
KFQGKVTITVDKSSTSAYMELNSLTSDDTAVYYCARDVYWGQGTTVTVSS

Light chain
DVVMTQTPLSLSVTPGQPASISCRSSQSLVHSNGKTYLQWYLQKPGQSPKLLIYKVSNRFSGV
PDRFSGSGSGTDFTLKISRVEAEDVGVYFCAQSTHVWTFGGGTKVEIKRA

2. Sequence-based “Relaxed” mode

A different humanized sequence was selected allowing more amino acid changes in the human sequence with respect to the murine sequence. In Figure 10 are indicated both the “strict” and “relaxed” sequences together with the murine original sequence. The proposed “relaxed” sequence is indicated below:

Heavy chain
QVQLVQSGAEVKKPGASVKVSCTASGYSFTDYIIYWVKQAHGQSLEWMGYIDPNNYNTRYSQ
KFQGKVTITVDKSSTSAYMELNSLTSDDTAVYYCARDVYWGQGTTVTVSS

Light chain
DVVMTQTPLSLSVTPGQPASISCRSSQSLVHSNGKTYLQWYLQKPGQSPKLLIYKVSNRFSGV
PDRFSGSGSGTDFTLKISRVEAEDVGVYFCAQSTHVWTFGGGTKVEIKRA

Example 4: CAR-CCR9 detection in transduced T cells

T cells transduced with mCAR-CCR9 (mur) and h1CAR-CCR9 (HUM1) and h2CAR-CCR9 (HUM2) could be detected by FACS. Figure 12 shows representative flow cytometry plots showing CAR construct detection in transduced T cells (GFP+) and anti-scFv. Histogram analysis of transduced cells show the murine (mur) and HUM2 CAR constructs are detectable with anti-F(ab')2 antibodies (anti-scFv).

Example 5: CAR-CCR9 cell line panel cytotoxicity

The capacity of CAR-CCR9 T-cells to eliminate target (T) CCR9+ MOLT4 and SupT1 cells was analyzed (Figure 13A). For this purpose, target cells (100.000 cells per condition) were labelled with 3 µM eFluor 670 and incubated with UT PBMCs and indicated CCR9-CAR-Ts at different effector (E):Target (T) ratios during 24 or 48 hours. The CCR9- MV4;11 cell line was employed as a control of specificity (Figure 13A). Cell viability was monitored by eFluor labelling of target cells and detection by FACS and after incubation with 7-amino actinomycin D (7-ADD) at each time point and E:T ratio. Percentage of cell populations were calculated by FACSDiva software analysis. Fraction of non-apoptotic cells were further quantified after incubation with Annexin V protein. The fraction of eFluor+/AnnexinV-/7ADD- was considered as non-apoptotic/death cells (Figure 13B) and analyzed with n=5 different donors. As shown in Figure 13B, murine and HUM2 CAR-CCR9-Ts are equally effective in killing MOLT4 or SupT1 cells but not MV4;11 cells. Killing activity of HUM1 was slightly lower. Altogether, these results clearly show that humanized CCR9--CAR T cells are as effective as murine CAR T-cells to promote cell death *in vitro*.

Example 6: CAR-CCR9 functionality *in vivo*

The activity of murine and humanized CCR9-CAR-Ts in vivo using Luc/GFP-expressing MOLT4 T-ALL cells was also evaluated (Figure 14). Immunodeficient mouse (NOD scid gamma) NSG mice were transplanted with $1{,}5x10^6$ Luc-expressing MOLT4 cells 3 days before intravenous (iv) infusion of either $4x10^6$ CCR9-CAR-Ts or UT PBMCs, and leukemia establishment was followed up bi-weekly by using bioluminescence (BLI) quantification (Figure 14A). To measure BLI, mice were given 150 mg/kg of D-luciferin intraperitoneally which, when interacting with luciferase, emitted a bioluminescent signal proportional to the number of tumor cells. This signal is measured

on a Xenogen IVIS 50 Imaging System (Perkin Elmer). The tumour burden was monitored at day 0 (day of CAR infusion), 3, 7, 11 and 13. Living Image software was used to visualise and calculate total luminescence as average radiance quantification (p/sec/cm2/sr). In contrast to the mice receiving UT cells, which showed massive tumor burden by BLI, those mice given CCR9-CAR-Ts had a lower disease progression until day 13 (Figure 14B). Quantification of BLI by IVIS showed no differences among the murine CAR-CCR9 and the humanized versions (Figure 14C).

For the analysis of tumour circulating cells and persistence of the transferred CARTs, the mice were sacrificed at the end of the experiment (day 13) and circulating peripheral blood (PB), hindlimbs and spleen were taken. BM cells were isolated by flushing the hindlimbs with PBS containing 2%FBS. Spleen cells were isolated by mincing the spleen by crushing with a sterile syringe piston and passing the cell suspension through a 70 µm cell strainer. For the FACS analysis, 100 µl of PB, BM and spleen cell suspension was stained with PBS/2%FBS containing fluorochrome ligated antibodies for the following markers: human leucocyte antigen (HLA)-ABC, CD45, CD3, CCR9, CD1a, CD38, for 30 min at 4 degrees. 1 ml of BD FACSTM fixing-lysis buffer (BD Biosciences) was added to each sample to eliminate erythrocytes. Samples were run in a FACSCantoTM-II flow cytometer. The % of blasts were determined as HLA-ABC+ CD45+ CD34+ CD3- and confirmed to be CD1a+ (and CCR9+). The results show a significant reduction of tumor burden in PB, BM and spleen in mice receiving CCR9 humanized or murine CAR-Ts related to UT (Figure 14D). Human CAR T-cells (HLA-ABC+ CD45+ CD3+) can be detected by FACS in the different compartments (Figure 14D). Overall, the above data demonstrates the efficacy of the murine and humanized CCR9-CAR T-cell products, showing a potent and specific antileukemic activity against T-ALL cell lines *in vitro* and potent antileukemic activity *in vivo*.

The activity of humanized 2 (HUM2) CCR9-CAR-Ts was further tested in vivo using a pre-clinical model of Luc/GFP-expressing patient-derived xenografts (PDX), initially created by implanting T-ALL blast cells of a human tumor into a mouse as previously described (45) (Figure 15). PDX mice largely retain the genetics of the human tumors from which they were initially created and is a valuable tool for optimizing CART therapy (46). In this case, PDX843 T-ALL keep high levels of CCR9 (Figure 15A) and after implantation, in contrast to the mice receiving UT cells, which showed massive tumor burden by bioluminescence, those mice given CCR9-CARTs had a lower disease progression until day 14 (Figure 15B and 15C). The results show a significant reduction of tumor burden in PB, BM and spleen in mice receiving humanized CAR-Ts related to UT (Figure 15D). Overall, the inventors could show the efficacy of the humanized CCR9-CAR T-cell products, showing a potent and specific antileukemic activity in vivo.

Example 7: Epitope Mapping clone 115

In order to assess to which epitope in CCR9 clone 115 binds, an epitope mapping was performed. The antibody for the mapping was produced as described in example 2. Peptides were synthesized with a purity>98% with an extra C-terminal Cys for BSA conjugation. As a positive control the peptide used for mice immunization was used MADDYGSESTSSMEDYVNFNF-C. Then, the next battery of peptides were synthesized covering the whole sequence from the N-terminal to the C-terminal part:

- Peptide 1: MADDYGSEST-C
- Peptide 2: GSESTSSMED-C
- Peptide 3: SSMEDYVNFN-C
- Peptide 4: SMEDYVNFNF-C

Binding of peptides to 115 antibodies were tested by ELISA titration. In brief, plates with each non-conjugated peptide (50µg/mL) or BSA-conjugated peptide (5µg/mL) were coated in 0.58M carbonate-bicarbonate buffer pH9.5, 100µL/well, at 4ºC O/N. After washing with PBS + Tween-20 0.05% v/v (0.05%PBST) and blocking with 3%milk-PBS, peptide coated wells were incubated with 115 purified antibodies at concentration ranging from 0 to 1µg/mL in PBS, washed and incubated with a secondary antibody (goat anti-mouse IgG-HRP). After washing, wells were incubated with the 3, 3′,5 ,5′-Tetramethylbenzidine (TMB) HRP substrate and reactions were stopped with 2M HCl and OD450 was recorded. Table 1 shows the obtained results.

Table 1: ELISA Results

Antibody dilution (µg/mL)	Peptide 1-BSA		Peptide 2-BSA		Peptide 3-BSA		Peptide 4-BSA		Positive control antigen peptide-BSA	
	repI	repII	repI	repII	repI	repII	repI	repII	repI	repII
1	0,04	0,03	0,03	0,03	2,17	2,16	2,73	2,75	2,58	2,68
0,5	0,03	0,02	0,02	0,02	2,03	2,06	2,67	2,65	2,44	2,57
0,25	0,02	0,02	0,02	0,02	1,95	2,05	2,46	2,53	2,29	2,42
0,125	0,02	0,01	0,01	0,01	1,97	1,97	2,23	2,39	2,15	2,31
0,0625	0,01	0,01	0,01	0,01	1,88	1,78	1,98	2,01	1,84	2,08
0,03125	0,01	0,01	0,01	0,01	1,66	1,57	1,44	1,53	1,28	1,48
0,01563	0,01	0,01	0,01	0,01	1,33	1,34	0,91	1,03	1,10	1,21
Blank (no Ab)	0,01	0,01	0,01	0,01	0,01	0,01	0,02	0,02	0,02	0,01

Antibody dilution (µg/mL)	Peptide 1		Peptide 2		Peptide 3		Peptide 4		Negative control BSA	
	repI	repII	repI	repII	repI	repII	repI	repII	repI	repII
1	0,02	0,02	0,03	0,02	2,06	2,10	2,49	2,46	0,02	0,02
0,5	0,01	0,02	0,02	0,02	1,94	1,83	2,29	2,37	0,02	0,02
0,25	0,02	0,02	0,02	0,01	1,89	1,80	2,09	2,24	0,01	0,02
0,125	0,01	0,02	0,01	0,01	1,77	1,77	1,69	1,84	0,01	0,01
0,0625	0,01	0,01	0,01	0,01	1,83	1,67	1,14	1,19	0,01	0,01
0,03125	0,01	0,01	0,01	0,01	1,65	1,62	0,67	0,76	0,01	0,01
0,01563	0,01	0,01	0,01	0,01	1,47	1,40	0,38	0,46	0,01	0,01
Blank (no Ab)	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01	0,01

The measured OD shows a strong binding against the complete antigen peptide MADDYGSESTSSMEDYVNFNF, used to generate the 115 antibody. In addition, the purified antibody 115 detect Peptide 3 (SSMEDYVNFN) and Peptide 4 (SMEDYVNFNF) in ELISA with strong signal but no signal was detected with Peptide 1 (MADDYGSEST) and Peptide 2 (GSESTSSMED). As a result, the 9 AA epitope sequence recognized by antibody 115 on the MADDYGSESTSSMEDYVNFNF peptide is highlighted in the full peptide antigen sequence: MADDYGSESTS**SMEDYVNFN**F.

Example 8: Comparative Affinity Studies

To assess the binding affinity of the antibodies of present invention compared to CCR9 antibodies of the prior art, the inventors synthesized the following antibodies as shown here below in CHO-S cells containing a 6xHis tag (bold and underlined) and determined the binding to the N-terminal peptide region of CCR9. All the scFvs were synthesized with the same signal peptide (underlined) and linker region between VH and VL to minimize differences. The sequences are:

Antibody of present invention: “CCR9_HUM2” (Signal peptide + SEQ ID NO:17 of present application + his-tag)

MGWSCIILFLVATATGVHSQVQLVQSGAEVKKPGASVKVSCTASGYSFTDYIIYWVKQA
HGQSLEWMGYIDPNNYNTRYSQKFQGKVTITVDKSSTSAYMELNSLTSDDTAVYYCAR
DVYWGQGTTVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQTPLSLSVTPGQPASI
SCRSSQSLVHSNGKTYLQWYLQKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFTLK
ISRVEAEDVGVYFCAQSTHVWTFGGGTKVEIKRA**HHHHHH**

Comparative antibody 1 (“Comp-1”): SEQ ID NO: 10 from WO2023/037125 A1

MGWSCIILFLVATATGVHSQVQLKESGPGLVQPSQTLSLTCTVSGFSVASYDMHWVRL
PPGKGLEWMGIIWANGNTHYNSGLKSRLSISRDTSKSQVFLKMNSLQTEDTAIYFCTRG
GFAYWGQGTLVTVSSGGGGSGGGGSGGGGSGGGGSDVVMTQTPVSLSVSLGGQVS
ISCRSSQSLVHNNGNTYLSWYLQKPGQSPQLLIYKVSNRFSGVSDRFSGSGSGTDFTL
KISRVEPDDLGVYYCGQGTQYPTFGGGTKLELK**HHHHHH**

(in this application identified as SEQ ID NO:57)

Comparative antibody 2 ("Comp-2"): "243LO326" (humanized optimized antibody, SEQ ID NO: 51 (VH) and SEQ ID NO: 52 (VL) of WO 2022/195028 A2)

MGWSCIILFLVATATGVHSQVQLVQSGAEVKKPGASVKVSCKASGYTFTSYWMHWVR
QAPGQGLEWIGVIYPGNSDTRYNQKFKGRVTITRDTSASTAYMELSSLRSEDTAVYYC
TRDYYSNYVYYYAMDYWGQGTTVTVSSGGGGSGGGGSGGGGSGGGGSDIVMTQTP
LSLPVTPGEPASISCRSSQSLVHPNRNTYLHWYLQKPGQSPQLLIYKVSNRFSGVPDRF
SGSGSGTDFTLKISRVEAEDVGVYYCSQSTHVPWTFGQGTKLEIK**HHHHHH**

(in this application identified as SEQ ID NO:58)

Comparative antibody 3 ("Comp-3"): "SR_92R" (Sequence obtained from Somovilla-Crespo Beatriz et al.; Frontiers in Immunology 2018; Figure 5)

MGWSCIILFLVATATGVHSEVKLEESGGGLVQPGGSMKLSCVASGFTFNKFWMNWVR
QSPEKGLEWVAEIRLKSNNYATHYAESVKGRFTISRDDSKSSVYLQMNNLRAEDTGIYY
CASDGWFAYWGQGTLVTVSAGGGGSGGGGSGGGGSGGGGSDVVMTQTPLSLPVSL
GDQASISCRSSQSLVHSNGNTYLNWCLQRPGQSPKSLIYKVSNRFSGVPDRFSGSGS
GTDFTLKISRVEAEDLGVYFCSQSTHFPRTFGGGTKLEIKR**HHHHHH**

(in this application identified as SEQ ID NO:59)

Recombinant proteins were purified by using Ni Magnetic Beads affinity purification and analyzed by SDS-PAGE, SEC-HPLC and tested to be endotoxin free. SDS-PAGE analysis of integrity and correct molecular weight of the different scFvs are indicated below (Figure 16).

Then the peptide corresponding to the N-terminal domain 1 of CCR9 aminoacids 1 to 48 was chemically synthesized with a purity >75% and verified by HPLC (Genscript). The sequence of the peptide is:

MTPTDFTSPIPNMADDYGSESTSSMEDYVNFNFTDFYCEKNNVRQFAS (Pep1-48)

(in this application identified as SEQ ID NO:60)

The affinity between the CCR9-N-terminal domain 1 of CCR9 and the different scFvs was determined by Bio-Layer Interferometry (BLI), a technique that allows quantitative and qualitative measurement of biomolecular interactions in real-time. Briefly, scFv were immobilized onto NTA Biosensor (Sartorius) and quantification was performed in an Octet RED384 (Sartorius). scFv were dissolved in PBS (150 mM NaCl, 38 mM Na2HPO4, 12 mM NaH2PO4, pH 7.2) and a buffer containing 0.03%Tween-20 in PBS, pH 7.2 was used for baseline steps. The assay was performed at 30°C and Pep1-48 was applied as analyte for association and dissociation steps at

concentrations from 6.25 nM to 200 nM. All the data were processed using the OctetR BLI Discovery version 12.2.2.26.

Figure 17 shows the results obtained, Table 10 summarizes assay conditions and Table 11 the calculation of Kinetic global parameters after fitting the binding to a 1:1 molecular interaction.

Table 10: NTA Assay conditions after optimization

Ligand	Ligand Conc. (µg/ml)	Immobilization Level (nm)	Analyte	Analyte Conc. (nM)	Association time (s)	Dissociation time (s)
CCR9_HUM2	10	~3.0	Pep1-48	100, 50, 25, 12.5, 6.25	90	200
Comp-1		~3.5		200, 100, 50, 25, 12.5	70	60
Comp-2		~3.5		100, 50, 25, 12.5	90	70
Comp-3		~3.5		100, 50, 25, 12.5, 6.25	90	100

Table 11: Kinetic global fitting (1:1 binding) results

Ligand	Analyte	Rmax	Ka (1/Ms)	Kdis (1/s)	KD (M)	Full X^2	Full R^2
CCR9_HUM2	Pep1-48	0.3406	2.678E05	1.675E-03	6.255E-09	0.0882	0.9983
Comp-3		0.1718	3.637E05	1.202E-02	3.306E-08	0.0688	0.9868
Comp-2		0.1037	2.477E05	1.520E-02	6.137E-08	0.0142	0.9824
Comp-1		N/A	N/A	N/A	N/A	N/A	N/A

The affinity test data were listed in Table 11 ranking from high to low.

The results indicate that “CCR9_HUM2” scFv shows the highest affinity (6.255 nM), “Comp-3” and “Comp-2” scFvs show lower affinity (33.06 nM and 61.37 nM, respectively) and the “Comp-1” scFv does not bind to this region of CCR9 at all.

Example 9: Comparative Epitope mapping

With the same constructs as described in Example 8 epitope mapping was performed on linear peptides based on Pep1-48 to determine the minimal binding region of the four scFv to N-terminal domain 1 of CCR9. For this, the sequence Pep1-48 (MTPTDFTSPIPNMADDYGSESTSS MEDYVNFNFTDFYCEKNNVRQFAS) was divided into two large peptides:

1a. MTPTDFTSPIPNMADDYGSESTSS (in this application identified as SEQ ID NO:61)

1b. MEDYVNFNFTDFYCEKNNVRQFAS (in this application identified as SEQ ID NO:62)

The peptides were synthesized naked or with a N-terminal Biotin modification with a purity of >98%. All four scFvs were verified by ELISA that can be detected with anti-His antibodies. ELISA

was performed for scFv-peptide binding validation. Briefly, naked peptides were coated (50µg/mL) in 0.58M carbonate-bicarbonate buffer pH9.5, 100µL/well, 4ºC O/N. Then after blocking (3% BSA-PBS, 300µL/well 37ºC 1.5h) and washing (PBS + Tween-20 0.05% v/v (0.05%PBST), wells were incubated with each purified scFv antibody at concentration of 1µg/mL in PBS at 37ºC 1h. After washing (0.05% PBST), wells were incubated with secondary antibodies (goat anti-His IgG-HRP) in PBS, 37ºC, 30min. After washing, and incubation with TMB substrate, reactions were stopped with 2M HCl and reading was quantified at OD450.

The results are shown in Table 12 and indicate that "CCR9_HUM2" and "Comp-2" both bind to peptide region amino acids 25 to 48 and that "Comp-3" binds to region 1-24. "Comp-1" does not bind to any of both, in agreement with BLI results (Table 11).

Table 12. ELISA results – each naked peptide in duplicate for each scFv. Rep, replicate.

Antibody concentration	MTPTDFTSPIPNMADDYGSESTSS		MEDYVNFNFTDFYCEKNNVRQFAS	
10 µg/mL	repI	repII	repI	repII
CCR9_HUM2	0,05	0,08	2,22	2,24
Comp-1	0,08	0,10	0,09	0,06
Comp-2	0,09	0,08	1,97	2,01
Comp-3	1,38	1,35	0,11	0,09
Blank (no Ab)	0,08	0,09	0,06	0,05

As a conclusion, the measured OD show a strong binding against the long peptide antigen divided into two large peptides confirming that three of tested scFvs "CCR9_HUM2", "Comp-2" and "Comp-3" can bind to the peptide antigen.

Then a library of overlapping peptides were designed and constructed separately to produce 16 different peptides, covering full antigen sequence of total 48 aa. Peptide size was 10aa, with 7aa overlapping and ELISA screening of scFv against each peptide was performed in duplicate. Screening was performed using naked peptides in a standard plate or biotinylated peptides using avidin-plates. The list of overlapping peptides was as follows:

MTPTDFTSPI (SEQ ID NO:63)
TDFTSPIPNM (SEQ ID NO:64)
TSPIPNMADD (SEQ ID NO:65)
IPNMADDYGS (SEQ ID NO:66)
MADDYGSEST (SEQ ID NO:67)
DYGSESTSSM (SEQ ID NO:68)
SESTSSMEDY (SEQ ID NO:69)
TSSMEDYVNF (SEQ ID NO:70)
SSMEDYVNFN (SEQ ID NO:71)
SMEDYVNFNF (SEQ ID NO:72)

MEDYVNFNFT (SEQ ID NO:73)
YVNFNFTDFY (SEQ ID NO:74)
FNFTDFYCEK (SEQ ID NO:75)
TDFYCEKNNV (SEQ ID NO:76)
YCEKNNVRQF (SEQ ID NO:77)
KNNVRQFAS (SEQ ID NO:78)

ELISA results using naked peptides showed that only "CCR9_HUM2" scFv binds three different naked peptides (SSMEDYVNFN, SMEDYVNFNF and MEDYVNFNFT), results that were in agreement with results shown in example 7 obtained with the full antibody. These results also indicate that scFv humanization does not change binding specificity of original murine CCR9 antibody clone 115.

Table 13 ELISA results of epitope mapping with naked peptides in duplicate for each scFv

Antibody concentration 1µg/mL	MTPTDFTSPI		TDFTSPIPNM		TSPIPNMADD		IPNMADDYGS		MADDYGSEST	
	repI	repII	repI	repII	repI	repII	repI	repII	repI	repII
CCR9_HUM2	0,04	0,04	0,05	0,04	0,03	0,02	0,02	0,02	0,03	0,03
Comp-1	0,05	0,04	0,03	0,06	0,05	0,05	0,01	0,01	0,02	0,02
Comp-2	0,03	0,03	0,04	0,05	0,02	0,02	0,02	0,02	0,02	0,02
Comp-3	0,02	0,04	0,07	0,08	0,02	0,04	0,02	0,04	0,02	0,04
Blank (no Ab)	0,04	0,03	0,03	0,04	0,03	0,04	0,02	0,02	0,02	0,03

Antibody concentration 1µg/mL	DYGSESTSSM		SESTSSMEDY		TSSMEDYVNF		SSMEDYVNFN		SMEDYVNFNF	
	repI	repII	repI	repII	repI	repII	repI	repII	repI	repII
CCR9_HUM2	0,02	0,02	0,03	0,03	0,03	0,03	0,90	0,99	1,54	1,48
Comp-1	0,02	0,02	0,03	0,04	0,03	0,03	0,07	0,09	0,02	0,02
Comp-2	0,02	0,02	0,03	0,02	0,03	0,03	0,10	0,09	0,03	0,03
Comp-3	0,03	0,03	0,03	0,04	0,04	0,03	0,09	0,08	0,05	0,07
Blank (no Ab)	0,03	0,03	0,03	0,03	0,03	0,02	0,07	0,06	0,02	0,03

Antibody concentration 1µg/mL	MEDYVNFNFT		YVNFNFTDFY		FNFTDFYCEK		TDFYCEKNNV		YCEKNNVRQF	
	repI	repII	repI	repII	repI	repII	repI	repII	repI	repII
CCR9_HUM2	0,84	0,86	0,03	0,02	0,04	0,03	0,05	0,03	0,04	0,03
Comp-1	0,05	0,06	0,05	0,04	0,02	0,02	0,03	0,03	0,02	0,04
Comp-2	0,04	0,04	0,02	0,01	0,03	0,03	0,03	0,03	0,05	0,07
Comp-3	0,05	0,06	0,03	0,01	0,05	0,07	0,03	0,04	0,04	0,03

Blank (no Ab)	0,03	0,03	0,02	0,03	0,03	0,04	0,03	0,03	0,02	0,04

Antibody concentration 1µg/mL	KNNVRQFAS		NC*	
	repI	repII	repI	repII
CCR9_HUM2	0,03	0,03	0,05	0,05
Comp-1	0,02	0,02	0,03	0,03
Comp-2	0,04	0,04	0,03	0,02
Comp-3	0,05	0,04	0,02	0,02
Blank (no Ab)	0,03	0,05	0,02	0,02

*NC = negative control

The experiment was also performed with biotin-peptides as the inventors cannot exclude that some naked peptides do not coat well. ELISA with biotin-conjugated peptides (5µg/mL) was performed very similar to described above with minor modifications.

Results are shown in Table 14 and clearly indicate that "CCR9_HUM2" scFv was shown to bind, as before, the same set of naked peptides (SSMEDYVNFN, SMEDYVNFNF and MEDYVNFNFT), in addition a positive binding was also obtained with TSSMEDYVNF. Positive binding using Biotin-peptides was also obtained this time for "Comp-3" (TSPIPNMADD and IPNMADDYGS) and for "Comp-2" (YVNFNFTDFY and FNFTDFYCEK).

Table 14. ELISA results of epitope mapping with biotinylated peptides in duplicate for each scFv

Antibody concentration 10 µg/mL	MTPTDFTSPI		TDFTSPIPNM		TSPIPNMADD		IPNMADDYGS		MADDYGSEST	
	repI	repII	repI	repII	repI	repII	repI	repII	repI	repII
CCR9_HUM2	0,09	0,09	0,12	0,09	0,09	0,06	0,14	0,10	0,09	0,16
Comp-1	0,08	0,07	0,06	0,08	0,13	0,15	0,16	0,14	0,08	0,07
Comp-2	0,08	0,10	0,10	0,12	0,04	0,05	0,10	0,09	0,08	0,09
Comp-3	0,10	0,13	0,11	0,11	1,60	1,69	1,59	1,47	0,09	0,13
Blank (no Ab)	0,09	0,08	0,08	0,09	0,10	0,09	0,08	0,09	0,09	0,09

Antibody concentration 10 µg/mL	DYGSESTSSM		SESTSSMEDY		TSSMEDYVNF		SSMEDYVNFN		SMEDYVNFNF	
	repI	repII	repI	repII	repI	repII	repI	repII	repI	repII
CCR9_HUM2	0,09	0,07	0,08	0,09	2,05	1,92	2,45	2,30	2,48	2,38
Comp-1	0,10	0,11	0,13	0,10	0,10	0,08	0,10	0,13	0,15	0,10

Comp-2	0,09	0,09	0,06	0,05	0,08	0,06	0,12	0,10	0,09	0,05
Comp-3	0,12	0,10	0,15	0,12	0,10	0,12	0,15	0,12	0,10	0,12
Blank (no Ab)	0,09	0,08	0,10	0,09	0,09	0,09	0,09	0,08	0,10	0,09

Antibody concentration 10 µg/mL	MEDYVNFNFT		YVNFNFTDFY		FNFTDFYCEK		TDFYCEKNNV		YCEKNNVRQF	
	repI	repII	repI	repII	repI	repII	repI	repII	repI	repII
CCR9_HUM2	2,56	2,32	0,17	0,21	0,05	0,06	0,08	0,06	0,07	0,06
Comp-1	0,16	0,14	0,10	0,09	0,06	0,05	0,08	0,05	0,06	0,06
Comp-2	0,10	0,08	1,93	2,06	1,45	1,35	0,06	0,05	0,09	0,10
Comp-3	0,10	0,11	0,10	0,13	0,09	0,07	0,08	0,09	0,11	0,09
Blank (no Ab)	0,09	0,08	0,09	0,09	0,07	0,08	0,07	0,05	0,08	0,09

Antibody concentration 10 µg/mL	KNNVRQFAS		NC*	
	repI	repII	repI	repII
CCR9_HUM2	0,07	0,09	0,09	0,08
Comp-1	0,05	0,06	0,11	0,12
Comp-2	0,08	0,07	0,14	0,11
Comp-3	0,09	0,08	0,12	0,10
Blank (no Ab)	0,07	0,08	0,09	0,09

*NC = negative control

A full curve ELISA was performed for each positive scFv/biotin-peptide pair in order to confirm previous results. Tables 15 - 17 show ELISA results of the dose-dependent full curve for each positive peptide with scFv “CCR9_HUM”, “Comp-2” and “Comp-3”, respectively. Specific binding is confirmed in all cases, corroborating previous results shown in Table 14.

Table 15. ELISA results. Each biotin-peptide in duplicate for scFv “CCR9_HUM”

scFv concentration (µg/mL)	TSSMEDYVNF		SSMEDYVNFN		SMEDYVNFNF		MEDYVNFNFT		NC*	
	repI	**repII**	**repI**	**repII**	**repI**	**repII**	**repI**	**repII**	**repI**	**repII**
10	1,46	1,36	1,90	1,87	1,79	1,85	1,72	1,79	0,08	0,06
5	1,47	1,38	1,86	1,82	1,78	1,79	1,71	1,72	0,05	0,04
2,5	1,42	1,40	1,81	1,78	1,76	1,77	1,66	1,64	0,04	0,03
1,25	1,18	1,23	1,74	1,77	1,73	1,72	1,54	1,49	0,05	0,04
0,625	0,78	0,67	1,73	1,74	1,71	1,67	1,53	1,46	0,03	0,06

0,3125	0,46	0,45	1,69	1,73	1,63	1,65	1,47	1,35	0,03	0,03
0,15625	0,22	0,18	1,64	1,59	1,63	1,60	1,41	1,37	0,05	0,04
Blank (no Ab)	0,04	0,03	0,08	0,05	0,04	0,05	0,05	0,06	0,04	0,03

*NC = negative control

Table 16. ELISA results. Each biotin-peptide in duplicate for scFv “Comp-2”

scFv concentration (µg/mL)	YVNFNFTDFY		FNFTDFYCEK		NC*	
	repI	**repII**	**repI**	**repII**	**repI**	**repII**
10	1,42	1,39	1,52	1,60	0,07	0,08
5	1,31	1,24	1,45	1,48	0,05	0,05
2,5	1,01	0,89	1,40	1,42	0,06	0,04
1,25	0,70	0,82	1,39	1,40	0,04	0,05
0,625	0,44	0,40	1,35	1,37	0,04	0,06
0,3125	0,28	0,31	1,23	1,27	0,04	0,03
0,15625	0,18	0,20	0,99	1,02	0,06	0,03
Blank (no Ab)	0,06	0,04	0,06	0,05	0,05	0,04

*NC = negative control

Table 17. ELISA results. Each biotin-peptide in duplicate for scFv “Comp-3”

scFv concentration (µg/mL)	TSPIPNMADD		IPNMADDYGS		NC*	
	repI	**repII**	**repI**	**repII**	**repI**	**repII**
10	1,84	1,89	1,76	1,75	0,21	0,18
5	1,74	1,78	1,78	1,72	0,14	0,09
2,5	1,46	1,45	1,51	1,49	0,07	0,08
1,25	1,21	1,26	1,22	1,23	0,06	0,05
0,625	0,77	0,79	0,95	0,94	0,06	0,04
0,3125	0,46	0,45	0,62	0,66	0,05	0,06
0,15625	0,25	0,19	0,38	0,41	0,05	0,04
Blank (no Ab)	0,05	0,06	0,05	0,04	0,09	0,08

*NC = negative control

As a summary, it can be concluded that each of the 3 purified scFv antibodies “CCR9_HUM”, “Comp-2” and “Comp-3” could bind to different peptides in ELISA with strong signal, the positive peptides for each scFv were the following:

- “CCR9_HUM”: TSSMEDYVNF, SSMEDYVNFN, SMEDYVNFNF and MEDYVNFNFT

- "Comp-2": YVNFNFTDFY and FNFTDFYCEK
- "Comp-3": TSPIPNMADD and IPNMADDYGS

The "Comp-1" purified scFv did not show any binding to the control peptides MEDYVNFNFTDFYCEKNNVRQFAS and MTPTDFTSPIPNMADDYGSESTSS, nor to any smaller peptide tested.

To conclude, the epitope sequence recognized by each of the three scFvs "CCR9_HUM", "Comp-2" and "Comp-3" on the full antigen peptide (MTPTDFTSPIPNMADDYGSESTSSM EDYVNFNFTDFYCEKNNVRQFAS) and identified by this study is highlighted in bold in the peptide sequence below:

- "CCR9_HUM":
MTPTDFTSPIPNMADDYGSESTSS**MEDYVNFN**FTDFYCEKNNVRQFAS
- "Comp-2":
MTPTDFTSPIPNMADDYGSESTSSMEDYVN**FNFTDFY**CEKNNVRQFAS
- "Comp-3":
MTPTDFTSP**IPNMADDY**GSESTSSMEDYVNFNFTDFYCEKNNVRQFAS

Example 10: CCR9 and CD1a expression in T-ALL samples

170 primary T-ALL samples sourced from different hospitals/biobanks across Europe and the US were analyzed. The expression of CCR9 and CD1a was determined by flow cytometry as described for Figure 1 (CCR9) or Figure 18A (CCR9 and CD1a) using the fluorochrome-conjugated MoAbs anti-CD45, CD7, CD4, CD8, CCR9 and CD1a. 70% of the T-ALL samples are CCR9 positive and 59% are CD1a positive (Figure 18G) (cut-off >20% as defined in diagnostic labs). Of note, dual staining of CCR9 and CD1a reveals the presence of phenotypically different T-ALL clones including double negative T-ALLs, double positive T-ALLs and single (CCR9 or CD1a) T-ALLs analyzed in human samples (Figure 18B to G). It is also important to note the variable proportions of CD1a- and CCR9-expressing blasts within each patient (Figure 18), underpinning the clinical advantage of a dual targeting of both CD1a and CCR9 that will cover 85% of patients that represents a significantly higher proportion of T-ALL blasts (Figure 18G). Moreover, dual CD1a and CCR9 therapy will also contribute to reduce immunescape of those patients co-expressing both antigens (44%) (Figure 18G).

Example 11: Efficacy and safety of a dual CAR-T targeting strategy against CCR9 and CD1a.

In order to test the efficacy and safety of a dual CAR-T targeting strategy against CCR9 and CD1a double positive cells, knock-out cells for CD1a (CD1aKO), knock-out for CCR9 (CCR9KO) and double knock-out cells for both CD1a and CCR9 (dKO) were generated by using CRISPR Cas-9

technology (Figure 19A). Elimination of these genes does not affect proliferation of cells compared to the MOLT4 wt counterpart (Figure 19B). Efficacy of single or dual-cotransduced CAR-Ts against CD1a and CCR9 is similar as demonstrated after incubation of target wt cells (T) with effector CAR-Ts cells (E) at different E:T ratios, compared to untransduced cells (UT) (Figure 19C). Specificity and efficacy of single CAR-Ts compared with the dual therapy as a result of co-infection of single CARs is also demonstrated by crossed experiments as shown in Figure 19C. Single CAR-Ts against CCR9 eliminate MOLT4 CD1a KO but not CCR9 KO cells, and the opposite occurs with single CAR-Ts against CD1a. The dual combination of both CARs, after co-transduction, eliminates wt and both CCR9 or CD1a KO cells with minimal potential loss compared to the single treatments. Moreover, the dual therapy does not show a significant increase of toxicity by using single Knock-out cells for CCR9 or CD1a or double-knock out cells.

Example 12: Efficacy of a dual CAR-T targeting strategy against CCR9 and CD1a vs. single therapy.

In order to test the efficacy of a dual CAR-T targeting strategy against CCR9 and CD1a versus the single therapy, a population of MOLT4 cells co-expressing both antigens (wt cells) or expressing only CCR9 (CD1a KO cells) or only CD1a (CCR9 KO cells) were artificially mixed. This complex population of tumor cells faithfully represents the % of blast cells found in a patient, as shown in Figure 20A (comparative expression analysis of CCR9 and CD1a in a T-ALL patient and after mixing MOLT4 wt, CCR9+/CD1a- and CCR9-/CD1a+ cells). Dual therapy done as a co-transduction of single CARs is superior in specific cytotoxicity compared with the use of single CARs, as shown in Figure 20B at different E:T ratios or a fixed E:T ratio and a different time-points. Dual therapy is able to eliminate 100% of cells at 48 h compared to single CAR-T treatments, untransduced (UT) or non-effector (NE) cells.

Furthermore, the amount of released proinflammatory cytokines after co-culture of wt, CCR9+/CD1a- and CCR9-/CD1a MOLT4 cell lines with HUM2 CCR9 CAR T-cells, CD1a CAR T-cells or cells co-transduce with both, was determined at 24 h. The production of the proinflammatory cytokines interleukin (IL)-2, tumor necrosis factor α (TNFα), and interferon γ (IFNγ) was measured by using an enzyme-linked immunosorbent assay (ELISA) in supernatants harvested after 24 hours of co-culture. As shown in Figure 20C, IL-2, TNFα, and IFNγ cytokines were produced at similarly high levels with single CD1a or CCR9 CARs, compared with co-infected cells (CO, co CCR9_CD1a CAR) in MOLT4 cells with not a significant increase of dual vs single therapy. This fact of killing a double population of cells positive for both CCR9 and CD1a without increasing the release of proinflammatory cytokines is important to do not increase the toxicity of dual therapy that may cause CRS, as an undesirable side-effect.

Example 13: In vivo activity of single CCR9 and CD1a and dual CAR-T therapy.

The activity of single CCR9 and CD1a and dual CAR-Ts in vivo using Luc/GFP-expressing MOLT4 T-ALL cells was evaluated as indicated in Example 6 and Figure 14 (Figure 21). All NSG mice were injected with a mix of MOLT4 wt/CD1aKO/CCR9KO and randomized 3 days later by groups having similar initial luciferase activity and then injected with $3x10^6$ CAR T cells/mouse. Effector T cells UT, untransduced, single CCR9 and CD1a CAR-Ts and Mix-CD1a/CCR9 CAR-Ts (dual therapy, 50% mix of CD1a + 50% CCR9 CAR T cells) were administered. Surprisingly, as occurs with UT cells, those mice given single CCR9-CARTs or CD1a-CARTs have massive tumor burden and are not statistically significant between them, suggesting that single CARs are unable to control the disease progression when a mix of blast cells expressing both antigen targets are present. However, the group of mice receiving de dual therapy (Mix-CD1a/CCR9 CAR-Ts) had minimal disease progression until day 26 (Figure 21). Quantification of BLI by IVIS showed no differences among the UT and CAR-CCR9 or CAR-CD1a, whose BLI levels were significantly higher than those of mice receiving the dual therapy (Figure 21A and C). As expected, the results also show a significant reduction of tumor burden in BM in mice receiving the dual CAR-T therapy related to the single or UT (Figure 21B). Overall, the inventors could show the efficacy of the dual CCR9-CD1a-CAR T-cell therapy, showing a potent, synergistic and specific antileukemic activity against T-ALL expressing both CD1a and CCR9 in vivo. Interestingly, the synergistic effect in vivo is surprising compared with the additive effect observed in vitro in Figure 20B and may not be anticipated by the skilled person. In addition to this, very similar results were obtained using as a dual therapy the co-transduction of both CCR9 and CD1a CARs (Co CCR9_CD1a) as shown in Figure 21D (Bioluminescence quantification) and E (tumor burden in BM). Overall, these results demonstrate that the dual therapy both as mix of single CAR-Ts population or as single population co-transduced with both CARs is able to show a synergistic and specific antileukemic activity in vivo.

Example 14: Single vs. dual CAR-T therapy in patient-derived xenografts (PDX).

The activity of single CCR9 or CD1a CAR-T therapy was tested in vivo and compared with dual therapy after the co-transduction of single CARs at a MOI of 20. We used a pre-clinical model of patient-derived xenografts (PDX), initially created by implanting T-ALL blast cells of a human tumor into a mouse as previously described (Ref: https://haematologica.org/article/view/9640. In this case, PDX2 T-ALL keep high levels of CCR9 and CD1a, similarly to those found in patients co-expressing both targets (see Figure 22A). After tumor implantation, mice received a lower dose of CAR-Ts in comparison to the experiment shown in Figure 15 ($2x10^6$, instead of $4x10^6$ of experiment shown in Figure 15) in order to demonstrate long-term efficacy of treatments. In contrast to the mice receiving UT cells, which showed massive tumor burden by blast quantitation in PB, those mice given single CCR9 or CD1a CARTs or dual CCR9-CD1a CAR-T (co-transduction of single CARs) had a much lower disease progression until week 8 (Figure 22B).

As expected, the effect of single CARs is considerable higher than the experiment showed in Figure 21 because tumoral cells co-expressed both antigens and we may expect similar efficacy. Moreover, after long-term observation at week 8, the dual combination of CARs showed a significant reduction of tumor burden in PB, BM and spleen, compared with mice receiving single CCR9 or CD1a CAR-Ts related to UT, despite the co-expression of both antigens (Figure 22C to E). The skilled person may anticipate that in this model, the effectiveness of single vs dual therapy should be similar. However, these results clearly show the advantage of dual therapy vs monotherapy in particular when long-term progression is monitored. At the end of the experiment, tumor burden reduction in PB and SP is quite evident in those mice receiving the dual therapy (Figure 22, C and D). Overall, the inventors could show the efficacy of the dual CCR9/CD1a-CAR-T therapy product, as a mix of single CAR-Ts (Figure 21) or after co-transduction of single CARs (Figures 21 and 22), showing a potent and specific antileukemic activity of dual therapy in vivo that is stronger compared to monotherapy.

Example 15: Bicistronic CARs co-expressing CCR9 and CD1a.

The activity of single CAR-Ts against CD1a and CCR9 (monotherapy) was compared with a dual-therapy approach by using the co-transduction of single CARs (Co) or the use of different bicistronic CAR versions co-expressing CCR9 and CD1a (Figure 23A). Four bicistronic versions that differ in having different signal peptides, order of scFvs, and CAR DNA sequences were tested and compared between them. As expected, expression of bicistronic constructs is lower than obtained with single CARs or after co-transduction of single CARs, as shown by lower GFP levels around 20% (Figure 23B), as previously described. We introduced F2A sequences in between the two CARs that cause ribosomal “skipping” during translation, which results in a missing peptide bond and effectively separation of the two CAR fusion proteins. To determine the functionality of bicistronic in comparison with co-transduction constructs, we performed cytotoxicity assays with MOLT4 cells expressing both CCR9 and CD1a or with the different knock-out versions, as a control of specificity. A representative experiment is shown in Figure 23 (panels C to F), where is clearly observed that the different bicistronic CAR-Ts are as potent as the ones obtained by co-transduction, resulting in similar killing efficiency. The cytotoxic activity of bicistronic CAR-Ts against single positive cells for CD1a or CCR9 is as effective as the monotherapy and the co-transduction approach, as shown by using the corresponding CCR9 or CD1a KO cells, respectively. This necessary implies that bicistronic CARs are co-expressed with similar efficiencies to target single knock-out cells, but not double knock-out cells, and that F2A cleavage is efficiently produced between the two CARs.

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CLAIMS

1. A CCR9 targeting moiety that specifically binds to the amino acid sequence of SEQ ID NO:1 (SMEDYVNFN).

2. A CCR9 targeting moiety comprising an antibody, F(ab')2, Fab, scFab or scFv, said antibody, F(ab')2, Fab, scFab or scFv comprising
   a. a light chain domain (VL) comprising at least one complementarity determining region (CDR) selected from
      (i) a CDR comprising the amino acid sequence shown in SEQ ID NO:2 [LCDR-1], or a variant thereof;
      (ii) a CDR comprising the amino acid sequence shown in SEQ ID NO:3 [LCDR-2], or a variant thereof; and
      (iii) a CDR comprising the amino acid sequence shown in SEQ ID NO:4 [LCDR-3], or a variant thereof; and
   b. a heavy chain domain (VH) comprising at least one complementarity determining region (CDR) selected from
      (i) a CDR comprising the amino acid sequence shown in SEQ ID NO:5 [HCDR-1], or a variant thereof;
      (ii) a CDR comprising the amino acid sequence shown in SEQ ID NO:6 [HCDR-2], or a variant thereof; and
      (iii) a CDR comprising the amino acid sequence shown in SEQ ID NO:7 [HCDR-3], or a variant thereof.

3. The CCR9 targeting moiety of claims 1 or 2, comprising
   a. a VL domain consisting of SEQ ID NO:8 and a VH domain consisting of SEQ ID NO:9; or
   b. a VL domain consisting of SEQ ID NO:10 and a VH domain consisting of SEQ ID NO:11;
      or
   c. a VL domain consisting of SEQ ID NO:12 and a VH domain consisting of SEQ ID NO:13.

4. The CCR9 targeting moiety according to any one of the preceding claims, wherein the CCR9 targeting moiety is a scFv comprising
   a. a VL domain consisting of SEQ ID NO:8 and a VH domain consisting of SEQ ID NO:9; or

b. a VL domain consisting of SEQ ID NO:10 and a VH domain consisting of SEQ ID NO:11;
or
c. a VL domain consisting of SEQ ID NO:12 and a VH domain consisting of SEQ ID NO:13.

5. The CCR9 targeting moiety according to any one of the preceding claims, wherein the CCR9 targeting moiety comprises the amino acid sequence of SEQ ID NO:16, SEQ ID NO:17 or SEQ ID NO:18.

6. A chimeric antigen receptor (CAR) comprising:
a. an extracellular domain comprising a CCR9 targeting moiety according to any one of claims 1 to 5;
b. a transmembrane domain; and
c. an intracellular signaling domain.

7. The CAR according to claim 6, wherein
a. 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, preferably wherein the transmembrane domain comprises the transmembrane domain of CD8; and/or
b. the intracellular signaling domain comprises the intracellular domain of CD3ζ, FcRγ, CD3γ, CD3δ, CD3ε, CD5, CD22, CD79a, CD79b or CD66b, preferably of CD3ζ; and/or
c. 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, or CD276, preferably of CD137.

8. The CAR according to any one of claims 6 to 7 consisting of the amino acid sequence according to SEQ ID NO:22, SEQ ID NO:23 or SEQ ID NO:24.

9. The CCR9 targeting moiety or CAR of any one of the preceding claims further comprising a second targeting-moiety, wherein said second targeting moiety is selected from a CD3, CD4, CD5, CD7, CD37, CD30, CD33, CD99, CCR7, CDR3, TRBC1/2, or CD1a targeting moiety, preferably wherein said second targeting moiety is a CD1a targeting moiety.

10. The CCR9 targeting moiety or CAR of any one of the preceding claims, wherein the CD1a targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:31 or SEQ ID NO:33 and a VH domain consisting of SEQ ID NO:32 or SEQ ID NO:34.

11. A dual CAR comprising a first and a second CAR, wherein the first CAR is a CAR according to any one of claims 6 to 8 and the second CAR is a CAR comprising a CD1a targeting moiety, preferably wherein the CD1a targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:31 or SEQ ID NO:33 and a VH domain consisting of SEQ ID NO:32 or SEQ ID NO:34.

12. A nucleic acid encoding the CAR according to any one of claims 6-11.

13. A cell comprising the nucleic acid according to claim 12 and/or the CAR according to any one of claims 6-11, preferably wherein the cell is a T-cell.

14. A pharmaceutical composition comprising a plurality of cells according to claim 13 and a pharmaceutically acceptable carrier or diluent.

15. The cell according to claim 13 or the pharmaceutical composition according to claim 14 for use in a method of treating a CCR9-positive cancer, wherein the method comprises administering the cell or composition to a patient in need thereof, preferably wherein the CCR9-positive cancer is T-cell acute lymphoblastic leukemia or T-cell lymphoma, more preferably relapsed/refractory T-cell acute lymphoblastic leukemia.

16. The cell according to claim 13 or the pharmaceutical composition according to claim 14 for use in a method of treating a CD1a-positive cancer, preferably a CD1a and CCR9-positive cancer, wherein the method comprises administering the cell or composition to a patient in need thereof, preferably wherein the CD1a-positive cancer is T-cell acute lymphoblastic leukemia, preferably cortical T-cell acute lymphoblastic leukemia, more preferably relapsed/refractory cortical T-cell acute lymphoblastic leukemia.

17. The cell according to claim 13 or the pharmaceutical composition according to claim 14 for use in the method of treating a CD1a-positive and/or a CCR9-positive cancer, preferably a CD1a and CCR9-positive cancer, the method further comprising administering simultaneously or subsequently a CD1a CAR comprising a CD1a targeting moiety, a transmembrane domain and an intracellular signaling domain.

18. The cell according to claim 13 or the pharmaceutical composition according to claim 14 for use in the method of claim 17, wherein the CD1a targeting moiety is a scFv comprising a VL domain consisting of SEQ ID NO:31 or SEQ ID NO:33 and a VH domain consisting of SEQ ID NO:32 or SEQ ID NO:34.