Selective homing of CAR-CIK cells to the bone marrow niche enhances control of the Acute Myeloid Leukemia burden

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Marta Biondi (Tettamanti Center, Fondazione IRCCS San Gerardo dei Tintori, Italy) Sarah Tettamanti (Tettamanti Center, Fondazione IRCCS San Gerardo dei Tintori, Italy) Stefania Galimberti (University of Milano - Bicocca, Monza, Italy) Beatrice Cerina (Tettamanti Center, Fondazione IRCCS San Gerardo dei Tintori, Italy) Chiara Tomasoni (Tettamanti Center, Fondazione IRCCS San Gerardo dei Tintori, Italy) Rocco Piazza (Hematology, Fondazione IRCCS San Gerardo dei Tintori, Italy) Samantha Donsante (Sapienza University, Italy) Simone Bido (Division of Neuroscience, San Raffaele Scientific Institute, Italy) Vincenzo Perriello (Insitute of Hematology, University of Perugia, Italy) Vania Broccoli (National Research Council (CNR), Institute of Neuroscience, Milan, Italy) Andrea Doni (Istituto Clinico Humanitas, Italy) Francesco Dazzi (King's College London, United Kingdom) Alberto Mantovani (Department of Biomedical Sciences, Humanitas University, Pieve Emanuele, Milan, Italy) Gianpietro Dotti (Lineberger Comprehensive Cancer Center, University of North Carolina (UNC), United States) Andrea Biondi (Centro Ricerca Tettamanti Clinica pediatrica Universita degli Studi di Milano-Bicocca, Italy) Alice Pievani (Tettamanti Center, Fondazione IRCCS San Gerardo dei Tintori, Italy) Marta Serafini (Tettamanti Center, Fondazione IRCCS San Gerardo dei Tintori, Italy)

Abstract:
Acute myeloid leukemia (AML) is a hematological malignancy derived from neoplastic myeloid progenitor cells characterized by abnormal clonal proliferation and differentiation. Although novel therapeutic strategies have recently been introduced, the prognosis of AML remains unsatisfactory. So far, the efficacy of chimeric antigen receptor (CAR)-T therapy in AML has been hampered by several factors including the poor accumulation of the blood-injected cells in the leukemia bone marrow (BM) niche, where chemotherapy-resistant leukemic stem cells reside. Thus, we hypothesized that overexpression of CXCR4, whose ligand CXCL12 is highly expressed by BM stromal cells within the niche, could improve T cell homing to the BM and consequently enhance their intimate contact with BM-resident AML cells facilitating disease eradication. Specifically, we engineered conventional CD33.CAR-cytokine induced killer cells (CIKs) with the wild-type CXCR4 and the variant CXCR4^{R334X}, responsible for leukocyte sequestration in the BM of WHIM syndrome patients.

Overexpression of both CXCR4^{wt} and CXCR4^{mut} in CD33.CAR-CIKs resulted in significant improvement of chemotaxis toward recombinant CXCL12 or BM stromal cell conditioned medium with no observed impairment of cytotoxic potential *in vitro*. Moreover, CXCR4-overexpressing CD33.CAR-CIKs showed enhanced *in vivo* BM homing, associated with a prolonged retention for the CXCR4^{R334X} variant.

However, only CD33.CAR-CIKs co-expressing CXCR4^{wt} but not CXCR4^{mut} exerted a more sustained *in vivo* antileukemic activity and extended animal survival, suggesting a non-canonical role for CXCR4 in modulating CAR-CIK functions independent of BM homing. Taken together, these data suggest that arming CAR-CIKs with CXCR4 may represent a promising strategy for increasing their therapeutic potential for AML.

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Marta Biondi,[1,2] Sarah Tettamanti,[1] Stefania Galimberti,[3] Beatrice Cerina,[1] Chiara Tomasoni,[1] Rocco Piazza,[2,4] Samantha Donsante,[5] Simone Bido,[6] Vincenzo Maria Perriello,[7] Vania Broccoli,[6,8] Andrea Doni,[9] Francesco Dazzi,[10#] Alberto Mantovani,[9,11,12] Gianpietro Dotti,[13] Andrea Biondi,[2,14§] Alice Pievani,[1*] and Marta Serafini[1*§]

[*]Equal contribution;

[§]Corresponding Authors

[#]Currently member of Astra Zeneca

[1]Tettamanti Center, Fondazione IRCCS San Gerardo dei Tintori, Monza, Italy.

[2]Department of Medicine and Surgery, University of Milano-Bicocca, Monza, Italy.

[3]Bicocca Bioinformatics Biostatistics and Bioimaging B4 Center, School of Medicine and Surgery, University of Milano-Bicocca, Monza, Italy.

[4]Hematology, Fondazione IRCCS San Gerardo dei Tintori, Monza, Italy.

[5]Department of Molecular Medicine, Sapienza University, Rome, Italy.

[6]Division of Neuroscience, San Raffaele Scientific Institute, Milan, Italy.

[7]Insitute of Hematology, University and Hospital of Perugia, Perugia, Italy.

[8]National Research Council (CNR), Institute of Neuroscience, Milan, Italy.

[9]IRCCS Humanitas Research Hospital, Rozzano, Milan, Italy.

[10]School of Cardiovascular Sciences, King's College London, London, UK.

[11]Department of Biomedical Sciences, Humanitas University, Pieve Emanuele, Milan, Italy.

[12]William Harvey Research Institute, Queen Mary University, London, UK.

[13]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC, USA.

[14]Pediatrics, Fondazione IRCCS San Gerardo dei Tintori, Monza, Italy.

Correspondence:

Marta Serafini, Tettamanti Center, Fondazione IRCCS San Gerardo dei Tintori, Via Pergolesi 33, 20900 Monza, Italy. Tel: +390392333661; E-mail: serafinim72@gmail.com

Andrea Biondi, Pediatrics, Fondazione IRCCS San Gerardo dei Tintori, Via Pergolesi 33, 20900 Monza, Italy. Tel: +390392336816; E-mail: abiondi.unimib@gmail.com

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Abstract

Acute myeloid leukemia (AML) is a hematological malignancy derived from neoplastic myeloid progenitor cells characterized by abnormal clonal proliferation and differentiation. Although novel therapeutic strategies have recently been introduced, the prognosis of AML is still unsatisfactory. So far, the efficacy of chimeric antigen receptor (CAR)-T therapy in AML has been hampered by several factors including the poor accumulation of the blood-injected cells in the leukemia bone marrow (BM) niche, where chemotherapy-resistant leukemic stem cells reside. Thus, we hypothesized that overexpression of CXCR4, whose ligand CXCL12 is highly expressed by BM stromal cells within the niche, could improve T cell homing to the BM and consequently enhance their intimate contact with BM-resident AML cells facilitating disease eradication. Specifically, we engineered conventional CD33.CAR-cytokine induced killer cells (CIKs) with the wild-type CXCR4 and the variant CXCR4^{R334X}, responsible for leukocyte sequestration in the BM of WHIM syndrome patients. Overexpression of both CXCR4^{wt} and CXCR4^{mut} in CD33.CAR-CIKs resulted in significant improvement of chemotaxis toward recombinant CXCL12 or BM stromal cell conditioned medium with no observed impairment of cytotoxic potential *in vitro*. Moreover, CXCR4-overexpressing CD33.CAR-CIKs showed enhanced *in vivo* BM homing, associated with a prolonged retention for the CXCR4^{R334X} variant. However, only CD33.CAR-CIKs co-expressing CXCR4^{wt} but not CXCR4^{mut} exerted a more sustained *in vivo* antileukemic activity and extended animal survival, suggesting a non-canonical role for CXCR4 in modulating CAR-CIK functions independent of BM homing. Taken together, these data suggest that arming CAR-CIKs with CXCR4 may represent a promising strategy for increasing their therapeutic potential for AML.

Key Points

- CAR-T therapy in AML is hampered by the limited accumulation of the therapeutic cells in the bone marrow niche, where leukemic stem cells are nested.
- Engineering CAR-CIK cells with CXCR4 promotes their homing in the BM and improves their antileukemic efficacy in AML.

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Introduction

The therapeutic potential of chimeric antigen receptor (CAR)-T cells for patients suffering from acute myeloid leukemia (AML) is still far from being realized due to multiple hurdles. The main reasons include the lack of leukemic stem cells (LSCs)/AML-specific target antigens, the disease heterogeneity and the leukemia-induced remodeling of the bone marrow (BM) microenvironment. So far, efforts to potentiate CAR-T cell therapy in AML have mostly focused on identifying optimal antigens and CAR structures to promote more specific targeting, and on counteracting the leukemia-induced immune suppression by combination therapy using immune checkpoint blocking antibodies[1].

However, fundamental to the optimization of CAR-T cell clinical outcome is also the requirement for blood-injected cells to efficiently reach and persist at the tumor site. Such a challenge, well documented in solid tumors, has been extended in the last few years to hematological malignancies, in which the BM is the primary location for acute leukemia initiation, maintenance, progression and chemoresistance. This is mainly relevant in the AML context, as innovative curative treatments must have the potential to eliminate LSCs residing in the BM niche and there persisting after conventional treatments. Accumulating evidence suggests that CAR-T homing ability to the BM microenvironment is a prerequisite to mediate consistent therapeutic activity in AML.

For instance, a small date set from a phase I clinical study with anti-Lewis (Le)-Y.CAR-T cells seems to suggest that the accumulation of CAR-T cells in the BM correlates with better clinical outcome supporting the hypothesis that improving T-cell trafficking to the BM may increase their therapeutic effects [2].

Chemokines and their receptors play a crucial role in the migration and homing of lymphocytes. A key requirement for efficient migration of cytotoxic T cells is that they express chemokine receptors matching the chemokines produced by tumor or tumor-associated cells. However, culture conditions for expansion of human lymphocytes dampen the expression of chemokine receptors and may negatively influence their homing ability to BM[3,4]. Therefore, CAR-T cells may be modified to express chemokine receptors to potentiate their migratory and infiltrative capacity. Notably, this approach has shown promising results in numerous preclinical tumor models[5–9]. Furthermore, some clinical trials using chemokine receptors CCR4 (NCT03602157) and CXCR4 (NCT04727008) to improve CAR-T cells trafficking to the tumor site are ongoing.

Trafficking of lymphocytes into the BM relies on the expression of CXCR4, a chemokine receptor that binds to the chemokine CXCL12, which is highly expressed by stromal and endothelial cells in

the BM. Specifically, in AML, blasts overexpressing CXCR4 interact with CXCL12, improving their homing to the protective BM microenvironment and obtaining pro-survival signals[10]. The CXCL12/CXCR4 interaction keeps leukemic blasts in close contact with supporting stromal cells and extracellular matrix constitutively releasing growth-promoting and anti-apoptotic signals that contribute to AML immune escape. Furthermore, blocking the CXCL12/CXCR4 interaction with the inhibitor plerixafor disrupts AML blast interaction with the BM microenvironment, causing AML cell mobilization into the circulation[11,12].

We tested the hypothesis that previously optimized CD33.CAR-cytokine induced killer cells (CIKs) that are *ex vivo* expanded T lymphocytes with a mixed T-NK phenotype endowed with MHC-unrestricted antitumor activity[13], overexpressing CXCR4 would acquire superior homing ability to the BM and therefore enhanced elimination of BM-resident AML cells. Furthermore, we also tested whether increasing the CD33.CAR-CIKs retention in the BM by co-expressing a gain-of-function (GOF) variant of CXCR4 (R334X mutation) described in the WHIM syndrome would further increase the antitumor effects of CD33.CAR-CIKs[14]. R334X mutation causes a C-terminus truncation impairing CXCR4 internalization upon CXCL12 binding and therefore results in increased receptor signaling compared to the wild-type counterpart[15].

Materials and methods

Generation of CAR constructs

Three bicistronic SB-transposon vectors were generated by modifying the pT4 Sleeping Beauty (SB) CD33-CH3-CD28-OX40-ζ CAR construct (hereafter named CD33.CAR) to encode both human CXCR4 and CD33.CAR: (1) CXCR4(I)CD33.CAR construct, in which both CD33.CAR and CXCR4 expression is driven by an internal ribosome entry site (IRES), and (2) CD33.CAR(2A)CXCR4[wt] and (3) CD33.CAR(2A)CXCR4[mut], in which the CXCR4 coding sequence, in its wild-type or R334X mutated form, is cloned downstream to the CD33.CAR sequence linked with self-cleaving peptide 2A.

CIKs differentiation and modification

CAR-CIKs were generated, as previously described[13,16], by sequentially adding IFN-γ (1,000U/ml, Dompè Biotec) at day 0 and IL-2 (300U/ml, Chiron BV) and anti-CD3 monoclonal antibody OKT3 (50ng/ml; Janssen-Cilag) at day 1 to peripheral blood mononuclear cells (PBMCs). At the end of the differentiation protocol, mature CIKs typically are almost entirely CD3^{+} T lymphocytes expressing CD8 and CD56 and display an effector memory phenotype. For the non-viral CIKs

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modification, the SB-pT4 vector and SB100X transposase were provided by Z. Izsvak (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany).

Transwell migration assay

CAR[+]-CIKs migration ability was assessed using 5μm pore 24-transwell plates (Corning). Advanced RPMI1640 medium (600ul) with 2% FBS containing 200 ng/ml human recombinant (rh)CXCL12 (Peprotech) was added to the lower chamber, and the upper chamber was filled with 100ul medium containing control CD33.CAR[+]- or CXCR4 overexpressing-CD33.CAR[+]-CIKs ($0.5x10^6$ cells). After 2 hours of incubation, cells of the lower chamber were collected, and, after adding 10μl of CountBright Absolute Counting Beads (Thermo Fisher Scientific) per tube, the number of migrated cells was quantified by FACS.

The same procedure was also followed using supernatants of mesenchymal stromal cells from healthy donors or AML patients (HD- or AML-MSCs) (see supplemental Methods) as stimuli. CXCR4 blocking was achieved by pretreating CAR[+]-CIKs with 100μM plerixafor (Mozobil®, Sanofi Oncology) for 30 minutes at 37°C prior to plating the migration assay. To test the capacity of CXCR4-overexpressing CD33.CAR[+]-CIKs to migrate towards murine CXCL12, 200ng/ml mouse recombinant CXCL12 (Peprotech Inc) or mouse BM supernatant were used as stimuli.

Conjugated stability assay

KG-1 cells or KG-1 cells CRISPR-Cas9 CD33 knockout (see supplemental Methods), were labeled with Cell Tracer Violet (Thermo Scientific) and incubated for 30 min at 37°C with CD33.CAR[+]-, CD33.CAR[+]-CXCR4[wt]- or CD33.CAR[+]-CXCR4[mut]-CIKs at a ratio of 1:1 to form conjugates. After this incubation, cells were allowed to migrate through 5μM pores to 200 ng/ml rhCXCL12 for 2 hours before FACS analysis.

In vitro CAR-related functional assays

CAR-CIKs *in vitro* effector functions were tested against CD33^{+} KG-1 cell line or primary AML cells performing cytotoxicity, cytokines production and proliferation assays, both in the presence or not of rhCXCL12 (see supplemental Methods). Primary AML cells were obtained from BM and PBMCs collected from AML patients, after written informed consent was obtained from patients or parents/legal guardians in agreement with the Declaration of Helsinki.

In vivo homing assay

The study was approved by the Italian Ministry of Health. Procedures involving animals were conformed to protocols approved by the Milano-Bicocca University in compliance with national and international law and policies. Six- to eight-week-old male NSG (NOD.Cg-Prkdc[scid]Il2rg[tm1Wjl]/SzJ) mice (Charles River Laboratories) were sublethally irradiated (200cGγ) 48 hours prior treatment with CAR-CIKs. Then, 10^7 sorted CD33.CAR[+]-CXCR4[wt]-, CD33.CAR[+]-CXCR4[mut]- and control CD33.CAR[+]-CIKs were intravenously infused. Mice were euthanized from 7 to 14 days after infusion and peripheral blood (PB), lungs, spleen, and BM were analyzed by FACS to assess CAR-CIKs engraftment.

In vivo antileukemic activity

NSG mice were intravenously injected with CD33^{+} KG-1 cells ($5x10^6$ cells/mouse). Two weeks later, mice were left untreated or infused with 10^7 sorted CD33.CAR^{+}-CXCR4[wt]-, CD33.CAR^{+}-CXCR4[mut]- and control CD33.CAR[+]-CIKs.

For endpoint experiments, mice were euthanized 25 days after CAR-CIK infusion and PB, spleen, and BM were analyzed by FACS and by immunohistochemistry to establish AML burden (see supplemental Methods).

For survival experiments, AML burden was monitored through BM aspiration and analyzed by FACS at the indicated time points.

Results

CXCR4 expression drops during CAR-CIKs expansion and can be stably restored using bicistronic Sleeping Beauty transposon vectors without phenotypic alterations.

The CXCL12/CXCR4 axis influences the AML LSC nesting into the protective BM niche[10]. We examined bulk RNA sequencing (RNA-seq) datasets obtained from AML patients available through the TCGA database, and confirmed that CXCR4 is highly expressed by AML blasts (n=173) as compared to normal peripheral blood mononuclear cells (PBMCs) (n=70; padj=7.73e-26; **Supplemental Figure 1A**). Furthermore, the analysis of two published single-cell RNA sequencing (scRNA-seq) datasets from AML BM (EGAS00001005593,[17]; GSE214914) revealed that CXCR4 expression decreases during myeloid cell maturation (**Supplemental Figure 1B,C**) and it is particularly high on immature cell subsets (>10th percentile on HSC/MPP, CMP and GMP) (**Supplemental Figure 1D,E**). In addition, a scRNA-seq dataset of murine BM (GSE128423,[18]) indicates that CXCL12 is strongly expressed by BM stromal cells of the *MLL-AF9* leukemia model (**Supplemental Figure 1F,G,H**). We corroborated these data showing the presence of high levels

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of CXCL12 in the serum collected from BM samples of AML patients, which is comparable to those observed in healthy controls (p=0.3011, **Supplemental Figure 1I**).

As *ex vivo* culturing of human lymphocytes can affect the expression of chemokine receptors, we analyzed the expression of CXCR4 in CIKs engineered to express the CD33.CAR[13]. We consistently observed that CD33.CAR-CIKs displayed significant reduction in CXCR4 expression at the end of culture compared to freshly isolated T-cells (n=16; p<0.0001) **(Figure 1A)**, in both CD4^{+} and CD8^{+} subpopulations (**Figure 1B**). This observation, consistent with previous studies[19], suggests that *ex vivo* expansion reduces the potential ability of CD33.CAR-CIKs to use the CXCL12 gradient to migrate to BM after adoptive transfer in patients. Thus, we explored whether CXCR4 overexpression obtained via gene transfer could restore CD33.CAR-CIKs homing to the BM.

Therefore, we designed two different bicistronic SB transposon vectors containing either IRES or 2A-like peptide, hereafter named CXCR4(I)CD33.CAR and CD33.CAR(2A)CXCR4 constructs, to co-express two transgenes under the control of a single promoter **(Supplemental Figure 2A)**. The monocistronic CD33.CAR construct was used as control. The expression of CXCR4 and CD33.CAR was assessed by FACS analysis at the end of three weeks of culture (**Supplemental Figure 2B**). CIKs transduced with either CD33.CAR(I)CXCR4 (n=8) or CXCR4(2A)CD33.CAR vectors (n=8) showed significantly higher expression of CXCR4 compared to CIKs transduced with the CD33.CAR construct alone (n=12; p=0.0005) **(Supplemental Figure 2C)**. Notably, we observed that CD33.CAR expression in CIKs transduced with the CD33.CAR(2A)CXCR4 was similar or even higher than that of CIKs transduced with the CD33.CAR construct (p=0.028). In contrast, CIKs engineered with the CXCR4(I)CD33.CAR, in which CAR expression is under the control of the IRES element, showed reduced CAR expression compared to CIKs transduced with the CD33.CAR construct (p=0.028) **(Supplemental Figure 2D)**. Accordingly, we selected the 2A peptide-based bicistronic construct to proceed with further investigations.

Several studies have shown that carboxy-terminus truncated forms of CXCR4 receptor, usually expressed on leukocytes of patients with WHIM syndrome, have improved chemotaxis responsiveness toward CXCL12[20]. Therefore, we postulated that the transduction of CD33.CAR-CIKs with the most common mutant version of CXCR4 (CXCR4[mut]) in WHIM patients, namely the R334X[21], might not only increase the efficiency of BM trafficking, but it might also promote CAR-CIKs retention within the BM niche. We thus developed an alternative 2A peptide-based bicistronic SB transposon vector to mediate the co-expression of CD33.CAR and CXCR4[mut] (**Figure 1C**). Transfection of CIKs with both bicistronic vectors resulted in an increased and stable CXCR4 expression compared to CD33.CAR-CIKs **(Figure 1C and 1D)**. Specifically, CD33.CAR-

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CXCR4[mut]-CIKs (n=19) displayed consistent increase of CXCR4 MFI compared to both CD33.CAR-CIKs (p<0.0001) and CD33.CAR-CXCR4[wt]-CIKs (p=0.0012) at the end of the culture **(Figure 1E)**. CD33.CAR expression was equally maintained among the three different constructs (n=19) **(Figure 1F)**. CD33.CAR-CIKs expressing either CXCR4[wt] or CXCR4[mut] retained the expression of typical phenotypic markers (CD4, CD8, CD56, CD45RO, CD62L), with a slight alteration in CD4^{+} and CD8^{+} subsets in CD33.CAR-CXCR4[wt] compared to CD33.CAR-CIKs **(Figure 1G)**. No differences were observed in memory phenotype **(Figure 1H)**.

CXCR4 R334X GOF mutation is associated with decreased receptor internalization and enhanced surface recovery of its expression after protracted CXCL12-induced activation[20,22]. We observed that CD33.CAR-CXCR4[mut]-CIKs displayed consistently reduced CXCR4 down-regulation after incubation with CXCL12 compared to the other CAR constructs (n=10, p<0.0001) (**Supplemental Figure 3A-B**). Accordingly, CXCL12 pre-treated CD33.CAR-CXCR4[mut]-CIKs (n=5) mediated a superior chemotactic response to sequential stimulation in comparison with CD33.CAR-CXCR4[wt]-(p=0.0068) and CD33.CAR-CIKs (p=0.0020) (**Supplemental Figure 3C**).

Overall, these data underline the relevance of the CXCL12 gradient generated within the BM in AML and the feasibility to genetically upregulate the CXCR4 on CD33.CAR-CIKs to promote their migration toward CXCL12.

CXCR4-overexpression on CD33.CAR-CIKs leads to enhanced chemotactic activity toward CXCL12, while retaining CAR-related effector functions.

Next, we evaluated the chemotactic properties of CD33.CAR-CXCR4[wt]- and CD33.CAR-CXCR4[mut]-CIKs toward the CXCL12 gradient in a transwell migration assay. To determine the effective contribution of the engineered CXCR4[wt] or CXCR4[mut] molecules on CD33.CAR-CIKs functionality, we selected CAR[+]-CIKs by immunomagnetic sorting (purity >99%; **Figure 2A**). Notably, both CD33.CAR[+]-CXCR4[wt]- (p=0.015) and CD33.CAR[+]-CXCR4[mut]-CIKs (p<0.0001) displayed improved migratory response toward rhCXCL12 compared to CD33.CAR-CIKs (n=10). Specifically, CD33.CAR[+]-CIKs expressing CXCR4[mut] migrated more efficiently than those expressing CXCR4[wt] (p=0.0362) (**Figure 2B**). Afterwards, to better mimic the conditions of the BM microenvironment, we used the supernatant of MSCs derived from HD or AML patients as chemotactic stimulus. No significant differences in CXCL12 levels were observed (**Figure 2C**). Remarkably, both CD33.CAR[+]-CXCR4[wt]- and CD33.CAR[+]-CXCR4[mut]-CIKs showed increased chemotactic response toward both HD-MSC and AML-MSC supernatants compared to control CD33.CAR[+]-CIKs (n=14, p<0.0001) (**Figure 2D**). This enhanced migration is strictly associated

with the presence of CXCL12 in the supernatant, as it was abrogated after pre-treatment with the CXCR4 antagonist plerixafor. Even in this case, CXCR4^{mut} promoted superior CXCL12-dependent chemotaxis compared to CXCR4^{wt} (p=0.0056 for HD-MSC and p=0.043 for AML-MSC supernatants).

To assess if CXCR4 overexpression may have an impact on CAR-related cytotoxic properties, we tested cytotoxicity, proliferation and cytokines production of CD33.CAR$^+$-CXCR4^{wt}- and CD33.CAR$^+$-CXCR4^{mut}-CIKs incubated with CD33$^+$ KG-1 AML cell line and patient-derived primary AML cells, in the presence or absence of CXCL12.

CXCR4-overexpressing CD33.CAR$^+$-CIKs retain specific cytotoxic activity against KG-1 cells and primary AML samples (**Figure 2E**). CD33.CAR$^+$-CIKs overexpressing either CXCR4^{wt} or CXCR4^{mut} proliferated in response to CD33$^+$ leukemic cells (**Figure 2F**). Moreover, the intracellular staining for IFN-γ and IL-2 revealed cytokine production after co-culture with CD33$^+$ target cells (**Figure 2G**). Of note, the presence of CXCL12 in the co-culture enhanced the proliferation and the cytokine release of CD33.CAR$^+$-CIKs expressing CXCR4^{mut}. The antileukemic activity of CD33.CAR$^+$-CXCR4^{wt}-CIKs toward the KG-1 cell line and primary AML blasts was superior to that exerted by CD33.CAR-CIKs in the presence of CXCL12 at low E:T ratios (KG-1: p=0.03 for E:T 0.25:1and 0.125:1, **Figure 2H**; AML#1: p=0.016 for E:T 0.125:1, **Figure 2I**). We then investigated the cytotoxic activity of CXCR4-overexpressing CD33.CAR-CIKs migrated toward the CXCL12 gradient. The enhanced migration along the CXCL12 gradient resulted in significantly higher lysis of primary blasts by CXCR4^{wt}-overexpressing CD33.CAR$^+$-CIKs compared to control CD33.CAR$^+$-CIKs (p=0.002; **Figure 2L**).

Overall, these data suggest that CXCR4 overexpression in CD33.CAR-CIKs increases their migration towards the CXCL12 gradient without compromising their cytotoxic activity against AML cell lines and primary blasts.

CXCR4-overexpressing CD33.CAR-CIKs possess superior in vivo homing ability to the BM.

To assess whether CXCR4-overexpressing CD33.CAR-CIKs acquire superior homing to the BM *in vivo*, we first established that CD33.CAR$^+$-CXCR4^{wt}- and CD33.CAR$^+$-CXCR4^{mut}-CIKs exhibit enhanced *in vitro* migration capacity in response to recombinant murine CXCL12 and mouse BM supernatant as compared to CD33.CAR-CIKs (data not shown and **Figure 3A**). Afterwards, NSG mice were infused intravenously with either CD33.CAR$^+$-CXCR4^{wt}-, CD33.CAR$^+$-CXCR4^{mut}- or CD33.CAR$^+$-CIKs (10^7 cells per mouse) (**Figure 3B**). The frequency and absolute number of hCD45$^+$ cells recovered from BM, PB, and spleen were determined by FACS analysis 7 days after

transplant. hCD45$^+$ cells frequency was higher in the BM of mice receiving CD33.CAR$^+$-CXCR4^{wt}-(n=10) and CD33.CAR$^+$-CXCR4^{mut}-CIKs (n=12) compared to those infused with CD33.CAR$^+$-CIKs (n=12, p=0.0038 and p=0.0006, respectively) (**Figure 3C-D**). Specifically, mice receiving CD33.CAR$^+$-CXCR4^{mut}-CIKs showed the highest number of hCD45$^+$ cells in the BM (p<0.0001 *vs* CD33.CAR$^+$-CIKs, p=0.0369 *vs* CD33.CAR$^+$-CXCR4^{wt}-CIKs) (**Figure 3D**). The frequency and the absolute number of CXCR4-overexpressing CD33.CAR$^+$-CIKs in PB and spleen displayed only minimal differences compared to control CD33.CAR$^+$-CIKs (**Figure 3E**).

We then assessed the kinetics of CXCR4-overexpressing CD33.CAR$^+$-CIKs in the niche by sampling the BM at day +10 and +14 after transplant. At day +10, mice treated with CD33.CAR$^+$-CXCR4^{mut}- (n=13) showed higher frequency and number of hCD45$^+$ cells in the BM compared to those treated with CD33.CAR$^+$-CIKs (n=12, p<0.0001) **(Figure 3F)**. These results were further confirmed on day +14, as CD33.CAR$^+$-CXCR4^{mut}- (n=10) were still present in the BM compartment at higher levels compared to CD33.CAR$^+$-CIKs (n=11, p<0.0001) (**Figure 3G**).

Overall, these data suggest that CXCR4 overexpression on CD33.CAR-CIKs increases their migration to the BM and, in the case of the mutated CXCR4, further prolongs their persistence.

CXCR4^{wt}-overexpressing CD33.CAR-CIKs promote better elimination of BM-resident AML cells and prolong survival of mice.

To verify if CXCR4-overexpression in CD33.CAR-CIKs confers superior antitumor activity, we established a leukemia xenograft model by injecting CD33$^+$ KG-1 AML cells into NSG mice. Fourteen days later, mice were treated with CD33.CAR$^+$-CXCR4^{wt}-, CD33.CAR$^+$-CXCR4^{mut}- or control CD33.CAR$^+$-CIKs. In the first set of experiments, mice were sacrificed 25 days after CAR-CIKs infusion and the frequency of residual hCD33$^+$ cells was monitored by FACS in BM, PB, and spleen **(Figure 4A)**. Animals treated with CD33.CAR$^+$-CIKs displayed a reduction of the frequency and absolute number of hCD33$^+$ cells in the BM. The treatment with CXCR4-overexpressing CD33.CAR$^+$-CIKs further decreased the amount of hCD33$^+$ KG-1 cells, specifically in the case of CD33.CAR$^+$-CXCR4^{wt}-CIKs (percentage: p=0.0106 *vs* CD33.CAR$^+$-CIKs, p=0.0336 *vs* CD33.CAR$^+$-CXCR4^{mut}-CIKs; absolute number: p=0.0156 *vs* CD33.CAR$^+$-CIKs, p=0.0418 *vs* CD33.CAR$^+$-CXCR4^{mut}-CIKs) (**Figure 4B-C)**. Similarly, immunohistochemistry analyses showed that CD33$^+$ AML cells were almost undetectable in the BM of the CD33.CAR$^+$-CXCR4^{wt}-CIKs-treated group (**Supplemental Figure 4**). Reduction of leukemic burden was observed also in the spleen, where CXCR4-overexpressing CD33.CAR$^+$-CIKs showed enhanced antitumor activity compared to CD33.CAR$^+$-CIKs (percentage: p=0.0003 for CD33.CAR$^+$-CXCR4^{wt}-CIKs and

p=0.0086 for CD33.CAR[+]-CXCR4[mut]-CIKs; absolute number: p=0.00107 for CD33.CAR^{+}-CXCR4[wt]-CIKs and p=0.033 for CD33.CAR^{+}-CXCR4[mut]-CIKs) (**Figure 4D**). Similarly, low hCD33^{+} AML residual cells were detected in the PB of mice treated with CXCR4-overexpressing CD33.CAR^{+}-CIKs and control CD33.CAR^{+}-CIKs (**Figure 4D**). We did not observe any accumulation of CD33.CAR[+]CXCR4[wt]- and CD33.CAR[+]-CXCR4[mut]-CIKs in tissues expressing CXCL12 as liver, heart, kidney and brain after i.v. administration. Moreover, there were no obvious signs of histological damage in these organs (data not shown).

In the second set of experiments, leukemia burden was monitored weekly in BM aspirates starting from 2 weeks after CAR-CIKs infusion, and mice survival was evaluated (**Figure 4E**). By week 7, CD33.CAR[+]-CXCR4[wt]-CIKs demonstrated a superior control of AML progression with lower AML cells amount in the BM than CD33.CAR[+]-CXCR4[mut]- and CD33.CAR-CIKs groups (n=4 mice per group, **Figure 4F**). In association with the reduced leukemic tumor burden, the survival of the KG-1 cell-inoculated mice treated with CD33.CAR[+]-CXCR4[wt]-CIKs was significantly prolonged (n=4 mice per group, p=0.0001, Log-rank test), with median survival time increased from 57.5, 77.5, and 87.5 days respectively in the untreated, CD33.CAR[+]-CIKs and CD33.CAR[+]-CXCR4[mut]-CIKs groups to 110 days in the CD33.CAR[+]-CXCR4[wt]-CIKs group (p=0.010 *vs* CD33.CAR[+]- and p=0.010 *vs* CD33.CAR[+]-CXCR4[mut]-CIKs) (**Figure 4G**). The unexpected underperformance of CD33.CAR[+]-CXCR4[mut]-CIKs could be ascribed to immunological synapse instability. Actually, it was reported that in the presence of competing CXCL12 signal the stability of the immune synapse of T lymphocytes from patients with WHIM-mutant CXCR4 is disrupted as a result of impaired recruitment of the mutant receptor[23]. Hence, we performed conjugate stability experiments in which CD33.CAR^{+}-CXCR4[wt]- and CD33.CAR^{+}-CXCR4[mut]-CIKs were brought into contact with labeled CD33^{+} or CD33^{-} KG-1 cells to form conjugates. When exposed to CXCL12, CD33.CAR^{+}-CXCR4[wt]-CIKs conjugated with CD33^{+} KG-1 were less responsive and migrated less efficiently than unengaged CD33.CAR^{+}-CXCR4[wt]-CIKs left in contact with CD33^{-} KG-1 cells. In contrast, CD33.CAR^{+}-CXCR4[mut]-CIKs conjugated with CD33^{+} KG-1 were still efficiently attracted and migrated toward the chemokine, suggesting an impaired conjugate stability in the presence of competing external CXCL12 signals (p = 0.00058 for CD33.CAR[+]-CXCR4[mut] vs CD33.CAR[+]-CXCR4[wt]-CIKs) (**Figure 4H**).

Overall, these findings demonstrated that CXCR4[wt] overexpression in CD33.CAR-CIKs favorably improves the antileukemic effect of these cells.

Discussion

We have developed a novel approach to engineer CIKs to co-express CD33.CAR and CXCR4, in both wild-type and WHIM syndrome-derived truncated forms. Both CXCR4-overexpressing CD33.CAR-CIKs displayed increased migration toward the chemokine CXCL12 and effectively killed CD33^{+} cell lines and primary AML cells. However, while CAR-CIKs engineered with the CXCR4[mut] variant demonstrated a prolonged retention in the BM compared to CAR-CIKs co-expressing the wild-type CXCR4, the latter led to superior disease control underlying that the physiological reclining of CXCR4 in CIKs may have an essential role in modulating both their accrual and release from the leukemic BM niche and their activation status.

Strategies to improve CAR-T cell recruitment to the tumor have recently raised interest not only in solid tumors but also in hematological malignancies. Specifically in AML, where the efficacy of CAR-T therapy is hampered, the homing of blood-injected effector cells in the BM is required for their intimate contact with BM-residing leukemia cells including LSCs, whose elimination is fundamental to achieve deep and durable remission.

In particular, we explored the possibility to exploit the CXCL12/CXCR4 axis, which is critical in the AML niche, in order to maximize CD33.CAR-CIK recruitment to the BM, thus possibly increasing their therapeutic efficacy. The CXCR4 receptor is involved in the homing of hematopoietic and immune cells into the BM niche, and therefore can be leveraged to drive CAR-T cells into the niche, in response to the CXCR4 ligand, CXCL12 produced by multiple cell types, such as stromal cells, endothelial cells and osteoblasts[24]. In a previous study, CXCR4 was co-expressed on c-kit CAR-T cells to maximize the HSC clearance within the BM[25]. Furthermore, CXCR4 upregulation on anti-EpCAM.CAR-T cells, obtained by *ex vivo* treatment with rapamycin, has shown increased BM infiltration and elimination of marrow-resident AML cells in a xenograft mouse model[4].

The CXCL12/CXCR4 axis is central to AML pathogenesis as it controls blasts adhesion into the protective BM niche, adaptation to the hypoxic environment, cellular migration and survival[10]. High levels of CXCR4 expression in blasts are associated with poor relapse-free and overall survival[26]. Furthermore, blocking CXCL12/CXCR4 axis is an attractive therapeutic strategy, since it can lead to AML cells mobilization from the BM into the circulation, depriving them of essential survival signals[10–12]. Moreover CXCL12/CXCR4 is implicated in the T_{reg} cells homing to the BM microenvironment in AML[27,28].

To enhance migration but also to increase CAR-CIK retention in the BM, bringing them in closer contact with marrow-residing LSCs, we also postulated the use of gain-of-function (GOF) variants of CXCR4. GOF mutations of *CXCR4*, with the most prevalent being the R334X, are a cause of WHIM syndrome, which results in panleukocytopenia in the PB but hyperplasia in the BM[14]. This

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is likely caused by the stronger binding of the C-terminal truncated CXCR4 WHIM-mutant to CXCL12, increasing intracellular signaling and preventing leukocytes release into the PB from the BM[15]. Levy et al showed that transfecting NK cells with GOF mutant variant of CXCR4 leads to superior BM homing[29]. Additionally, it was demonstrated that CXCR4[R334X] is effective in enhancing the controlling effects of anti-B cell maturation antigen (BCMA)CAR-NK cells on *in vivo* multiple myeloma growth[30].

In our study, we observed that CXCR4 is strongly down-regulated in CD33.CAR-CIKs during *ex vivo* expansion and demonstrated that CXCR4 expression can be stably increased in CD33.CAR-CIKs using a bicistronic SB transposon vector containing 2A-like peptide, without negatively impacting their phenotype.

Both CD33.CAR[+]-CXCR4[wt]- and CD33.CAR^{+}-CXCR4[mut]-CIKs showed improved chemotaxis toward rhCXCL12 or HD-/AML-MSC supernatants, compared to CD33.CAR^{+}-CIKs. Using as target CD33^{+} AML cell lines as well as primary AML cells, similar cytotoxic activity, proliferative response and IFN-γ or IL-2 secretion levels were observed in CD33.CAR[+]-CIKs, regardless of whether they overexpressed CXCR4[wt], CXCR4[mut] or none.

Finally, our data establish the feasibility and efficacy of single administration of CXCR4-overexpressing CD33.CAR[+]-CIKs to control leukemia *in vivo* in a xenograft model. First, both the CXCR4-engineered products displayed enhanced *in vivo* BM homing, along with a prolonged retention in the case of CXCR4[mut], as expected. However, only CD33.CAR[+]-CIKs co-expressing CXCR4[wt] exerted a superior control of AML progression if compared with the conventional CD33.CAR[+]-CIKs, significantly increasing the survival time of treated mice.

In addition to the enhanced BM homing, the increased antileukemic effects of CD33.CAR-CXCR4[wt]-CIKs could be determined by other factors. CXCR4 is known to affect T cell costimulation because CXCL12 facilitates the formation of the immunological synapse and amplifies the downstream TCR intracellular signaling [31,32]. This mechanism could explain at least in part our experimental observation that CXCL12 increases the cytotoxic effects of CD33.CAR-CXCR4[wt]-CIKs at lower E:T ratios. On the other hand, we found that CXCR4[mut]-CD33.CAR-CIKs have reduced antileukemic effects *in vivo*, which resembles the effects of the WHIM-mutant CXCR4 that delivers aberrant signals to T and B cells. First of all, in the presence of distracting CXCL12, CXCR4 mutant competes with the TCR signaling, therefore disrupting T cell-APC synapses, thus inhibiting T cell activation rather than enhancing it[23]. Consistently, we confirmed that CXCR4[mut] disrupts the stability also of CD33.CAR[+]-CIKs/target cell conjugates *in vitro* and this may dampen CAR-CIKs activation and their downstream effector functions. Furthermore, it was reported that WHIM-mutant CXCR4, due to its impaired CXCL12-induced down-regulation,

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may lead to aberrant T cell hyperactivation in response to CXCL12, reducing their survival[33]. Interestingly, in the presence of CXCL12, CXCR4[mut]-CD33.CAR-CIKs have shown *in vitro* increased proliferation and cytokine production in response to AML cells. Therefore, this hyperactivation mediated by CXCL12-stimulated WHIM-mutant CXCR4 coupled with the CAR signaling can lead *in vivo* to activation-induced cell death and CAR-CIK exhaustion, reducing their persistence. Differently from our data, Ng et al reported that CXCR4[R334X]-modified BCMA-targeting CAR-NK cells have improved control of multiple myeloma in the BM[30]. However, they electroporated the effector cells with a CXCR4[R334X]-encoding mRNA, obtaining an only transient expression of the receptor (less than 48h), thus avoiding the potential effects of CXCL12-mediated aberrant costimulation *in vivo*.

Regarding the potential off-target effects of this CXCR4-mediated approach, a GTEX database analysis on expression levels of CXCL12 showed its expression on healthy tissues other than BM, such as heart, liver, kidney and brain, and thus we cannot fully exclude potential side effects in patients. In our studies we did not observe any evidence of accumulation of CXCR4-modified CD33.CAR-CIKs in normal tissues, and we did not record side-effects in animals within the limitations of the xenograft model. We employed CD33 as the target for our studies, as a proof-of-principle, based on the previous preclinical experience with CAR-T cells for AML [13,34–37]. However, CD33 may cause on-target off-tumor toxicity on normal HSPCs. However, the concept of coupling CAR and CXCR4 expression in T cells to increase their BM homing remains broadly applicable to other AML-associated antigens.

In conclusion, this study provides a proof-of-concept demonstrating that arming anti-AML CAR-CIKs with the wild-type receptor CXCR4 is a feasible strategy to enhance control of the AML burden in the BM niche, showing striking efficacy *in vitro* and *in vivo*.

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Authorship

Contribution: M.B., G.D., A.P., and M.S. supervised the study and wrote the manuscript; M.B., S.T., G.D., A.P., and M.S. determined the methodology; M.B., B.C., C.T., S.D., S.B., V.P., A.D., and A.P. performed the investigation; M.B., S.D., S.B. A.D., and A.P. curated the data; M.B., S.G., and A.P. formally analyzed the data; S.G. performed statistical analysis; R.P. performed gene expression analyses; S.T., V.B., F.D., A.M., and A.B. critically discussed data and edited the manuscript; A.B. and M.S. provided the funding; F.D., G.D., A.P., and M.S. conceived the study.

Conflict-of-interest disclosure: G. Dotti has served as a consultant for Bellicum Pharmaceuticals and Catamaran. The other authors declare no conflict of interest.

Data Availability

The data generated in this study are available within the article and its supplemental data files. EGAS00001005593 data can be retrieved from the following repository: https://ega-archive.org/studies/EGAS00001005593 and can be interactively explored using a dedicated Abseq App (https://abseqapp.shiny.embl.de/). GSE214914 data can be retrieved from: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE214914. GSE128423 data can be retrieved from: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128423.

References

1.   Mardiana S, Gill S. CAR T Cells for Acute Myeloid Leukemia: State of the Art and Future Directions. *Front. Oncol.* 2020;10:.

2.   Ritchie DS, Neeson PJ, Khot A, et al. Persistence and Efficacy of Second Generation CAR T Cell Against the LeY Antigen in Acute Myeloid Leukemia. *Mol. Ther.* 2013;21(11):2122–2129.

3.   Zou Y, Li F, Hou W, et al. Manipulating the expression of chemokine receptors enhances delivery and activity of cytokine-induced killer cells. *Br. J. Cancer*. 2014;110(8):1992–1999.

4.   Nian Z, Zheng X, Dou Y, et al. Rapamycin pretreatment rescues the bone marrow AML cell elimination capacity of CAR-T cells. *Clin. Cancer Res.* 2021;clincanres.0452.2021.

5.   Di Stasi A, De Angelis B, Rooney CM, et al. T lymphocytes coexpressing CCR4 and a chimeric antigen receptor targeting CD30 have improved homing and antitumor activity in a Hodgkin tumor model. *Blood*. 2009;113(25):6392–6402.

6.   Craddock JA, Lu A, Bear A, et al. Enhanced tumor trafficking of GD2 chimeric antigen

receptor T cells by expression of the chemokine receptor CCR2b. *J. Immunother.* 2010;33(8):780–788.

7.   Moon EK, Carpenito C, Sun J, et al. Expression of a Functional CCR2 Receptor Enhances Tumor Localization and Tumor Eradication by Retargeted Human T cells Expressing a Mesothelin-Specific Chimeric Antibody Receptor. *Clin. Cancer Res.* 2011;17(14):4719–4730.

8.   Lesch S, Blumenberg V, Stoiber S, et al. T cells armed with C-X-C chemokine receptor type 6 enhance adoptive cell therapy for pancreatic tumours. *Nat. Biomed. Eng. 2021 511.* 2021;5(11):1246–1260.

9.   Cadilha BL, Benmebarek MR, Dorman K, et al. Combined tumor-directed recruitment and protection from immune suppression enable CAR T cell efficacy in solid tumors. *Sci. Adv.* 2021;7(24):1–12.

10. Ladikou EE, Chevassut T, Pepper CJ, Pepper AGS. Dissecting the role of the CXCL12/CXCR4 axis in acute myeloid leukaemia. *Br. J. Haematol.* 2020;189(5):815–825.

11. Nervi B, Ramirez P, Rettig MP, et al. Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. *Blood.* 2009;113(24):6206–6214.

12. Uy GL, Rettig MP, Motabi IH, et al. A phase 1/2 study of chemosensitization with the CXCR4 antagonist plerixafor in relapsed or refractory acute myeloid leukemia. *Blood.* 2012;119(17):3917–3924.

13. Rotiroti MC, Buracchi C, Arcangeli S, et al. Targeting CD33 in Chemoresistant AML Patient-Derived Xenografts by CAR-CIK Cells Modified with an Improved SB Transposon System. *Mol. Ther.* 2020;28(9):1974–1986.

14. McDermott DH, Murphy PM. WHIM syndrome: Immunopathogenesis, treatment and cure strategies. *Immunol. Rev.* 2019;287(1):91–102.

15. Balabanian K, Lagane B, Pablos JL, et al. WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. *Blood.* 2005;105(6):2449–2457.

16. Magnani CF, Turazzi N, Benedicenti F, et al. Immunotherapy of acute leukemia by chimeric antigen receptor-modified lymphocytes using an improved Sleeping Beauty transposon platform. *Oncotarget.* 2016;7(32):51581–51597.

17. Triana S, Vonficht D, Jopp-Saile L, et al. Single-cell proteo-genomic reference maps of the hematopoietic system enable the purification and massive profiling of precisely defined cell states. *Nat. Immunol.* 2021;22(12):1577.

18. Baryawno N, Przybylski D, Kowalczyk MS, et al. A Cellular Taxonomy of the Bone Marrow Stroma in Homeostasis and Leukemia. *Cell*. 2019;177(7):1915-1932.e16.

19. Beider K, Nagler A, Wald O, et al. Involvement of CXCR4 and IL-2 in the homing and retention of human NK and NK T cells to the bone marrow and spleen of NOD/SCID mice. *Blood*. 2003;102(6):1951–1958.

20. Gulino AV, Moratto D, Sozzani S, et al. Altered leukocyte response to CXCL12 in patients with warts hypogammaglobulinemia, infections, myelokathexis (WHIM) syndrome. *Blood*. 2004;104(2):444–452.

21. Hernandez PA, Gorlin RJ, Lukens JN, et al. Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. *Nat. Genet.* 2003;34(1):70–74.

22. Kawai T, Choi U, Whiting-Theobald NL, et al. Enhanced function with decreased internalization of carboxy-terminus truncated CXCR4 responsible for WHIM syndrome. *Exp. Hematol.* 2005;33(4):460–468.

23. Kallikourdis M, Trovato AE, Anselmi F, et al. The CXCR4 mutations in WHIM syndrome impair the stability of the T-cell immunologic synapse. *Blood*. 2013;122(5):666–673.

24. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity*. 2006;25(6):977–988.

25. Arai Y, Choi U, Corsino CI, et al. Myeloid Conditioning with c-kit-Targeted CAR-T Cells Enables Donor Stem Cell Engraftment. *Mol. Ther.* 2018;26(5):1181–1197.

26. Spoo AC, Lübbert M, Wierda WG, Burger JA. CXCR4 is a prognostic marker in acute myelogenous leukemia. *Blood*. 2007;109(2):786–791.

27. Zou L, Barnett B, Safah H, et al. Bone marrow is a reservoir for CD4+CD25+ regulatory T cells that traffic through CXCL12/CXCR4 signals. *Cancer Res.* 2004;64(22):8451–8455.

28. Wang R, Feng W, Wang H, et al. Blocking migration of regulatory T cells to leukemic hematopoietic microenvironment delays disease progression in mouse leukemia model. *Cancer Lett.* 2020;469(October 2019):151–161.

29. Levy E, Reger R, Segerberg F, et al. Enhanced bone marrow homing of natural killer cells following mRNA transfection with gain-of-function variant CXCR4R334X. *Front. Immunol.* 2019;10(JUN):1–13.

30. Ng YY, Du Z, Zhang X, Chng WJ, Wang S. CXCR4 and anti-BCMA CAR co-modified natural killer cells suppress multiple myeloma progression in a xenograft mouse model. *Cancer Gene Ther. 2021 295*. 2021;29(5):475–483.

31. Molon B, Gri G, Bettella M, et al. T cell costimulation by chemokine receptors. *Nat. Immunol.* 2005;6(5):465–471.

32. Kumar A, Humphreys TD, Kremer KN, et al. CXCR4 Physically Associates with the T Cell Receptor to Signal in T Cells. *Immunity*. 2006;25(2):213–224.

33. Roselli G, Martini E, Lougaris V, et al. CXCL12 mediates aberrant costimulation of B lymphocytes in warts, hypogammaglobulinemia, infections, myelokathexis immunodeficiency. *Front. Immunol.* 2017;8(SEP):1–12.

34. Marin V, Pizzitola I, Agostoni V, et al. Cytokine-induced killer cells for cell therapy of acute myeloid leukemia: improvement of their immune activity by expression of CD33-specific chimeric receptors. *Haematologica*. 2010;95(12):2144–2152.

35. Pizzitola I, Anjos-Afonso F, Rouault-Pierre K, et al. Chimeric antigen receptors against CD33/CD123 antigens efficiently target primary acute myeloid leukemia cells in vivo. *Leukemia*. 2014;28(8):1596–1605.

36. Dutour A, Marin V, Pizzitola I, et al. In vitro and in vivo antitumor effect of anti-CD33 chimeric receptor-expressing EBV-CTL against CD 33 + acute myeloid leukemia. *Adv. Hematol.* 2012;2012:.

37. O'Hear C, Heiber JF, Schubert I, Fey G, Geiger TL. Anti-CD33 chimeric antigen receptor targeting of acute myeloid leukemia. *Haematologica*. 2015;100(3):336–344.

Figure Legends

Figure 1. *Ex vivo* expansion of CD33.CAR-CIKs is associated with down-regulation of CXCR4, and its expression can be stably increased using bicistronic Sleeping Beauty transposon vectors without phenotypic alterations.

Data are presented as individual values and the mean ± SD. **A, B** Summary of the percentage of CXCR4 expression on circulating CD3^{+} T cells and on CD33.CAR-CIKs at the end of *ex vivo* expansion. The analysis was performed on all CD3^{+} cells (n=16 different donors, ****p<0.0001 by paired *t* test) (**A**) and on CD4^{+} and CD8^{+} subpopulations (n=8 different donors, ***p = 0.0003 for CD4^{+} and ***p = 0.00011 for CD8^{+} by *t* test) (**B**). **C** Schematic representation of the vectors encoding CD33.CAR and CXCR4[wt] or CXCR4[mut]. Representative flow cytometry plots showing CD33.CAR and CXCR4 expression in CIKs transduced with CD33.CAR, CD33.CAR(2A)CXCR4[wt] or CD33.CAR(2A)CXCR4[mut] transposon vectors. **D, E** Summary of CXCR4 expression percentage in culture during time (**D**) and of CXCR4 MFI at the end of the

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culture (**E**) on CD33.CAR-, CD33.CAR CXCR4^{wt}- and CD33.CAR CXCR4^{mut}-CIKs (n=19 different donors, ****p<0.0001, ***p = 0.0003, **p = 0.0012 by paired *t* test). **F** Summary of CD33.CAR expression percentage (left) and MFI (right) on CD33.CAR-, CD33.CAR-CXCR4^{wt}- and CD33.CAR-CXCR4^{mut}-CIKs after *ex vivo* expansion (n=19 different donors). **G** Percentage of CD4^{+}, CD8^{+}, CD56^{+} cells on CD3^{+} for CD33.CAR-, CD33.CAR-CXCR4^{wt}- and CD33.CAR-CXCR4^{mut}-CIKs after *ex vivo* expansion (n=13 different donors, *p = 0.015 for CD4^{+}, *p = 0.031 for CD8^{+} by paired *t* test). **H** Different memory cell subsets (Naïve, CM, EM, and EMRA cells) on CD3^{+} for CD33.CAR-, CD33.CAR-CXCR4^{wt}- and CD33.CAR-CXCR4^{mut}-CIKs after *ex vivo* expansion (n=13 different donors).

Figure 2. CXCR4-overexpressing CD33.CAR-CIKs have improved migration capability toward CXCL12 and retain antitumor activity *in vitro*.

Data are presented as individual values and the mean ± SD. **A** Representative flow cytometry plots showing CD33.CAR and CXCR4 expression on purified CD33.CAR^{+}-, CD33.CAR^{+}-CXCR4^{wt}- and CD33.CAR^{+}-CXCR4^{mut}-CIKs (see "Supplemental Methods"). **B** Percentage of migration of CXCR4-overexpressing CD33.CAR-CIKs in response to CXCL12 in transwell assays (n=10 independent experiments using CAR-CIKs generated from 10 different donors, ****p < 0.00001, *p = 0.015 for CD33.CAR^{+} vs CD33.CAR^{+}-CXCR4^{wt}, *p = 0.0362 for CD33.CAR^{+}-CXCR4^{wt} vs CD33.CAR^{+}-CXCR4^{mut} by paired *t* test). **C** CXCL12 levels measured in the culture supernatant of BM-derived mesenchymal stromal cells from healthy donors (HD-MSCs, n = 6 different donors) or AML patients (AML-MSCs, n = 10 different donors). **D** Percentage of migration of CXCR4-overexpressing CD33.CAR^{+}-CIKs in response to culture supernatant of HD-MSCs (left) or AML-MSCs (right) in the absence or presence of plerixafor. For HD-MSCs: n=12 experiments using CAR-CIKs generated from 6 different donors and supernatant samples from 6 different HD-MSCs, ****p<0.0001 and **p=0.0056 by paired *t* test. For AML-MSCs: n=14 experiments using CAR-CIKs generated from 6 different donors and supernatant samples from 10 different AML-MSCs, ****p<0.0001 and *p=0.043 by paired *t* test. **E** Cytotoxicity (E:T ratio of 5:1) of CXCR4-overexpressing CD33.CAR^{+}-CIKs against CD33^{+} KG-1 cell line and primary AML cells in the absence or presence of 200 ng/ml CXCL12 (for KG-1 with 200 ng/ml CXCL12: **p = 0.006 by paired *t* test). **F** Proliferation of CXCR4-overexpressing CD33.CAR^{+}-CIKs in response to CD33^{+} KG-1 cell line and primary AML cells in the absence or presence of 200 ng/ml CXCL12 (for AML#1 with 200 ng/ml CXCL12: *p = 0.012, **p = 0.003; for AML#2 with 200 ng/ml CXCL12: *p = 0.012; for AML#3 with 200 ng/ml CXCL12: **p = 0.006 for CD33.CAR^{+}-CXCR4^{mut}- *vs* CD33.CAR^{+}-CXCR4^{wt}-CIKs, **p = 0.002 for CD33.CAR^{+}-CXCR4^{mut}- *vs* CD33.CAR^{+}-CIKs. A

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paired *t* test was used). **G** Cytokine release of CXCR4-overexpressing CD33.CAR$^+$-CIKs in response to CD33$^+$ KG-1 cell line and primary AML cells in the absence or presence of 200 ng/ml CXCL12 (*IFNγ*: *p = 0.012; *IL-2*: for KG-1 with 200 ng/ml CXCL12, *p = 0.029; for AML#1 with 200 ng/ml CXCL12, *p = 0.034, **p = 0.005; for AML#2 and AML#3 with 200 ng/ml CXCL12, **p = 0.001. A paired *t* test was used.). For **E**, **F**, **G** n=9 (for KG-1) and n=8 (for primary AML cells) independent experiments using CAR-CIKs generated from different donors. **H, I** Quantification of CD33$^+$ KG-1 cell line (**H**) and primary AML cells (**I**) lysis after 24 hours of coculture with CXCR4-overexpressing CD33.CAR$^+$-CIKs at low E:T cell ratios, in the presence of 200 ng/ml CXCL12 (for KG-1 at E:T 0.25:1 and 0.125:1: *p = 0.03 for CD33.CAR$^+$- vs CD33.CAR$^+$-CXCR4^{wt}-CIKs; for AML#1 at E:T 0.125:1: *p = 0.016 for CD33.CAR$^+$-CXCR4^{wt}- vs CD33.CAR$^+$- and CD33.CAR$^+$-CXCR4^{mut}-CIKs. A paired *t* test was used). N=5 (for KG-1) and n=6 (for AML#1) independent experiments using CAR-CIKs generated from different donors. **L** Cytotoxicity of CXCR4-overexpressing CD33.CAR$^+$-CIKs post-chemotaxis toward CXCL12 gradient. Migrated CIKs were harvested and co-cultured for 4 hours with primary AML cells (For AML#1: **p = 0.002 for CD33.CAR$^+$-CXCR4^{wt}- vs CD33.CAR$^+$-CIKs, *p = 0.012 for CD33.CAR$^+$-CXCR4^{wt}- vs CD33.CAR$^+$-CXCR4^{mut}-CIKs; for AML#2: **p = 0.002 for CD33.CAR$^+$-CXCR4^{wt}- vs CD33.CAR$^+$-CIKs, **p = 0.009 for CD33.CAR$^+$-CXCR4^{wt}- vs CD33.CAR$^+$-CXCR4^{mut}-CIKs. A paired *t* test was used). For each primary AML, n=8 independent experiments using CAR-CIKs generated from 8 different donors.

Figure 3. CXCR4-overexpressing CD33.CAR-CIKs have enhanced *in vivo* BM homing ability. Data are presented as individual values and the mean ± SD. **A** Percentage of migration of CXCR4-overexpressing CD33.CAR$^+$-CIKs in response to mouse BM supernatant (n = 5 independent experiments using CAR-CIKs generated from one donor and supernatant samples from 5 different mice, *p=0.027 by paired *t* test). **B** Scheme of CD33.CAR-CIKs homing model using NSG mice inoculated via tail vein injection with 10^7 CD33.CAR$^+$-, CD33.CAR$^+$-CXCR4^{wt}- or CD33.CAR$^+$-CXCR4^{mut}-CIKs. Mice were euthanized 7, 10 or 14 days after infusion, and human CD45$^+$CD3$^+$ T cells were enumerated in BM, PB and spleen by flow cytometry. **C** Representative flow cytometry plots of human CD45$^+$ cell engraftment within mice BM at 7 days after infusion. **D, E** Summary of the percentage and absolute numbers of hCD45$^+$ in BM (**D**), PB and spleen (**E**) of mice at 7 days after infusion (n=12 mice in CD33.CAR$^+$ and CD33.CAR$^+$-CXCR4^{mut}, n=11 mice in CD33.CAR$^+$-CXCR4^{wt} group. 3 independent experiments using CAR-CIKs generated from 3 different donors. For percentage of hCD45$^+$ in BM: ***p=0.0006 and **p=0.0038. For absolute number of hCD45$^+$ in BM: ****p < 0.0001, **p=0.0035 and *p=0.0369. For absolute number of hCD45$^+$ in spleen:

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*p=0.0393. A mixed effect model was used). **F, G** Summary of the percentage and absolute number of hCD45^{+} in BM in mice euthanized 10 days (**F**) or 14 days (**G**) after infusion (n=12 mice in CD33.CAR^{+}, n=10 mice in CD33.CAR^{+}-CXCR4[wt] and n=13 mice in CD33.CAR[+]-CXCR4[mut] group for experiments at 10 days, ****p < 0.0001. n=11 mice in CD33.CAR[+], n=8 mice in CD33.CAR[+]CXCR4[wt] and n=10 mice in CD33.CAR[+]-CXCR4[mut] group for experiments at 14 days, ****p < 0.0001, **p=0.0029 and *p = 0.0425. 3 independent experiments using CAR-CIKs generated from three different donors. A mixed effect model was used).

Figure 4. CXCR4[wt]-overexpressing CD33.CAR-CIKs have superior antileukemic activity against KG-1 cells in the BM.

Data are shown as mean values ± SD. **A** Scheme of the AML model using NSG mice inoculated via tail vein injection with 5 x10^{6} KG-1 cells and 14 days later with CD33.CAR[+]-, CD33.CAR^{+}-CXCR4[wt]- or CD33.CAR^{+}-CXCR4[mut]-CIKs (10[7] cells/mouse). Mice were euthanized 25 days after CD33.CAR-CIKs infusion and human CD45^{+}CD33^{+} leukemia cells were enumerated in BM, PB and spleen by flow cytometry. **B** Representative flow cytometry plots of human CD33^{+} cell engraftment in the BM of mice. **C, D** Summary of the percentage and absolute number of hCD33^{+} in BM (for percentage: *p = 0.0106 for CD33.CAR^{+}- vs CD33.CAR^{+}-CXCR4[wt]-CIKs and *p = 0.0336 for CD33.CAR^{+}-CXCR4[wt]- vs CD33.CAR^{+}-CXCR4[mut]-CIKs; for absolute number: *p = 0.0156 for CD33.CAR[+]- vs CD33.CAR[+]-CXCR4[wt]-CIKs and *p = 0.0418 for CD33.CAR[+]-CXCR4[wt]- vs CD33.CAR[+]-CXCR4[mut]-CIKs by a mixed effect model) (**C**), spleen and peripheral blood (for percentage in spleen: ***p = 0.0003 and **p = 0.0086; for absolute number in spleen: **p = 0.00107 and *p = 0.033 by a mixed effect model) (**D**), of mice at 25 days (n=11 mice/group. 3 independent experiments using CAR-CIKs generated from 3 different donors). **E** In a second experimental setting, using the same model shown in (**B**), femoral BM aspiration was performed on mice starting from day 14 after CD33.CAR-CIKs injection until survival, and percentage of hCD33^{+} leukemia cells in the BM was analyzed by flow cytometry. **F** Summary of percentage of hCD33^{+} cells in the BM, n=4 mice/group from 4 independent experiments using CAR-CIKs generated from 4 different donors. **G** Kaplan-Meier survival curves of the same mice in (**F**). Comparisons of survival curves were determined by Log-rank test. **H** Percentage of residual migration of conjugates formed by CD33.CAR^{+}-, CD33.CAR^{+}-CXCR4[wt]-, or CD33.CAR^{+}-CXCR4[mut]-CIKs with CD33^{+} KG-1 (stable conjugates) respect to CD33^{-} KG-1 cells (control). The conjugates were allowed to migrate toward CXCL12 in a transwell filter allowing the migration of single cells only (n=7 independent experiments using CAR-CIKs generated from 7 different donors. ***p = 0.0006 and **p = 0.00101 by *t* test).
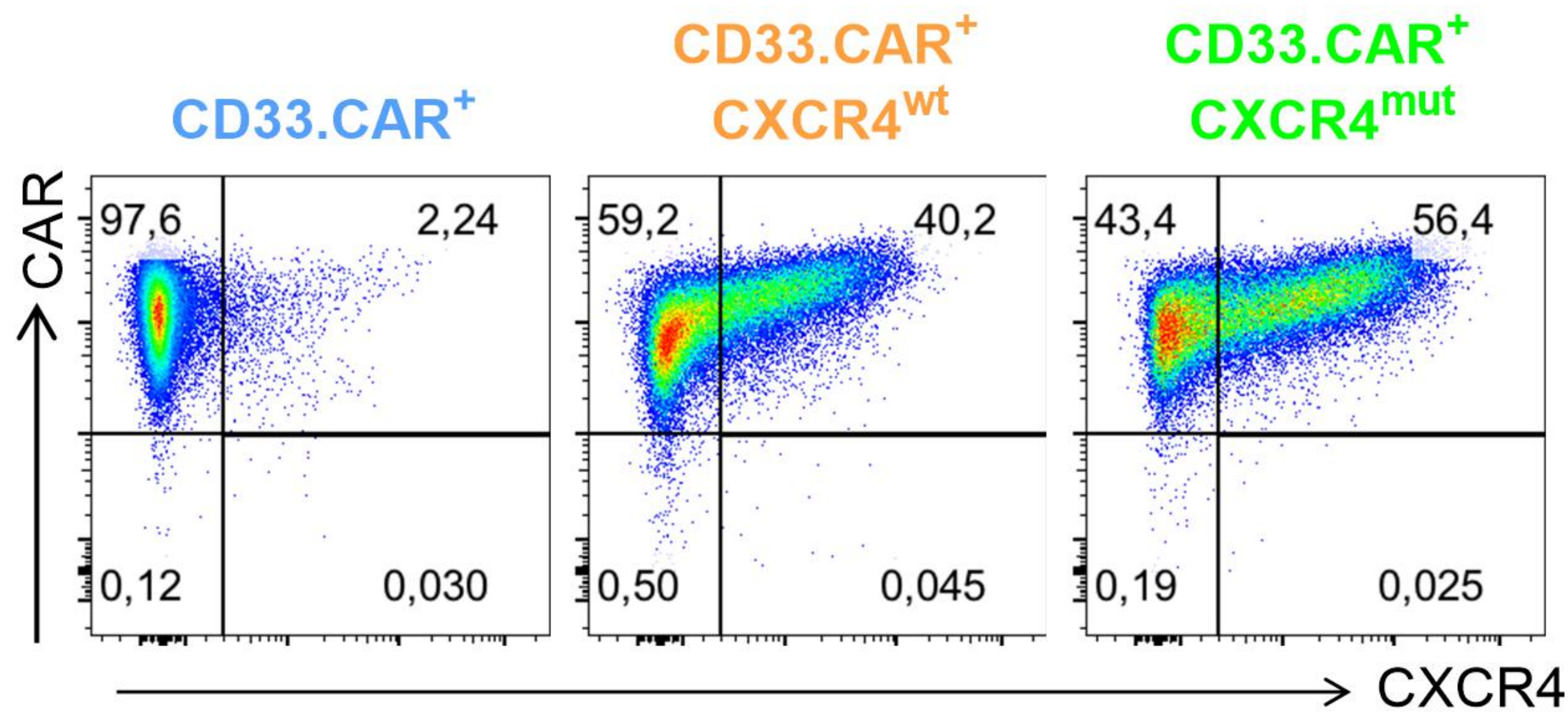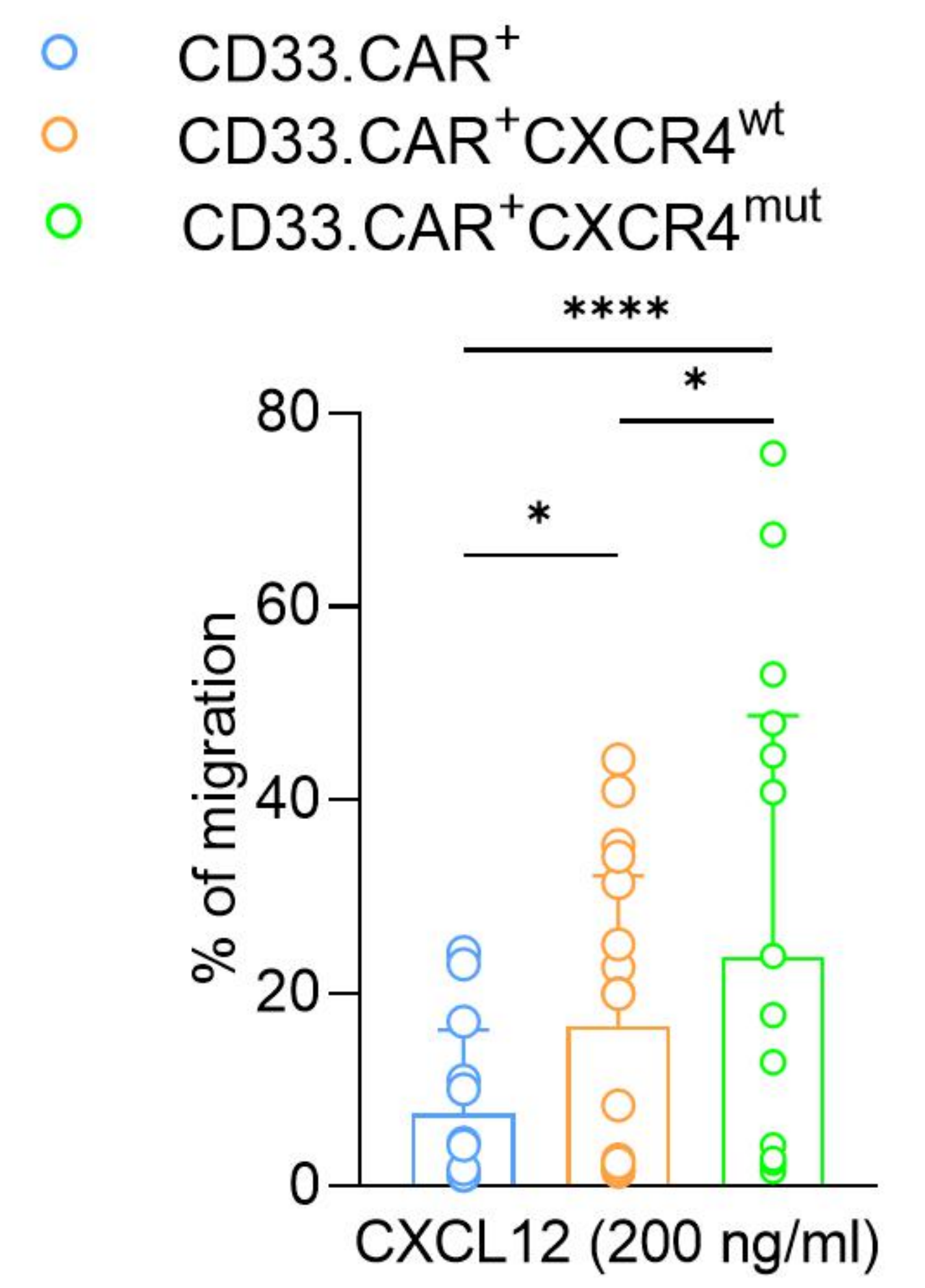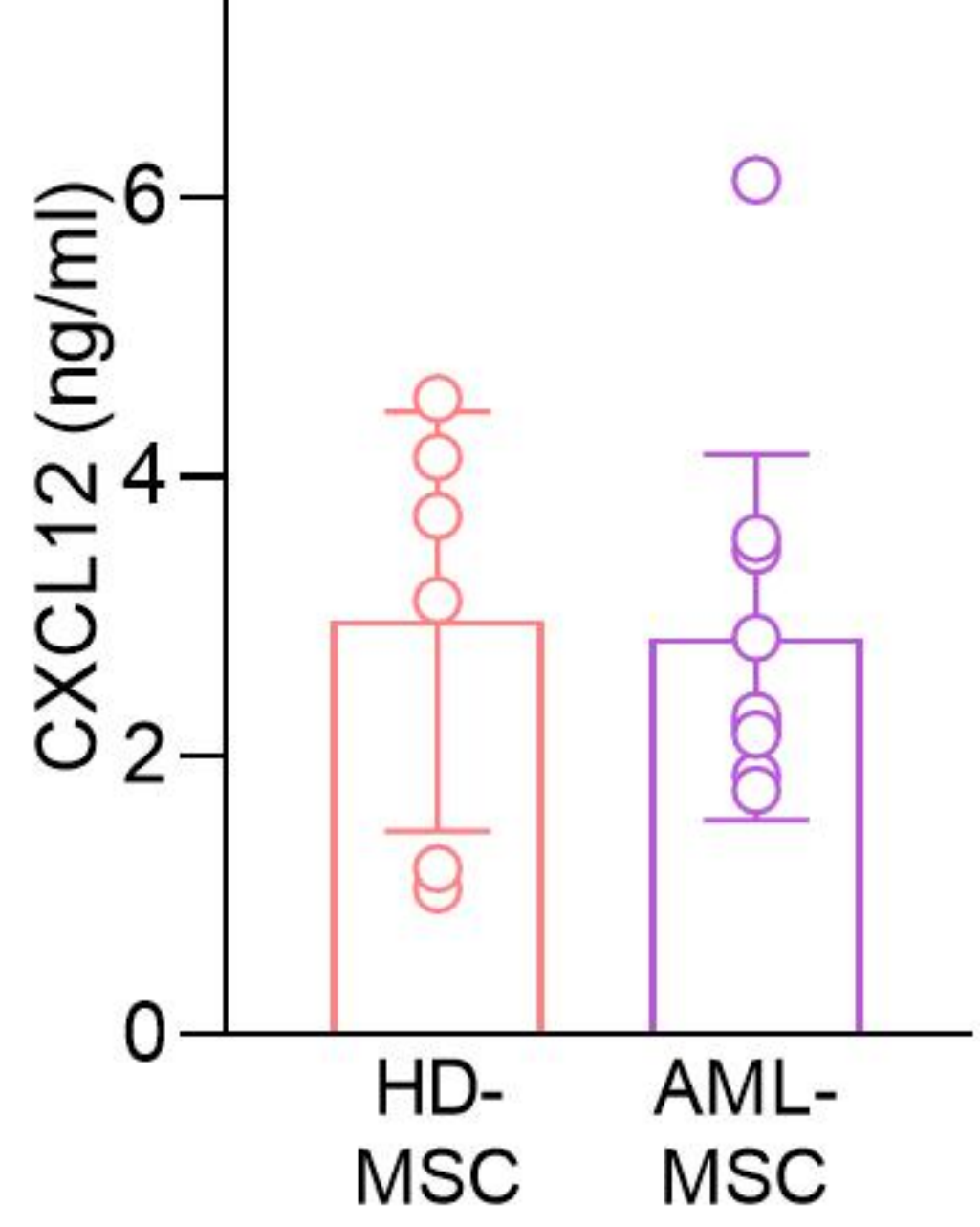
Figure 1

Figure 2

Figure 3

Figure 4

A

CD33.CAR⁺
CD33.CAR⁺CXCR4ʷᵗ
CD33.CAR⁺CXCR4ᵐᵘᵗ
i.v. 10⁷ cells

KG-1 i.v.
5x10⁶ cells

FACS analysis

Day 0   +14   +39

B

UNTR   CD33.CAR⁺   CD33.CAR⁺CXCR4ʷᵗ   CD33.CAR⁺CXCR4ᵐᵘᵗ

mCD45

76,7   4,60   0,031   0,51

hCD33

C bone marrow

○ UNTR   ○ CD33.CAR⁺CXCR4ʷᵗ
○ CD33.CAR⁺   ○ CD33.CAR⁺CXCR4ᵐᵘᵗ

% of hCD33⁺

hCD33⁺cell number/BM (x10³)

D spleen

% of hCD33⁺

hCD33⁺ cell number/spleen (x10⁴)
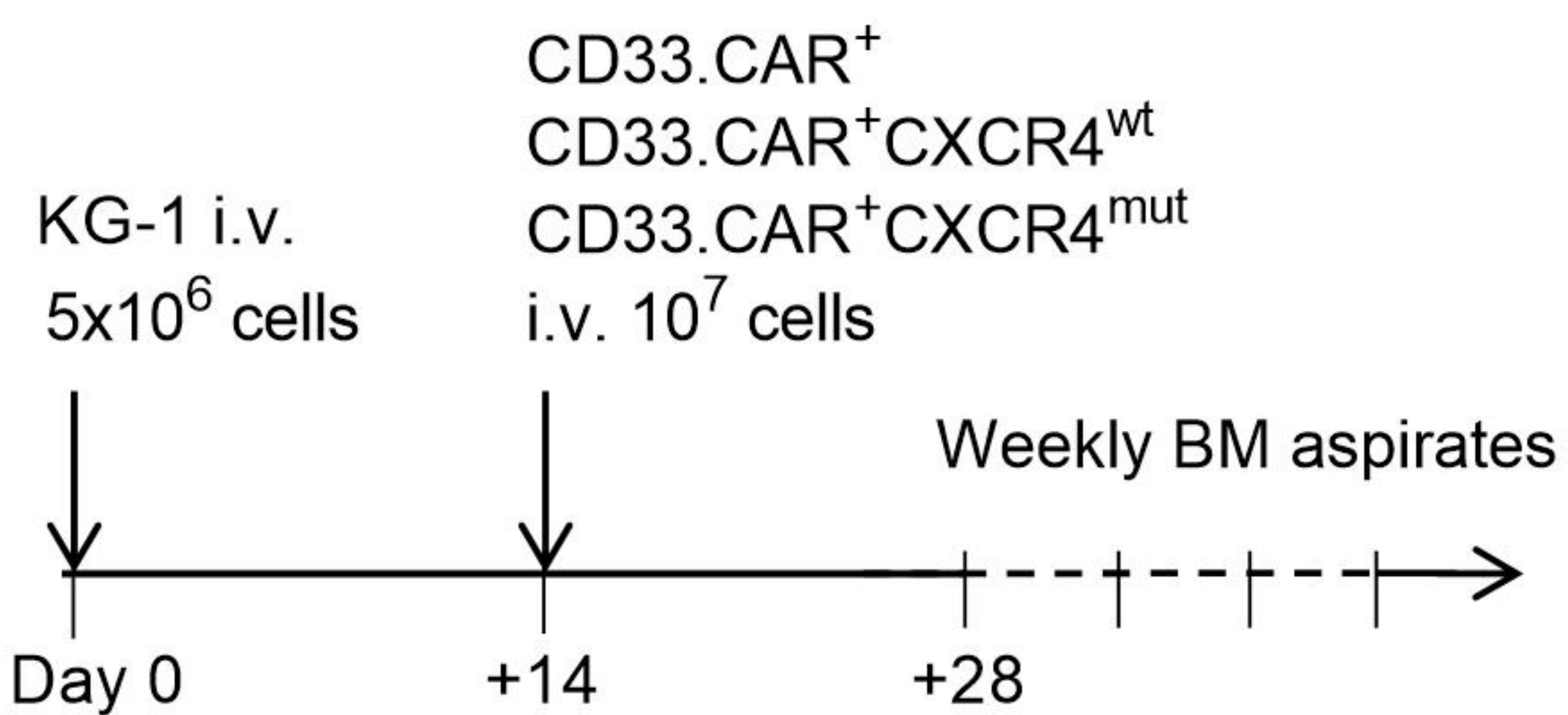
peripheral blood

% of hCD33⁺

hCD33⁺cell number/µl blood

E

CD33.CAR⁺
CD33.CAR⁺CXCR4ʷᵗ
CD33.CAR⁺CXCR4ᵐᵘᵗ
i.v. 10⁷ cells

KG-1 i.v.
5x10⁶ cells

Weekly BM aspirates

Day 0   +14   +28

F

% of hCD33⁺

Days after KG-1 infusion

G

CD33.CAR⁺CXCR4ʷᵗ vs CD33.CAR⁺: p =0.01
CD33.CAR⁺CXCR4ʷᵗ vs CD33.CAR⁺CXCR4ᵐᵘᵗ: p =0.01

p = 0.0001

Probability of Survival

Days after KG-1 infusion

H

% of conjugated migration