Generation and proof-of-concept for allogeneic CD123 CAR-Delta One T (DOT) cells in acute myeloid leukemia

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ABSTRACT
Background Chimeric antigen receptor (CAR)-T cells have emerged as a breakthrough treatment for relapse/refractory hematological tumors, showing impressive complete remission rates. However, around 50% of the patients relapse before 1-year post-treatment. T-cell ‘fitness’ is critical to prolong CAR-T persistence and activity. Allogeneic T cells from healthy donors are less dysfunctional or exhausted than autologous patient-derived T cells; in this context, Delta One T cells (DOTs), a recently described cellular product based on MHC/HLA-independent VΔ1-γδ T cells, represent a promising allogeneic platform.

Methods Here we generated and preclinically validated, for the first time, 4-1BB-based CAR-DOTs directed against the interleukin-3γc chain receptor (CD123), a target antigen widely expressed on acute myeloid leukemia (AML) blasts.

Results CD123CAR-DOTs showed vigorous, superior to control DOTs, cytotoxicity against AML cell lines and primary samples both in vitro and in vivo, even on tumor rechallenge.

Conclusions Our results provide the proof-of-concept for a DOT-based next-generation allogeneic CAR-T therapy for AML.

INTRODUCTION
Immunotherapy has promoted marked improvements in cancer treatment over the last decade. The immune system offers a wide range of alternatives, including cytotoxic lymphocytes—native or engineered—to eliminate treatment-resistant tumors. In this context, CD19-directed chimeric antigen receptor (CAR)-transduced T cell (CAR-T) therapies have shown impressive rates of complete remissions (CR) in relapse/refractory (r/r) B-cell malignancies, especially in B-cell acute lymphoblastic leukemia (ALL).1 2 However, 1-year progression-free survival remains ~50% due to frequent relapses.3 4 Antigen loss and phenotypic escape as well as CAR-T cell disappearance, dysfunctionality or exhaustion are commonly responsible for such failures.5 6 Thus, new therapeutic options based on ‘fitter’ effector T cells are being actively investigated in order to increase T-cell persistence and efficacy.

Allogeneic cytotoxic cells obtained from healthy donors (HD) are among the most attractive avenues for ‘off-the-shelf’ CAR-T cell therapies. In particular, MHC/HLA-independent γδ T cells have emerged as a promising candidate based on their high cytotoxic activity, stimulatory cytokine release and recruitment of other immune cells to the tumor site.7 8 Our data demonstrate the capacity of CAR-Delta One T cells (DOTs) to be a disruptive ‘off-the-shelf’ allogeneic cellular immunotherapy for acute myeloid leukemia.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ Allogeneic cytotoxic cells obtained from healthy donors are a very interesting alternative for next-generation ‘off-the-shelf’ chimeric antigen receptor (CAR)-T cell therapies. In particular, MHC/HLA-independent γδ T cells have emerged as a promising candidate based on their high cytotoxic activity, stimulatory cytokine release and recruitment of other immune cells to the tumor site.

WHAT THIS STUDY ADDS
⇒ Our data demonstrate the capacity of CAR-Delta One T cells (DOTs) to be a disruptive ‘off-the-shelf’ allogeneic cellular immunotherapy for acute myeloid leukemia.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ CAR-DOTs exhibit a combination of properties that may change allogeneic cell immunotherapy of hematological (and probably solid) tumors in the future.
preclinical models of solid and hematological malignancies,16 most notably acute myeloid leukemia (AML).14

AML, the most common acute leukemia in adults, is characterized by the accumulation of differentiation-defective immature and proliferative myeloid blasts in the bone marrow (BM) and PB.13 Unfortunately, patient overall survival has not improved significantly over the last decades, with relapses being frequent and presenting poor outcome, thus requiring hematopoietic stem cell transplantation as rescue therapy.16 Despite its molecular enormous heterogeneity,15 17 18 CD123, the α chain of the interleukin (IL)-3 receptor, is consistently expressed in >90% of AML blasts, in similar levels to leukemic stem cells, and its expression is maintained at relapse.19 20 Based on this rational, we previously showed robust efficacy of second generation (4-1BB-based) CD123-directed CAR-T cells in preclinical AML models21

Clinically implemented CAR-T cell approaches are based on abundant αβ T cells22–24 which have limited applicability in the allogeneic setting due to their MHC/HLA restriction and graft-versus-host potential.25 Importantly, immunological BM dysfunction and T-cell exhaustion has been reported in intensively chemotherapy-treated r/r AMLs.26 Thus, safety allowing, allogeneic T cells from HDs would represent an ‘off-the-shelf’ cost-efficient therapy to eliminate r/r AML. Building on these foundations, we prompted to explore the potential of MHC/HLA-independent DOT cells as vehicle for our second generation CD123CAR in preclinical AML models.

Here we describe, for the first time, the generation of CAR-expressing DOT cells (CAR-DOTs) and provide the proof-of-concept for their application in AML treatment. Retrovirally-transfected CD123CAR-DOTs potently eliminated AML cell lines and primary samples both in vitro and in vivo, increasing the efficacy of ‘naked’ (mock-transduced) DOTs in different models and conditions, without phenotypic alterations. Moreover, CD123CAR-DOTs released type 1 (antitumor) cytokines and chemokines on exposure to primary AML cells. In in vivo rechallenge experiments, CD123CAR-DOTs persisted and remained functional against AML, 9 weeks after their infusion into mice. IL-15 significantly improved their in vivo efficacy by sustaining longer action and enabling one-single-dose treatment. Our data demonstrate the potential of CAR-DOTs to represent a disruptive ‘off-the-shelf’ allogeneic cellular immunotherapy for AML.

**METHODS**

**DOT cell generation**

PB mononuclear cells (PBMCs) were isolated fromuffy coats from HDs by Ficoll-Hypaque gradient centrifugation. Buffy coats were obtained from the Barcelona Blood and Tissue Bank on Institutional Review Board-approval (HCB/2018/0030). PBMCs were incubated with anti-αβTCR Biotin mAb (Miltenyi Biotec) followed by incubation with anti-Biotin mAb microbeads to deplete αβ T cells by magnetic separation using autoMACS under depleteS protocol (Miltenyi Biotec, Biergisch Gladbach, Germany). DOT cells were generated from αβ-depleted PBMCs cultured in either 96-well plates or G-REX platform (Wilson Wolf Manufacturing), based on an adaptation from our previous protocol.10 11 Briefly, αβ-depleted PBMCs were resuspended in OpTmizer-CTS medium supplemented with 2.5% heat-inactivated human plasma (LifeSciences), 2 mmol/L L-glutamine (Thermo Fisher) and 50 U/mL/50 μg/mL of penicillin/streptomycin (Thermo Fisher) and cultured for 16–20 days. Animal-free human cytokines rIL-4 (100 ng/mL), rIFNγ (70 ng/mL), rIL-2 (7 ng/mL), and rIL-1β (15 ng/mL; all from PeproTech), and a soluble mAb anti-CD3 (clone OKT3, 140 ng/mL; BioLegend), were added to the medium at day 0. At day 7, cultures were supplemented with anti-CD3 (clone OKT3, 1 μg/mL or 2 μg/mL for 96-well plate protocol), rIL-21 (13 ng/mL) and rIL-15 (70 ng/mL; also from PrepoTech). On day 11, new medium was added to cultures, supplemented with anti-CD3 (1 μg/mL) and rIL-15 (100 ng/mL). Cells were incubated at 37°C and 5% CO2. DOT cells were harvested at the end of the culture and either used fresh (for in vitro assays) or cryopreserved (for in vivo experiments) in OpTmizer media+20% human plasma+10% DMSO, and stored in liquid nitrogen.

**CD123-CAR retroviral production and transduction**

To construct the retroviral transfer vector, the complete CD123-directed CAR (including CSL362 scFv, CD8 transmembrane domain, 4-1BB costimulatory domain, CD3ζ endodomain, and a T2A-eGFP cassette) previously described27 was cloned into an SFG retroviral backbone. The SFG vector expressing eGFP alone (mock vector) was used as a control. Viral particles pseudotyped with RD114 were generated using 293 T cells with GeneJuice transfection reagent (Sigma-Aldrich, Saint Louis, Missouri, USA) following manufacturer’s instructions with a 1:5:1:5:1 μg ratio of the SFG:Peq-Pam:RD114 DNA plasmids and concentrated using Retro-X Concentrator (Takara, Kusatsu, Japan) following manufacturer’s instructions. DOT cells were expanded for 7 days prior to viral transduction. Proper CAR transduction was checked by flow cytometry by eGFP expression and by using AffiniPure F(ab’)2 Fragment Goat Anti-Human IgG (H+L) (Jackson ImmunoResearch Laboratories). Other conditions tested during transduction optimization of DOT cells included the use of lentiviral particles as previously reported27 at multiplicities of infection between 10 and 50, as well as with retroviral particles with RetroNectin (Takara) following manufacturer’s instructions. All lentiviral transduction conditions were tested at days 7 and 11 of the DOT cell expansion protocol.

**Immunophenotyping of DOT cells, cell lines and primary AML samples**

Proper differentiation and activation of DOT cells was confirmed by surface staining with CD3-PB (UCHT1), CD3-APC-Gy7 (UCHT1), TCRγδ-PE (B1), TCRαβ-APC
(IP26), CD4-APCγγ7 (RPA-T4), CD8-BV510 (RPA-T8), CD25-APCγγ7 (BC96), CD69-BV510 (FN50), NKP30-BV421 (P30-15), NKG2D-BV510 (1D11), CD45RA-APC (HI100), CD27-PB (O323), CD62L-BV510 (DREG-56), DNAM-1-BV510 (11A8) from BioLegend (San Diego, California, USA) and TCR Vδ CD45RA- NKp30- Human Cytokine Antibody Array 4000 from RayBiotech. DOT/mock- The 7–12 weeks old non-AML patient-derived xenograft models In vivo samples. CD123 + AML blasts (primograft-ences). Cytokine production was measured by Gmined using Trucount absolute count beads (BD Biosciences). primar and housed under pathogen- animal facility of the Barcelona Biomedical Research Sánchez Martínez D, et al. J Immunother Cancer 2022;10:e005400. doi:10.1136/jitc-2022-005400Statistical analysis

Data from at least three individual donors are shown in all figures. All p values were calculated by unpaired two-tailed or one-tailed Student’s t test using Prism software (GraphPad). A p value *<0.05/***<0.01/****<0.001/*****<0.0001 was considered statistically significant.

RESULTS

CD123-directed DOT cells (CD123CAR-DOTs) expand robustly while preserving DOT phenotype

We set out to generate, for the first time, CAR-DOT cells, and purpose them for AML targeting. DOT cells were expanded and activated as described, and transduction was tested at days 7 and 11 using different retroviral (PeqPam/RD114) and lentiviral (VSV-G/pSAX2) conditions (online supplemental figure S1). Retro-X Concentrator at day 7 showed the highest rate of anti-CD123CAR expression and was chosen for downstream generation of CD123CAR-DOTs (figure 1A). CD123CAR transduction/expression did not impact γδ TCR and Vδ1+ T cell percentages, which were ~97% and ~70%, respectively, as in the original ‘naked’ DOT product. Likewise, total and Vδ1+ T-cell numbers at the end of the process were similar for mock-DOTs and CAR-DOTs (figure 1C). Retroviral particles infected the Vδ1+ and Vδ1γδ T-cell populations in similar proportions, and the CD123CAR expression as determined by GFP positivity correlated well with anti-ScFv labeling (figure 1B,C). CAR+ DOTS (n=14) presented a similar T-cell differentiation stage to CAR+ DOTS, as demonstrated by CD62L/CD45RA staining by FACS. The majority of DOTs were effector T cells (Teff), with an expected degree of variability between donors, followed by effector memory (Teff) cell numbers at the end of the process were similar for mock-DOTs and CAR-DOTs (figure 1C). Retroviral particles infected the Vδ1+ and Vδ1γδ T-cell populations in similar proportions, and the CD123CAR expression as determined by GFP positivity correlated well with anti-ScFv labeling (figure 1B,C). CAR+ DOTS (n=14) presented a similar T-cell differentiation stage to CAR+ DOTS, as demonstrated by CD62L/CD45RA staining by FACS. The majority of DOTs were effector T cells (Teff), with an expected degree of variability between donors, followed by effector memory (Teff) DOTs,
Figure 1  CD123CAR-DOT cell generation and phenotypic characterization. (A) CD123CAR-DOT cell production scheme. αβ-depleted PBMCs were cultured under OKT3 antibody and cytokine stimulation for 3 weeks and transduced with γ-retroviruses at day 7. (B) Upper panels, γδ-TCR+ and Vδ1+ T cell percentages evolution from day 0 to end-product for mock DOTs and CAR-DOTs. Lower panels, representative FACS dot plots depicting the expression of Vδ1/Vδ2 and Vδ1/CD3 in end-products. (C) Upper panels, cell number for total cells and Vδ1+ T cells along the CAR-DOT generation process. Lower panels, representative FACS dot plot for Vδ1/GFP and anti-ScFv/GFP expression. (D) Left-middle panels, scheme and representative FACS plot depicting TCM, TNSCM, TEM, TEFF T-cell subsets as defined by CD62L versus CD45RA staining. Right panel, percentage of TCM, TNSCM, TEM, TEFF T-cell subsets for CAR’DOT and CAR DOT subpopulations (n=14). (E) NKG2D, NKP30, NKP44, DNAM-1 and PD-1 expression (n=10), and (F) perforin (Pfr), granzyme A (GzmA) and granzyme B (GzmB) expression (n=6) in CAR DOT versus CAR’DOT cells. CAR, chimeric antigen receptor; DOT, Delta One T cells; PBMCs, peripheral blood mononuclear cells; TCM, central memory; TEM, effector memory; TNSCM, naive/stem central memory.
and lower contributions of central memory (T_{CM}) and naïve/stem central memory (T_{N/SCM}) DOTs (figure 1D). Furthermore, both CAR' and CAR' DOTs expressed their natural killer (NK) cell receptor signature according to the published hierarchy: NKG2D (90%) > DNAM-1 (>60%) > Nkp30 and Nkp44. By contrast, the immune checkpoint programmed cell death protein-1 (PD-1) was strikingly absent from both DOT populations (figure 1E). The expression of the key components of cytotoxic granules, perforin, granzyme (Gzm) A and GzmB, was also not affected by CAR transduction (figure 1F). Collectively, these data demonstrate the feasibility of CAR-DOT generation, and further show that retroviral infection and CD123CAR expression on the cell surface does not modify DOT expansion properties or phenotype, thus generating NK-like cytotoxic effector γδ T cells highly enriched in Vδ1’ T cells.

**CD123CAR-DOTs specifically augment cytotoxicity against AML cell lines and primary blasts in vitro**

In order to test the anti-AML activity of CAR-DOTs, we performed in vitro cytotoxicity assays against the CD123' AML cell lines MOLM13 and THP-1, and the CD123' T-cell ALL line Jurkat, as negative control. CD123CAR-DOTs specifically eliminated CD123' AML cells in an E:T ratio-dependent manner, with substantially increased cytotoxic activity compared with mock-DOTs. Even at low E:T ratios (1:16, 1:8, 1:4) we achieved 20–60% more AML cell lysis than with mock-DOTs in 48 hours and 24 hours assays (figure 2, online supplemental figure S2). Importantly, CD123CAR-DOTs showed similar potency to conventional CD123CAR-Ts (online supplemental figure S3), thus allowing us to concentrate on the allogeneic advantages of CD123CAR-DOTs. Next, to evaluate their capacity to kill primary tumors, CD123CAR-DOTs were co-cultured with primary CD123' AML samples, with different percentages of CD123'CD33' blasts (figure 2B). CD123CAR-DOTs showed enhanced cytotoxicity over mock-DOTs against CD123' AML primary blasts in 48 hours assays (figure 2C). Moreover, compared with mock-DOTs, CD123CAR-DOTs produced significantly higher levels of master antitumor mediators, namely the cytokines tumor necrosis factor-α and interferon-γ, as well as IL-13, recently implicated in orchestrating tumor surveillance in mice. The T-cell costimulator 4-1BB, a major determinant of γδ T-cell-mediated tissue surveillance; the signal transducer Axl, shown to maximize IL-15R signaling in human NK cell differentiation; the myeloid differentiation factor, granulocyte-macrophage colony-stimulating factor (GM-CSF); and the chemokines MIP-1α (CCL3), CCL5, CXCL13 and lymphotactin (XCL1), all important in mobilizing multiple leukocyte subsets in cancer immunity. While not significantly augmented, IL-2 and FasL, key mediators of T-cell proliferation and cytotoxicity, respectively, also showed a tendency for higher expression in CD123CAR-DOT cells (figure 2D). These data demonstrate an enhanced effector potential of CD123CAR-DOT cells in direct comparison with naked/mock-DOT cells.

**Serial infusions of CD123CAR-DOTs exhibit robust anti-leukemic effect in vivo**

CD123CAR-DOT function was evaluated in vivo by employing Luc-expressing AML-PDX^{LUC} NSG mice were transplanted with 2.5×10^5 AML-PDX^{LUC} cells and, based on our previous extensive experience with DOTs in AML xenograft models, infused with 10×10^6 (CARDOTs or mock-DOTs) DOTs in three serial infusions (one injection per week (day 7, 14, 20), starting 1 week post-tumor injection), and leukemia progression was followed by BLI (figure 3A). CD123CAR-DOTs controlled AML tumor burden between days 20 and 47 significantly better than mock-DOTs which only managed to delay tumor growth and could not prevent an eventual logarithmic tumor growth from day 47 onwards (figure 3B). At this point, we decided to provide an additional fourth infusion at day 53, which resulted in complete control of leukemia in CD123CAR-DOT mice, in stark contrast with continued tumor growth in mock DOT-treated mice (figure 3B). Furthermore, FACS analyses of PB and BM samples collected at days 47 and 67 confirmed the BLI data. Leukemic burden, as determined by HLA-ABC/CD45/CD123/CD33 expression, showed high, intermediate and absent blast cells in untreated, mock-DOT and CAR-DOT-treated mice, respectively (figure 3C). These data highlight the therapeutic advantage over 12 weeks of CD123CAR-DOTs over mock-DOTs in in vivo AML-PDX models.

**Provision of IL-15 supports single-dose CD123CAR-DOT activity in vivo**

Having achieved the initial proof-of-concept for CD123CAR-DOT activity in AML xenografts, we next aimed to maximize it through provision of key DOT survival factors, particularly since the murine milieu lacks human cytokines. Given that the DOT protocol relies on IL-15 as the critical cytokine in the second stage of expansion/differentiation, we combined CD123CAR-DOTs with intraperitoneal IL-15 administration every 3–4 days. In this set of experiments, NSG mice were transplanted with 2.5×10^5 AML-PDX^{LUC} cells, and starting 1 week later, they received one, two or three doses of 10×10^6 CD123CAR-DOTs, with or without IL-15 injection (figure 4A). Leukemia engraftment was followed weekly by BLI and by flow cytometry analysis of BM and PB samples. In the absence of exogenous IL-15, three doses of CD123CAR-DOT (CAR3) were clearly more efficacious than one (CAR1) or two (CAR2) doses, since these only managed to delay tumor burden (compared with untreated control mice) (figure 4B,C). Strikingly, the provision of IL-15 every 3–4 days overrode all such differences, thus allowing complete leukemia control 60 days after even with a single CD123CAR-DOT infusion (figure 4B,C). These results demonstrate a critical
Figure 2  CD123CAR-DOT cells specifically target and eliminate CD123+ AML cell lines and primary samples in vitro. (A) Cytotoxicity of CAR-DOTs and mock-DOTs against CD123+ AML (MOLM13 and THP-1) and CD123− T-ALL (Jurkat, negative control) cell lines at the indicated E:T ratios in 48 hours assays (n=5). Small insets, CD123 expression in each cell line. (B) AML primary sample (Pt, patient) phenotype showing CD33/CD123 analysis by FACS. AML blasts are highlighted in blue. (C) Absolute counts of alive eFluor+CD123+ AML blasts measured by FACS in 48-hour cytotoxicity assays at 1:1/2:1 E:T ratios. (D) Cytokine array determination in supernatants obtained from four donors of CAR-DOTs/mock-DOTs exposed to two different AML primary samples for 48 hours. Relative expression was normalized to mock-DOT levels. *p<0.05, **p<0.01, ***p<0.001.

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CAR, chimeric antigen receptor; DOTs, Delta One T cells; E:T, Effector:Target.
Figure 3  CD123CAR-DOT cells fully control growth of primary CD123+ AML blasts in a PDX model. (A) Schematic of the AML-PDX model. NSG mice (n=4/group) were intravenously injected with 2.5×10⁵ AML-PDXLUC cells and then received three intravenous injections (one per week, starting 7 days after tumor injection) of 10×10⁶ CAR-DOT or mock-DOT cells. Tumor burden was monitored weekly by BLI using IVIS imaging. At day 53, mock-treated and DOT-treated mice received an extra infusion of 10×10⁶ mock-DOTs or CAR-DOTs, respectively. (B) IVIS imaging of tumor burden monitored by BLI at the indicated time points (scale: 5×10⁴-1×10⁷). Right panel shows the total radiance quantification (p/sec/cm²/sr) at the indicated time points. †: sacrifice. (C) Tumor burden analyzed by FACS in PB and BM at days 47 and 67. Primary AML blasts are shown in blue. Mouse cells are shown in black. *p<0.05, **p<0.01, ***p<0.001. AML, acute myeloid leukemia; BLI, bioluminescence; BM, bone marrow; CAR, chimeric antigen receptor; DOTs, Delta One T cells; PB, peripheral blood; PDX, patient-derived xenograft.
Adjuvant effect of exogenous IL-15 on CD123CAR-DOT therapy in AML-PDX in vivo.

Daily provision of IL-15 maximizes DOT-cell therapeutic efficacy in vivo

Building on the observed major impact of IL-15 on CD123CAR-DOT activity, we decided to evaluate side-by-side how IL-15 provision, in various regiments, would boost single-dose CD123CAR-DOT versus mock-DOT activities in vivo. In this experimental design, CD123CAR-DOTs or mock-DOTs were infused (in a single-one dose) 1 week after intravenous injection of $2.5 \times 10^5$ AML-PDX$^{LUC}$ cells, followed by three different IL-15 administration regimens: daily, every 3–4 days, or none at all (figure 5A). Strikingly, mock-DOTs plus daily IL-15 showed similar leukemia
Figure 5  Daily-infused IL-15 increases DOT cell persistence and enables elimination of primary CD123+ AML blasts in a PDX model. (A) Schematic of the AML-PDX model. NSG mice (n=4/group) were intravenously injected with 2.5×10⁵ AML-PDX LUC cells followed 7 days after by a one-single dose intravenous injection of 10×10⁶ CAR-DOTs or mock-DOTs. Mock-DOT and CAR-DOT-treated mice were followed in the absence or presence of IL-15, intraperitoneal administered either daily or every 3–4 days. (B) IVIS imaging of tumor burden monitored weekly by BLI at the indicated time points (scale: 5×10⁴–1×10⁶). (C) Total radiance quantification (p/sec/cm²/sr) at the indicated time points. (D) Tumor burden was monitored by FACS in BM at day 30. (E) Dot plots of representative Vδ1+ and Vδ2+ T cells determined in CAR-DOT/ mock-DOT-treated mice in BM at day 30. (F) Total γδ T cells were monitored by FACS in BM at day 30. AML, acute myeloid leukemia; BLI, bioluminescence; BM, bone marrow; CAR, chimeric antigen receptor; DOTs, Delta One T cells; IL, interleukin; PB, peripheral blood; PDX, patient-derived xenograft.
control to all groups of CD123CAR-DOT-treated mice, as evaluated by BLI up to day 42 (figure 5B,C). The daily provision of IL-15 was critical to enhance mock-DOT cell activity, since the 3–4 day IL-15 regimen barely reduced tumor burden compared with control groups (figure 5B,C). These data were consolidated by flow cytometry analysis of BM aspirates, which showed that AML cell engraftment correlated with IVIS imaging (figure 5D). Importantly, the infused (CAR- or mock-) DOTs were only detected in the BM at the endpoint of the experiment when daily IL-15 was administered, thus highlighting the importance of in vivo persistence of the T$_{19F}$ (figure 5E,F). These data unveil the importance of in vivo IL-15 administration regimens on DOT-based therapies in AML.

**Optimized CD123CAR-DOT treatment controls AML growth on in vivo rechallenge**

Persistence is a key goal and a major challenge of adoptive cellular immunotherapy. Aiming to determine the sustained functionality and potency of (CAR- or mock-) DOTs after >40 days of controlling AML progression, we then performed a tumor rechallenge experiment. Disease-free mice that eliminated the previous AML graft (figure 5), either under all CD123CAR-DOT treatments or under mock-DOTs-daily IL-15, were rechallenged with an additional AML-PDX$^{LUC}$ intravenous infusion at day 47 (figure 6A). In stark contrast to the other groups, CD123CAR-DOTs plus daily IL-15 was the only condition in which tumor growth kept controlled; in fact, AML cells were barely detectable by BLI or FACS analysis of PB and BM samples (figure 6B–E). These results firmly demonstrate a vigorous and persistent anti-leukemia effect of CD123CAR-DOTs plus daily IL-15, sustained over 70 days and even on tumor rechallenge, thus providing seminal proof-of-concept for their application for adoptive cellular immunotherapy in AML.

**DISCUSSION**

In the last few years, treatments for hematological neoplasias have undergone a revolution due to immunotherapy. In B-cell malignancies, autologous CAR-T cell therapies have rescued r/r patients on the path to palliative care, obtaining very impressive CR rates. The number of CAR-T clinical trials keeps increasing, aiming to implement alternatives for bad prognosis tumors and expanding their horizons to other types of diseases (HIV, COVID-19, autoimmune diseases, cardiac damage, etc.). However, CAR-T cells are still at the beginning of their clinical implementation, and a significant fraction (around 50%) of the patients relapse after treatment in a relative short period of time. Factors like the low number of T cells retrieved from the patient’s leukapheresis, tumor blast contamination in the starting material, or T-cell exhaustion in multitreated patients, can all explain autologous CAR-T failures stemming from the manufacturing process.

The BM of patients with leukemia is exposed to several cycles of chemotherapy and immunosuppressants, which alter the status of normal T cells. Particularly in patients with AML, T cells have been characterized with an exhausted phenotype at relapse. In this context, allogeneic T cells represent an attractive alternative, since circulating T cells from HDs have not suffered disease-induced or therapy-induced dysfunction. While various groups have reported the generation of allogeneic αβ T cells, these cells must undergo genetic manipulation, generally CRISPR/Cas9 editing, to eliminate the endogenous T-cell receptor (TCR) since it would otherwise drive a (potentially severe) graft-versus-host reaction. Elegant studies have reported the feasibility of these TCR$^{30}$ CAR-T cells in preclinical models and even in pediatric patients. However, a relevant recent paper has reported that TCR elimination in CAR-T cells leads to a decrease in persistence. Furthermore, from a clinical point-of-view, CRISPR/Cas9 handling would still have to solve complex regulatory requirements.

Since the vast majority of γδ T cells are HLA-independent, they do not require genetic manipulation to be used as allogeneic therapies. It was with this purpose that we previously developed DOT cells, a Vδ1 T-cell-enriched cell product characterized by the upregulation of multiple activating/cytotoxicity-associated NK-cell receptors (NKG2D, DNAM-1, Nkp30, Nkp44), and showed their anti-leukemic potential. However, our previous data in AML xenograft models left a significant margin for improvement in terms of control of tumor burden. Here we investigated whether DOT transduction with a CD123-directed CAR would improve efficacy and lead to complete and durable leukemia remissions. We show that, compared (side-by-side) with control (mock-)DOTs, CAR123-DOTs are substantially more potent at eliminating AML blasts (both cell lines and primary samples) in vivo, release increased levels of key cytokines involved in immune orchestration and amplification of the antitumor response; and are more efficient at controlling tumor growth in vivo, in AML-PDX experiments conducted over 70 days.

We also found that on administration of IL-15 in our AML-PDX models, CD123CAR-DOTs (and also mock-DOTs) persisted better, thus allowing a single dose to maintain mice free of leukemia. Critically, daily-infused IL-15 enabled CD123CAR-DOTs to sustain their anti-leukemic activity for more than 70 days, even after AML rechallenge. These results are in agreement with recent elegant publications showing CAR-Vδ1 T-cell efficacy and the adjuvant effect of IL-15 in B-cell lymphoma and hepatocellular carcinoma models. Of note, our study is the first to compare CAR-transduced with mock-transduced Vδ1 T cells, thus being able to ascertain the increased potency provided by CAR targeting. Only the clinical setting can establish the real benefit of IL-15 co-administration (potentially with an inducible ‘switch off’ system) with DOTs or CD123CAR-DOTs. Obviously, our (and other) preclinical mouse xenograft models lack...
endogenous human IL-15; thus, basal circulating IL-15 levels in patients could suffice to sustain (CD123CAR-) DOT persistence and activity/efficacy in the clinical setting. Importantly, lymphodepletion regimens that are used prior to CAR T-cell infusions were shown to increase the levels of IL-15 in patients. Therefore, although described as safe in non-human primates, the need for exogenous (adjuvant) IL-15 should be carefully examined since it caused some toxicities in a recent clinical trial in patients with cancer. This notwithstanding, this clinical trial employed a continuous bolus of IL-15 as single therapy, and thus, lower and/or intermittent IL-15 dosing may be a promising solution.
An important final aspect to discuss is the potential advantage of using DOTs as effector cells for CAR transduction. Compared with conventional αβ-dominated T-cell products, DOTs offer several advantages: first and foremost, the previously mentioned suitability for allogeneic use, without any need for additional genetic engineering, and without any loss in potency (online supplemental figure S3). Additionally, the immune checkpoint PD-1 is absent in DOTs and CD123CAR-DOTs (figure 1E), whereas the upregulated NK-associated cytotoxicity machinery (including DNAM-1, Nkp30 and Nkp44) may be critical on CAR antigen loss as observed with CD19 in B-ALL. Importantly, this enhanced NK-like cytotoxicity is also an advantage over other γδ T cell-based products being developed for adoptive immunotherapy of cancer. On the other hand, in comparison with NK cells, which obviously share such activating NK-cell receptors, DOTs offer as key advantages the previously documented absence of inhibitory KIRs, namely KIR2DL1, KIR2DL2, KIR2DL4, KIR2DL5A and KIR3DL1, and the additional input of TCR-dependent activation and expansion (up to very high yields as reported). In fact, the presence of a stochastically recombined and highly polyclonal TCR repertoire, allowing HLA-unrestricted recognition of stress-induced molecules, also contrasts tumor immune evasion (via CAR antigen loss or HLA downregulation, eg). Moreover, the ‘trophic’ (homeostatic) signals received through γδ TCR binding to butyrophilins constitutively expressed in multiple tissues may be beneficial for in vivo persistence, and may underlie the recent description of a striking Vδ1 T cell-associated CD19CAR-T cell expansion (from a very low initial frequency), over 10 years in a patient with disease-free Chronic Lymphocytic Leukemia (CLL). Furthermore, although allogeneic CD19-directed CAR-NK cells have recently shown promising clinical results, their ‘off-the-shelf’ implementation may be difficult due to the reported substantial decrease in viability after the freezing/thawing process, which contrasts with the robustness we observed with CD123CAR-DOTs. We therefore strongly believe that CAR-DOTs combine a set of properties that may revolutionize allogeneic cell immunotherapy of hematological (and possibly also solid) cancers in the near future.

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REFERENCES


SUPPLEMENTARY FIGURES

Figure S1. CD123CAR-DOT transduction protocol establishment. (A) CD123CAR-DOT transduction scheme. The cells were transfected at days 7 or 11 of the DOT protocol. (B) MOI (Multiplicity of infection) 25 and 50 for lentiviruses, RetroNectin and Retro-X Concentrator for retroviruses, were tested at day 7 or 11 to transfect DOT cells.
Figure S2. CD123CAR-DOTs specifically target and eliminate CD123+ AML cell lines in vitro. (A) Cytotoxicity of CAR-DOTs and mock-DOTs against CD123+ AML (MOLM13 and THP-1) and CD123- T-ALL (Jurkat, negative control) cell lines at the indicated E:T ratios in 24-hour assays (n=5). Small insets, CD123 antigen density in each cell line. *p<0.05, **p<0.01, ***p<0.001.
Figure S3. CD123 CAR-DOT cells show similar potency to CD123 CAR-T cells against CD123+ AML cells in vitro. Two cell lines were used as targets cells. Cytotoxicity assays were performed at E:T of 1:2 and 1:8 with readouts at 4h and 24h.
Figure S4. CD123 CAR-DOT cells eliminate CD34+ hematopoietic stem/progenitor cells

*in vitro*. Alive CD34+ cells were quantified with Trucount tubes after 24h of incubation at 2:1 E:T.
Figure S5. FACS analysis of Vδ1 γδT cells in each independent PB donor before and after DOT expansion. Vδ1 vs CD3 labeling at day 0 (A) and day 21 (B) of DOT protocol expansion (Fig 1B).
Figure S6. FACS determination of AML-PDX$^{LUC}$ cells in mock/CAR-DOT-treated mice.

Representative FACS dot plots at the time of sacrifice of four independent mice treated with mock-DOTs or CAR-DOTs + daily IL15. AML-PDX$^{LUC}$ cells were identified as hHLA-ABC$^+$hCD45$^+$hCD33$^+$hCD123$^+$ (blue). Black cells are hHLA-ABC$^-$hCD45$^-$ mouse cells.