


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-3α 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

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.

T cells are being actively investigated in order to increase T-cell persistence and efficacy.

Allogeneic cytotoxic cells obtained from healthy donors (HD) are an especially attractive avenue for ‘off-the-shelf’ next-generation CAR-T cell therapies. In particular, MHC/HLA-independent γδ T cells have emerged as a promising candidate based on their potent cytotoxic activity and release of cytokines stimulating and recruiting other immune cells to the tumor site.^{7–9} While the low abundance of γδ T cells in the human peripheral blood (PB) has hindered their clinical application, recent advances in protocol development have enabled their *ex vivo* expansion to large numbers.^{10–13} In particular, we have characterized Delta One T (DOT) cells, a Vδ1 T cell-enriched cellular product expressing enhanced levels of natural cytotoxicity receptors, and demonstrated that they constitute a safe and efficient effector platform to eliminate cancer cells in *in vitro* and *in vivo*



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preclinical models of solid and hematological malignancies,¹⁰ 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.¹⁵ 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 rationale, we previously showed robust efficacy of second generation (4-1BB-based) CD123-directed CAR-T cells in preclinical AML models.²¹

Clinically implemented CAR-T cell approaches are based on abundant $\alpha\beta$ T cells^{22–24} 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 from buffy 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- $\alpha\beta$ TCR Biotin mAb (Miltenyi Biotec) followed by incubation with anti-Biotin mAb microbeads to deplete $\alpha\beta$ T cells by magnetic separation using autoMACS under

depleteS protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). DOT cells were generated from $\alpha\beta$ -depleted PBMCs cultured in either 96-well plates or G-REX platform (Wilson Wolf Manufacturing), based on an adaptation from our previous protocol.^{10,14} Briefly, $\alpha\beta$ -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 μ g/mL of penicillin/streptomycin (Thermo Fisher) and cultured for 16–20 days. Animal-free human cytokines rIL-4 (100 ng/mL), rIFN- γ (70 ng/mL), rIL-21 (7 ng/mL), and rIL-1 β (15 ng/mL; all from PeproTech), and a soluble mAb anti-CD3 (clone OKT-3, 140 ng/mL; BioLegend), were added to the medium at day 0. At day 7, cultures were supplemented with anti-CD3 (clone OKT-3, 1 μ g/mL or 2 μ g/mL for 96-well plate protocol), rIL-21 (13 ng/mL) and rIL-15 (70 ng/mL; also from PeproTech). On day 11, new medium was added to cultures, supplemented with anti-CD3 (1 μ g/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, CD3z endodomain, and a T2A-eGFP cassette) previously described²¹ 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 μ g 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-APCCy7 (UCHT1), TCR $\gamma\delta$ -PE (B1), TCR $\alpha\beta$ -APC

(IP26), CD4-APCCy7 (RPA-T4), CD8-BV510 (RPA-T8), CD25-APCCy7 (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 V δ 1-PE (REA173), TCR V δ 2-APC (123R3) and NKp44-APC (2.29) from Miltenyi Biotec. Briefly, 2.5×10^5 cells were incubated with the antibodies for 30 min at 4°C and then washed. For intracellular staining, DOT cells were incubated after membrane labeling with anti-perforin-BV241 (dG9) and anti-granzyme B-BV510 (GB11) from BD Biosciences, and anti-granzyme A-APC (CB9) from BioLegend (San Diego, California, USA), using FIX&PERM Sample Kit (Nordic MUBio) under manufacturer's instructions. Non-reactive, isotype-matched fluorochrome-conjugated mAbs were systematically used to set the gates. Dead cells were discarded by 7-AAD staining.

The immunophenotyping of AML cell lines and primary samples (obtained from Hospital Clínic of Barcelona) was done by surface staining with CD45-PE (HI30), CD33-BV421 (HIM3-4) and CD123-APC (7G3) (BD Biosciences, Franklin Lakes, New Jersey, USA). A FACS-Canto-II flow cytometer equipped with FACSDiva software (BD Biosciences) was used for the analysis.²⁷

In vitro cytotoxicity assays and cytokine release determination

The cell lines MOLM13, THP-1 and Jurkat were purchased from DSMZ (Germany) and expanded according to DSMZ recommendations. Target cells (cell lines and primary AML blasts) were labeled with 3 μ M eFluor 670 (eBioscience) and incubated with CAR-DOTs or mock-DOTs at different Effector:Target (E:T) ratios for the indicated time periods in complete DOT media. CAR-DOT-mediated cytotoxicity was determined by analyzing the residual alive (7-AAD⁻) eFluor 670⁺ target cells at each time point and E:T ratio. Absolute cell counts were determined using Trucount absolute count beads (BD Biosciences).²⁸ Cytokine production was measured by G-Series Human Cytokine Antibody Array 4000 from RayBiotech in supernatants harvested after 48 hours-incubation CAR-DOT/mock-DOT (four donors) with two primary AML samples.

In vivo AML patient-derived xenograft models

The 7–12 weeks old non-obese diabetic *Cg-Prkdc^{scid} Il2rg^t m1Wjl/SzJ* (NSG) mice (Jackson Laboratory) were bred and housed under pathogen-free conditions in the animal facility of the Barcelona Biomedical Research Park (PRBB). Mice were intravenously transplanted in the tail with 2.5×10^5 Luc-GFP-expressing primary CD123⁺ AML blasts (primograft-expanded) (579AML-patient-derived xenograft (PDX)^{LUC}).²⁹ CAR-DOTs or mock-DOTs were thawed and resuspended in complete media and mice were intravenously infused in the tail vein with 10×10^6 cells once, two times or three times 7, 14 and 21 days post-tumor inoculation, respectively. Where

indicated, mice were injected intraperitoneally with 1 μ g of hrIL-15 every 3–4 days or daily from day 7 to the end of the experiment, as a strategy to support DOT-cell persistence *in vivo*. Tumor burden was followed by bioluminescence (BLI) using the Xenogen IVIS 50 Imaging System (PerkinElmer). To measure luminescence, mice received 150 mg/kg of D-luciferin intraperitoneally, and tumor burden was monitored at the indicated time points as we previously described.^{27,30} Living Image software (PerkinElmer) was used to visualize and calculate total luminescence. Besides, tumor burden was followed-up at different time points by bleeding and BM aspirate using fluorescence activated cell sorting (FACS) analysis. Mice were sacrificed when control and/or mock-DOT-treated animals were leukemic, and tumor burden (hHLA-ABC⁺hCD45⁺hCD33⁺hCD123⁺ graft) and effector DOT persistence (hHLA-ABC⁺hCD45⁺hCD3⁺V δ 1⁺V δ 2⁺) were analyzed in BM and PB by FACS. In rechallenge experiments, leukemia-free animals were reinfused with 2.5×10^5 CD123+AML primary cells, and disease reappearance was followed-up by BLI and FACS as above. All procedures were performed in compliance with the institutional animal care committee of the PRBB (DAAM9624).

Statistical 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,^{10,14} and transduction was tested at days 7 and 11 using different retroviral (PepPam/RD114) and lentiviral (VSV-G/psPAX2) 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 $\gamma\delta$ TCR⁺ and V δ 1⁺ T cell percentages, which were ~97% and ~70%, respectively, as in the original 'naked' DOT product.^{10,14} Likewise, total and V δ 1⁺ T-cell numbers at the end of the process were similar for mock-DOTs and CAR-DOTs (figure 1C). Retroviral particles infected the V δ 1⁺ and V δ 1⁺ $\gamma\delta$ 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 (T_{EFF}), with an expected degree of variability between donors, followed by effector memory (T_{EM}) DOTs,

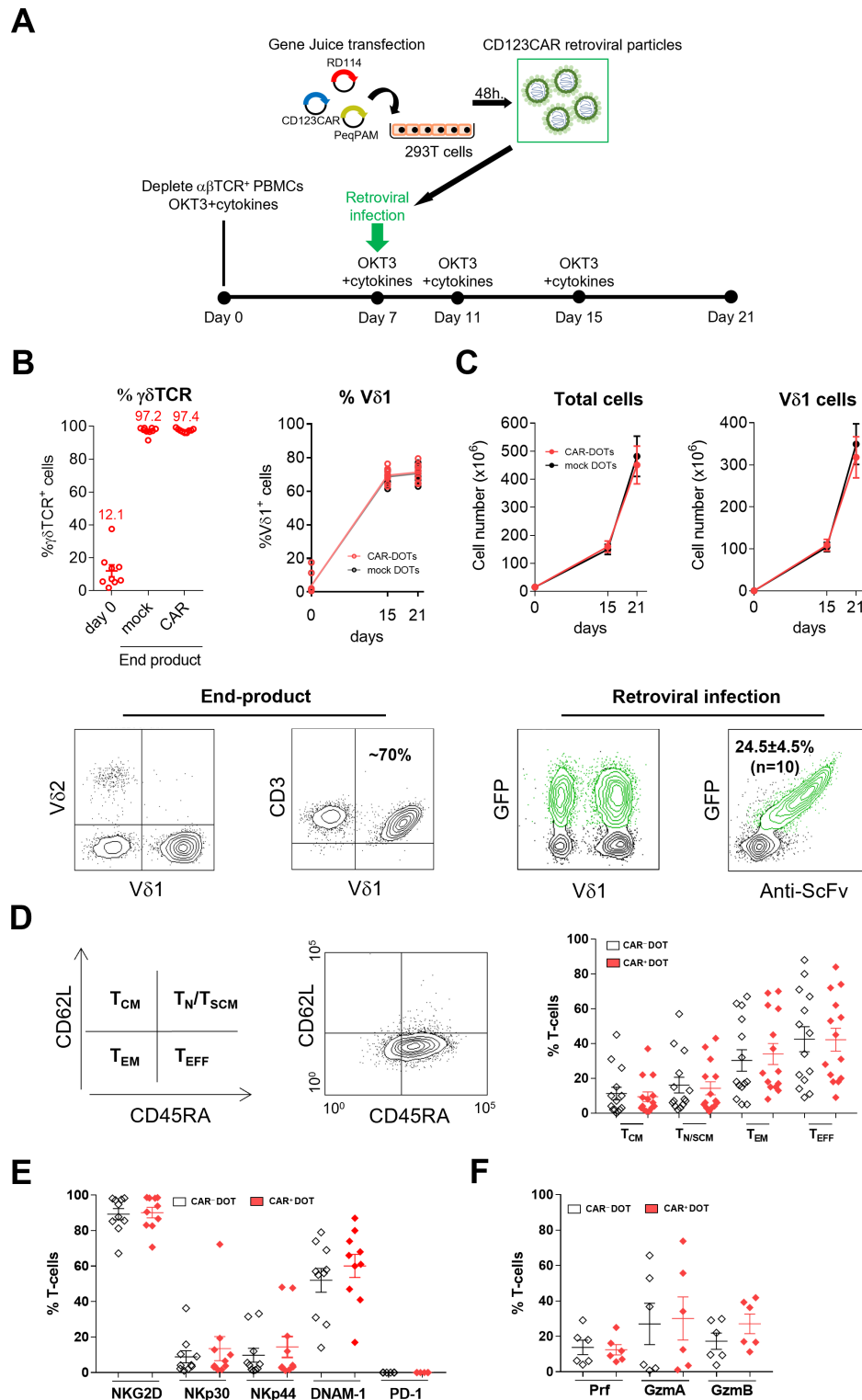


Figure 1 CD123CAR-DOT cell generation and phenotypic characterization. (A) CD123CAR-DOT cell production scheme. $\alpha\beta$ -depleted PBMCs were cultured under OKT3 antibody and cytokine stimulation for 3 weeks and transduced with γ -retroviruses at day 7. (B) Upper panels, $\gamma\delta$ -TCR+ and $V\delta 1$ +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 $V\delta 1/V\delta 2$ and $V\delta 1/CD3$ in end-products. (C) Upper panels, cell number for total cells and $V\delta 1$ +T cells along the CAR-DOT generation process. Lower panels, representative FACS dot plot for $V\delta 1/GFP$ and anti-ScFv/GFP expression. (D) Left-middle panels, scheme and representative FACS plot depicting T_{CM} , $T_{N/SCM}$, T_{EM} , T_{EFF} T-cell subsets as defined by CD62L versus CD45RA staining. Right panel, percentage of T_{CM} , $T_{N/SCM}$, T_{EM} , T_{EFF} 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 (Prf), 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; T_{CM} , central memory; T_{EFF} effector T cells; T_{EM} , effector memory; $T_{N/SCM}$, naïve/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 published¹⁰ 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 $\gamma\delta$ T cells highly enriched in $V\delta 1^+$ 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 mice³¹; the T-cell costimulator 4-1BB, a major determinant of $\gamma\delta$ T-cell-mediated tissue surveillance³²; the signal transducer Axl, shown to maximize IL-15R signaling in human NK cell differentiation³³; the myeloid differentiation factor, granulocyte-macrophage colony-stimulating factor (GM-CSF); and the chemokines MIP-1a (CCL3), CCL5, CXCL13 and lymphotactin (XCL1), all important in mobilizing multiple leukocyte subsets in cancer immunity.³⁴ 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,¹⁴ infused with 10×10^6 (CAR-DOTs 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,^{10 14} 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

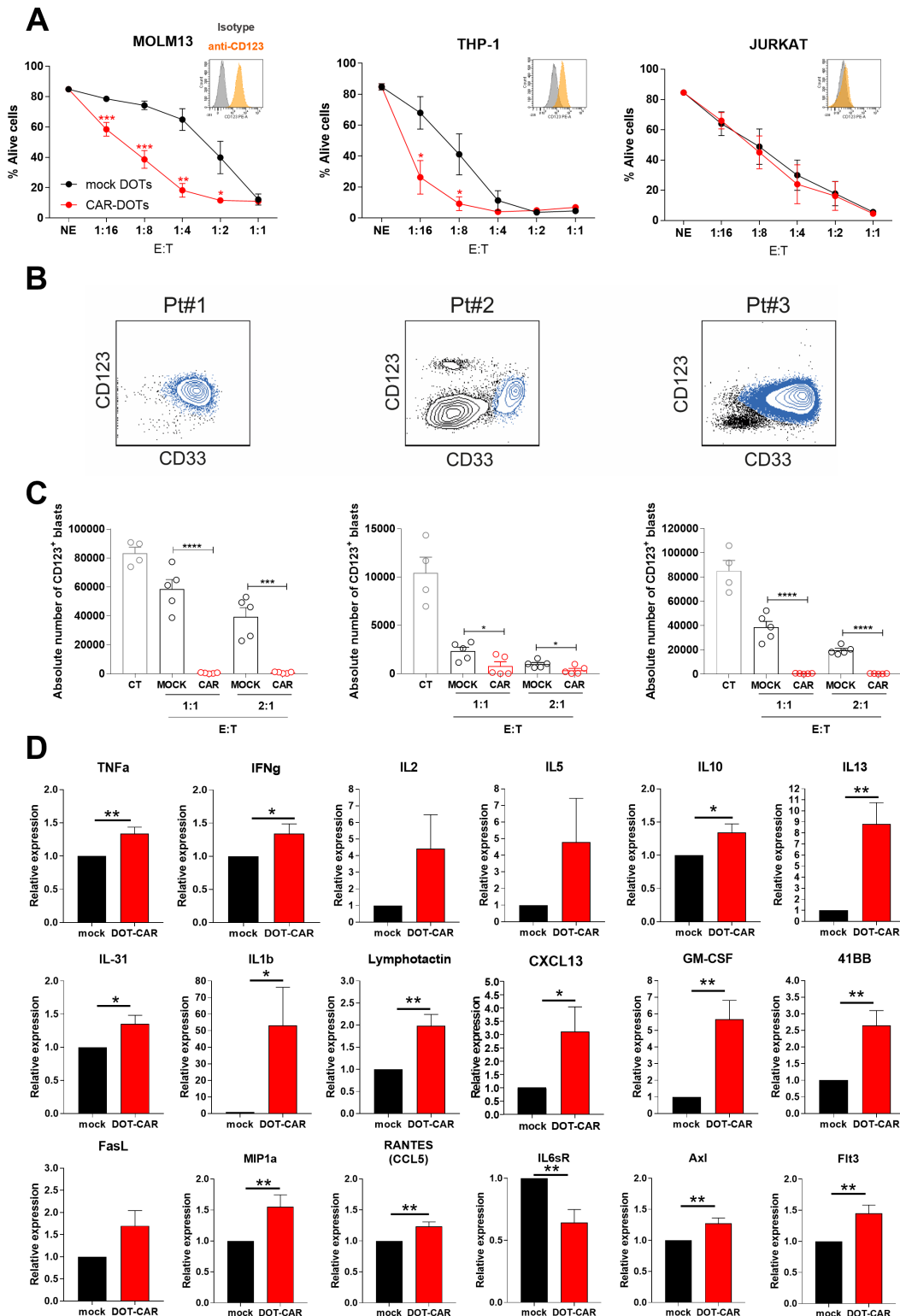


Figure 2 CD123CAR-DOT cells specifically target and eliminate CD123+ AML cell lines and primary samples *in vitro*. (A) Cytotoxicity of CAR-DOTs and mock-DOTs against CD123+ AML (MOLM13 and THP-1) and CD123- T-ALL (Jurkat, negative control) cell lines at the indicated E:T ratios in 48 hours assays (n=5). 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.

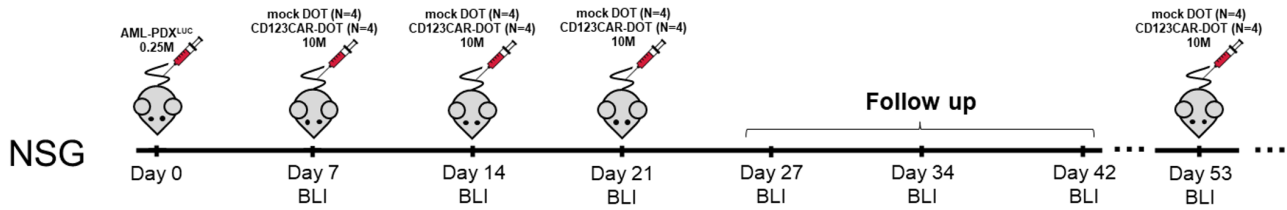
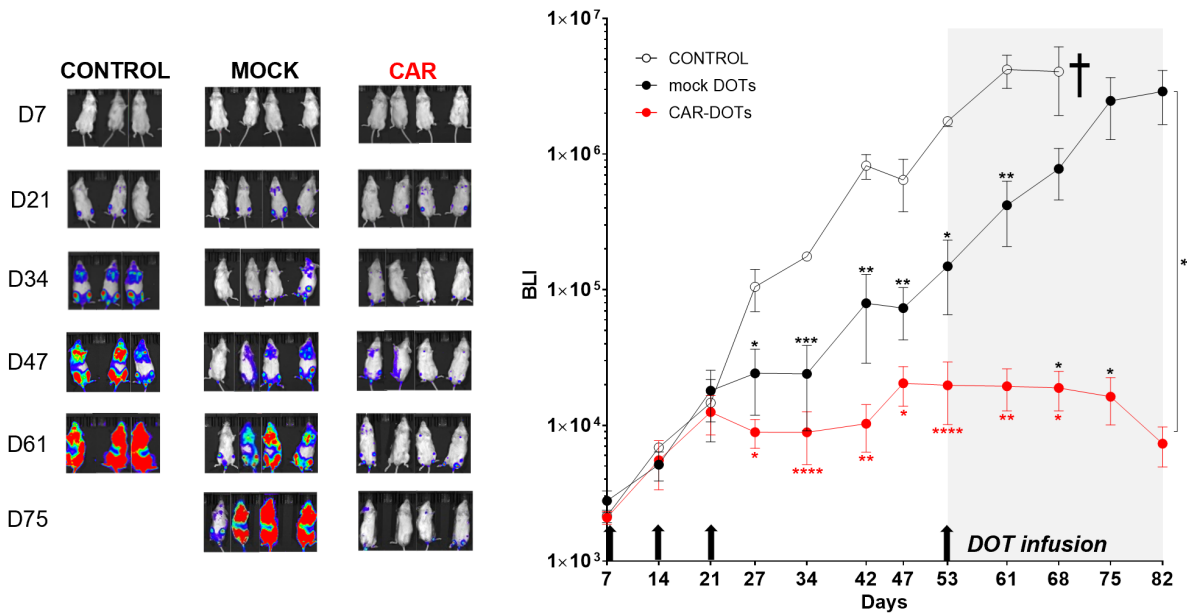
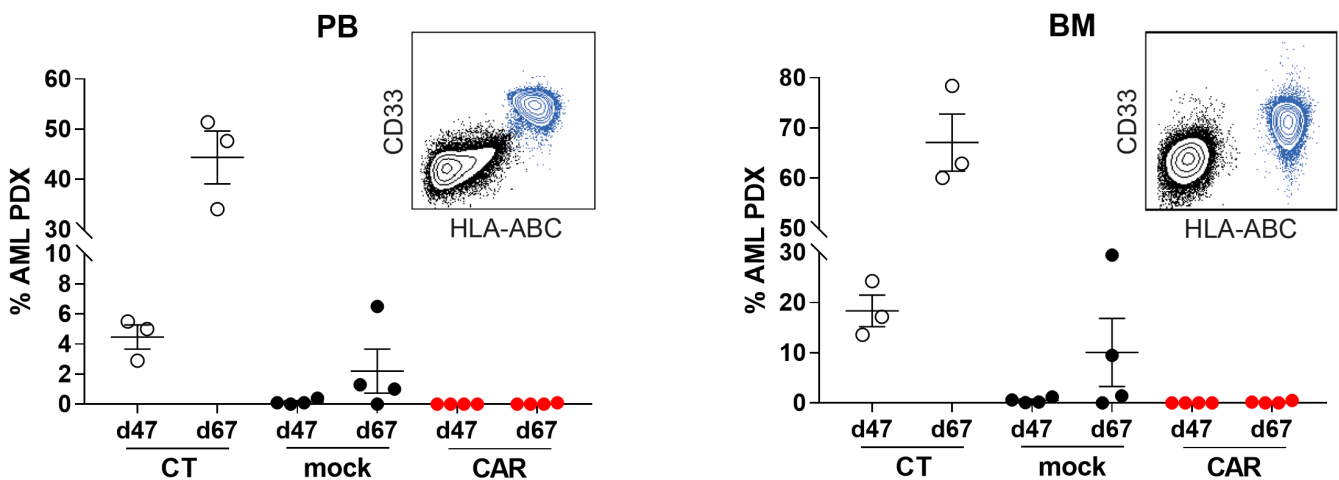
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Figure 3 CD123CAR-DOT cells fully control growth of primary CD123+AML blasts in a PDX model. (A) Schematic of the AML-PDX model. NSG mice ($n=4/\text{group}$) were intravenously injected with 2.5×10^5 AML-PDX^{LUC} cells and then received three intravenous injections (one per week, starting 7 days after tumor injection) of 10×10^6 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 10×10^6 mock-DOTs or CAR-DOTs, respectively. (B) IVIS imaging of tumor burden monitored by BLI at the indicated time points (scale: 5×10^4 – 1×10^6). 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.

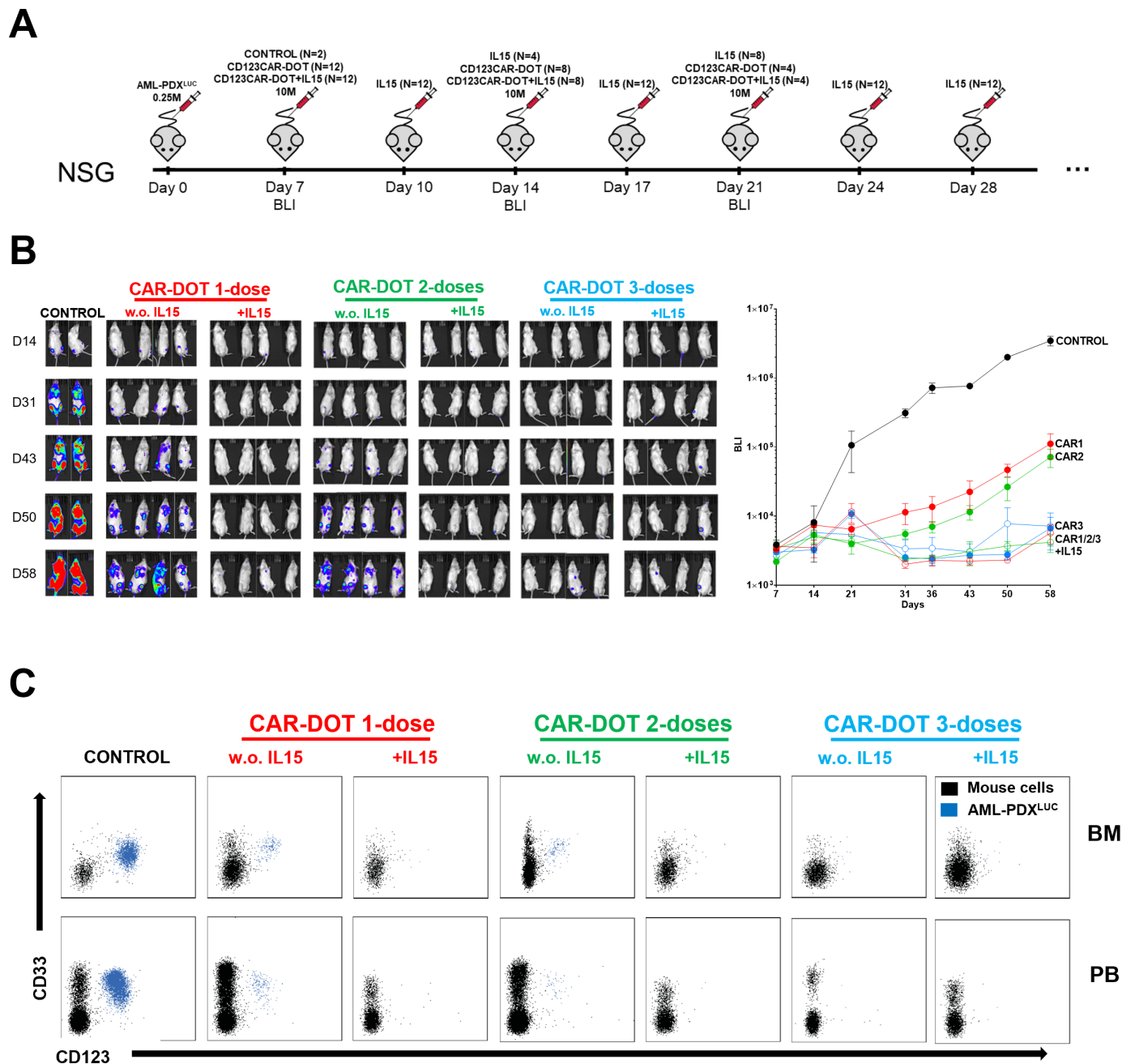


Figure 4 A single-dose of CD123CAR-DOT treatment combined with administration of IL-15 suffices to fully abolish AML progression in a PDX model. (A) Schematic of the AML-PDX model. NSG mice ($n=4/\text{group}$) were intravenously injected with 2.5×10^5 AML-PDX^{LUC} cells followed 7 days after by one, two or three intravenous injections (one per week) of 10×10^6 CAR-DOT cells, with or without administration of IL-15 intraperitoneally. (B) IVIS imaging of tumor burden monitored by BLI at the indicated time points (scale: 5×10^4 – 1×10^6). Right panel shows the total radiance quantification (p/sec/cm²/sr) at the indicated time points. (C) Tumor burden analyzed by FACS in PB and BM at the end of the experiment. AML blasts are highlighted in blue. 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.

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 regimens, 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 2.5×10^5 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

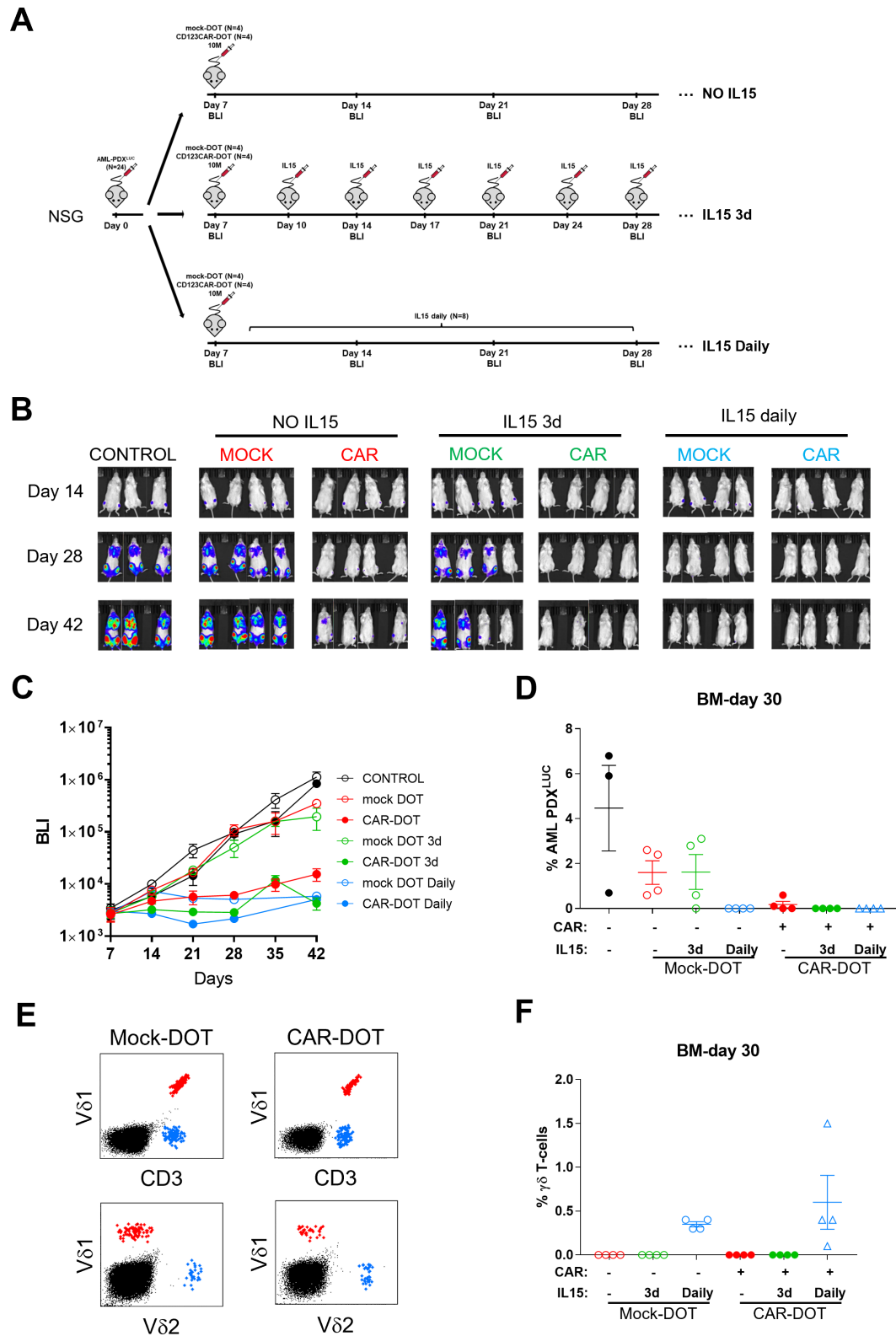


Figure 5 Daily-infused IL-15 increases DOT cell persistence and enables elimination of primary CD123+AML blasts in a PDX model. (A) Schematic of the AML-PDX model. NSG mice ($n=4/\text{group}$) were intravenously injected with 2.5×10^5 AML-PDX^{LUC} cells followed 7 days after by a one-single dose intravenous injection of 10×10^6 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: 5×10^4 – 1×10^6). (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 V δ 1+ and V δ 2+ T cells determined in CAR-DOT/ mock-DOT-treated mice in BM at day 30. (F) Total $\gamma\delta$ 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_{EFF} (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.^{1,2} 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).^{35–37} 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.^{3,4} 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.³⁸

The BM of patients with leukemia is exposed to several cycles of chemotherapy and immunosuppressants, which alter the status of normal T cells.^{5,39,40} Particularly in patients with AML, T cells have been characterized with an exhausted phenotype at relapse.²⁶ 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 $\alpha\beta$ 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^{KO} CAR-T cells in preclinical models and even in pediatric patients.^{41–43} However, a relevant recent paper has reported that TCR elimination in CAR-T cells leads to a decrease in persistence.⁴⁴ Furthermore, from a clinical point-of-view, CRISPR/Cas9 handling would still have to solve complex regulatory requirements.

Since the vast majority of $\gamma\delta$ T cells are HLA-independent,⁴⁵ they do not require genetic manipulation to be used as allogeneic therapies. It was with this purpose that we previously developed DOT cells, a V δ 1 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.^{10,14} However, our previous data in AML xenograft models left a significant margin for improvement in terms of control of tumor burden.¹⁵ 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 vitro*; 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-V δ 1 T-cell efficacy and the adjuvant effect of IL-15 in B-cell lymphoma and hepatocellular carcinoma models.^{12,46} Of note, our study is the first to compare CAR-transduced with mock-transduced V δ 1 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

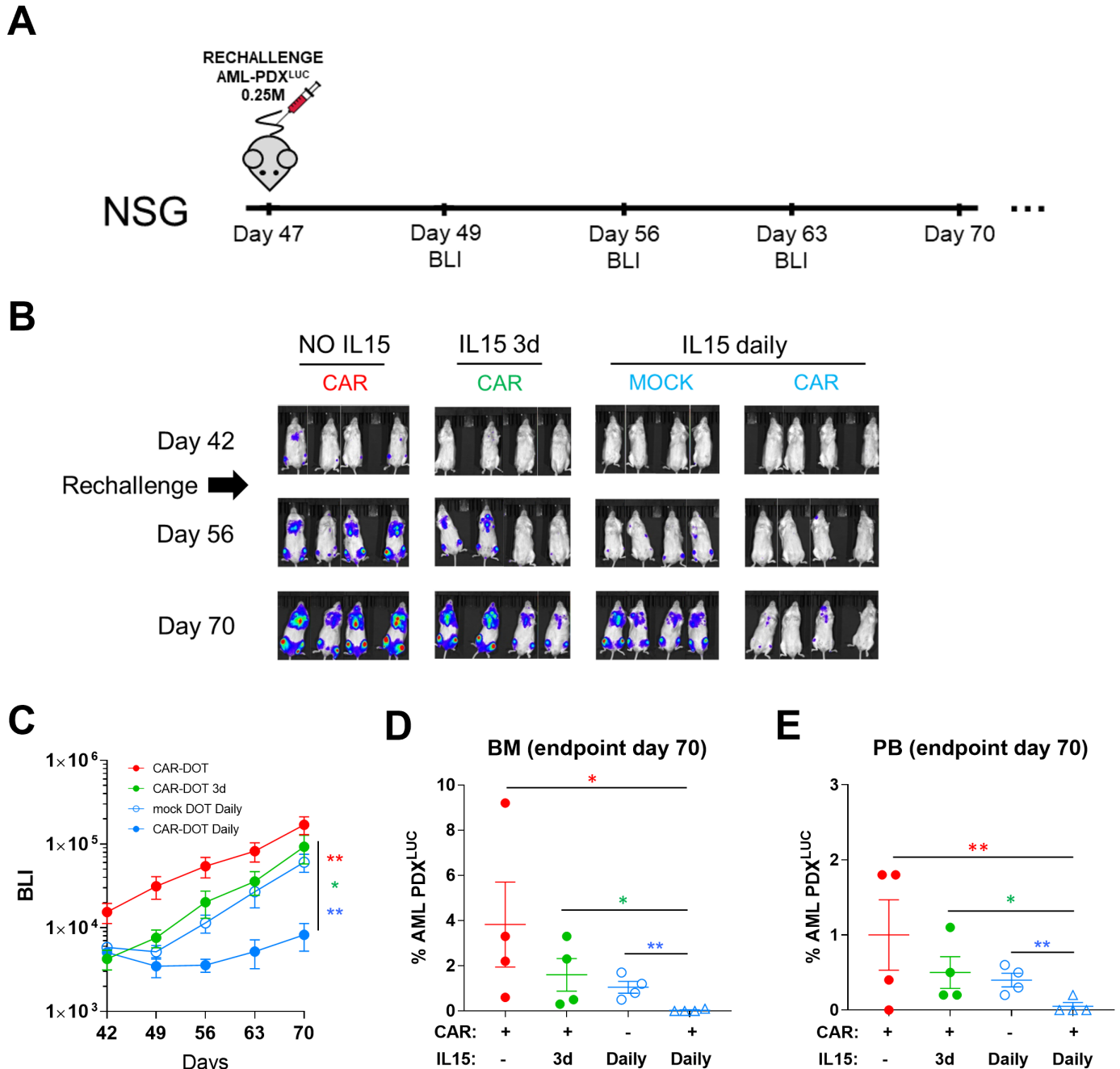


Figure 6 A single-dose of CD123CAR-DOT cells plus daily IL-15 sustain the ability to control AML progression on rechallenge in a PDX model. (A) Schematic of the tumor rechallenge AML-PDX^{LUC} experiments. Mock-DOTs plus daily IL-15-treated mice and CAR-DOTs-treated mice (regardless of IL-15 regimen) were rechallenged with 2.5×10^5 primary CD123+AML, 47 days after initial (CAR- or mock-) DOT cell infusion. (B) IVIS imaging of tumor burden monitored by BLI at the indicated time points (scale: 5×10^4 – 1×10^6). (C) Total radiance quantification (p/sec/cm²/sr) over time in mice rechallenged with AML-PDX^{LUC}. Tumor burden was monitored at endpoint analysis (day 70) by FACS in BM (D) and PB (E). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with one-tailed Student's t-test. 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.

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.⁴⁷ Therefore, although described as safe in non-human primates,⁴⁸

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.⁴⁹ 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 solutions.^{50 51}

An important final aspect to discuss is the potential advantage of using DOTs as effector cells for CAR transduction. Compared with conventional $\alpha\beta$ -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,⁵² and without any loss in potency (online supplemental figure S3). Additionally, the immune checkpoint PD-1 is absent in DOTs¹⁰ and CD123CAR-DOTs (figure 1E), whereas the upregulated NK-associated cytotoxicity machinery (including DNAM-1, Nkp30 and Nkp44)^{10,14} may be critical on CAR antigen loss as observed with CD19 in B-ALL.⁶ Importantly, this enhanced NK-like cytotoxicity is also an advantage over other $\gamma\delta$ T cell-based products being developed for adoptive immunotherapy of cancer.^{7–9, 12, 46} On the other hand, in comparison with NK cells, which obviously share such activating NK-cell receptors, DOTs offer as key advantages^{7, 8, 53} the previously documented¹⁰ 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).^{10, 14} In fact, the presence of a stochastically recombined and highly polyclonal TCR repertoire,¹⁴ allowing HLA-unrestricted recognition of stress-induced molecules,⁷ may also counteracts tumor immune evasion (via CAR antigen loss or HLA downregulation, eg). Moreover, the ‘trophic’ (homeostatic) signals received through $\gamma\delta$ TCR binding to butyrophilins constitutively expressed in multiple tissues⁵⁴ may be beneficial for *in vivo* persistence, and may underlie the recent description of a striking $V\delta 1^+$ 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).⁵⁵ Furthermore, although allogeneic CD19-directed CAR-NK cells have recently shown promising clinical results,⁵⁶ their ‘off-the-shelf’ implementation may be difficult due to the reported substantial decrease in viability after the freezing/thawing process,^{57, 58} 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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