Clinical Cancer Research

# Delta One T Cells for Immunotherapy of Chronic Lymphocytic Leukemia: Clinical-Grade Expansion/Differentiation and Preclinical Proof of Concept

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## Abstract

**Purpose:** The Vδ1$^+$ subset of γδ T lymphocytes is a promising candidate for cancer immunotherapy, but the lack of suitable expansion/differentiation methods has precluded therapeutic application. We set out to develop and test (preclinically) a Vδ1$^+$ T-cell–based protocol that is good manufacturing practice compatible and devoid of feeder cells for prompt clinical translation.

**Experimental design:** We tested multiple combinations of clinical-grade agonist antibodies and cytokines for their capacity to expand and differentiate (more than 2–3 weeks) Vδ1$^+$ T cells from the peripheral blood of healthy donors and patients with chronic lymphocytic leukemia (CLL). We characterized the phenotype and functional potential of the final cellular product, termed Delta One T (DOT) cells, *in vitro* and *in vivo* (xenograft models of CLL).

**Results:** We describe a very robust two-step protocol for the selective expansion (up to 2,000-fold in large clinical-grade cell culture bags) and differentiation of cytotoxic Vδ1$^+$ (DOT) cells. These expressed the natural cytotoxicity receptors, NKp30 and NKp44, which synergized with the T-cell receptor to mediate leukemia cell targeting *in vitro*. When transferred *in vivo*, DOT cells infiltrated tumors and peripheral organs, and persisted until the end of the analysis without showing signs of loss of function; indeed, DOT cells proliferated and produced abundant IFNγ and TNFα, but importantly no IL17, *in vivo*. Critically, DOT cells were capable of inhibiting tumor growth and preventing dissemination in xenograft models of CLL.

**Conclusions:** We provide a clinical-grade method and the preclinical proof of principle for application of a new cellular product, DOT cells, in adoptive immunotherapy of CLL. *Clin Cancer Res; 22(23); 5795–804. ©2016 AACR.*



## Introduction

Among various lymphocyte subsets being considered for cellular immunotherapy of cancer are γδ T cells, which provide robust and durable antitumor responses (1) and can combine T-cell-specific (TCR-mediated) and NK-characteristic mechanisms to recognize and target tumor cells (2, 3) without being constrained by MHC presentation of tumor-associated peptides (1, 4). Most strikingly, tumor-infiltrating γδ T cells recently emerged as the most significant favorable prognostic immune population in a collection of 39 cancer types (5). Although the clinical application of γδ T cells has been limited to its predominant circulating Vδ2$^+$ subset, yielding thus far modest clinical results (3), we have instead concentrated our efforts on their Vδ1$^+$ counterparts, which are less susceptible to activation-induced cell death and can persist long-term as tumor-reactive lymphocytes (6).

Vδ1-expressing γδ T cells constitute typically 10% to 30% of all γδ T cells in the peripheral blood, but their major fraction in epithelial tissues (6). Moreover, Vδ1$^+$ T cells are usually predominant (over Vδ2$^+$) in tumor infiltrates, and Vδ1$^+$ TIL-derived lines generally outperformed Vδ2$^+$ TIL lines in *in vitro* tumor cytotoxicity assays (7, 8). We previously identified a means to enhance leukemia targeting by peripheral blood Vδ1$^+$ (compared with Vδ2$^+$ T cells), through selective induction of natural cytotoxicity receptors (NCRs: NKp30, NKp44, and NKp46) upon stimulation with TCR agonists and cytokines *in vitro* (9). This expanded repertoire of activating/cytotoxicity receptors (2), together with their increased resistance to activation-induced cell death and exhaustion upon continuous stimulation (6), make Vδ1$^+$ T cells very attractive candidates for adoptive cell therapy (ACT) of cancer. However, difficulties in selectively expanding them to large numbers in good manufacturing practice (GMP) conditions have hindered the clinical application of Vδ1$^+$ T cells.

Current protocols to expand Vδ1$^+$ T cells *in vitro* make use of mitogenic plant lectins [phytohemagglutinin (PHA) or

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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## Translational Relevance

Vδ1$^+$ T cells, a subset of γδ T lymphocytes with strong tropism for tissues, is endowed with potent antitumor properties but has never been tested in the clinic due to lack of suitable expansion/differentiation protocols. Here we have developed a new and robust (highly reproducible) clinical-grade method, devoid of feeder cells, for selective and large-scale expansion and differentiation of cytotoxic Vδ1$^+$ T cells; and tested their therapeutic potential in preclinical models of chronic lymphocytic leukemia (CLL). Our cellular product, named Delta One T (DOT) cells, does not involve any genetic manipulation; and specifically targets leukemic but not healthy cells *in vitro*; and prevents wide-scale tumor dissemination to peripheral organs *in vivo*, without any signs of healthy tissue damage. Our results provide new means and the proof of principle for clinical application of DOT cells in adoptive immunotherapy of cancer.

concanavalin-A (ConA)] and unsafe materials that cannot be directly applied in the clinic (6). Also, recent findings on tumor-promoting effects of Vδ1$^+$ T cells producing IL17 (3, 10) have raised concerns about their application and stressed the need for a detailed characterization of effector Vδ1$^+$ lymphocytes that might be considered for ACT. These limitations and concerns led us here to devise solutions to expand and differentiate type 1 (IFNγ-producing) Vδ1$^+$ T cells expressing activating/cytotoxicity receptors using clinical-grade reagents to obtain sufficient numbers for ACT; and to provide robust preclinical proof-of-principle of their antitumor specificity and efficacy.

Among the various tumor targets of Vδ1$^+$ T cells (6), we focused on B-cell chronic lymphocytic leukemia (CLL; ref. 9). Although CLL treatment has recently seen substantial progress (11–13), most of the newly approved agents fail to induce complete tumor remissions. However, CAR-T cell immunotherapy has produced some complete remissions in recent CLL clinical trials (14, 15), but at the cost of the entire B-cell lineage. Our ACT strategy is based on Vδ1$^+$ T cells, thus aimed to selectively target transformed, but not normal B lymphocytes. Circulating Vδ1$^+$ T cells are typically increased in up to 60% of CLL patients (16) and have been associated with stable disease in low-risk CLL patients (17). Moreover, peripheral blood Vδ1$^+$ T cells were shown to recognize ULBP3-expressing B-CLL cells (17); and to exhibit specific cytotoxicity against CLL-derived cells *in vitro* (18).

Here we report the definition, optimization, and characterization of a Vδ1$^+$ T-cell-enriched ACT product (from herein designated DOT—Delta One T cells) for immunotherapy of CLL. We provide a clinical-grade two-stage protocol that enables the generation of large numbers of type 1 DOT effectors that selectively target tumor cells via the combined action of TCR and NCRs; and the proof of concept of their efficacy in inhibiting tumor growth and dissemination *in vivo*.

## Materials and Methods

### Leukemia patient samples

B-cell CLL cells were obtained from the peripheral blood of patients at first presentation, after informed consent and institutional review board approval. Sample preparation is described in Supplementary Methods.

### Leukemia cell line

The MEC-1 CLL cell line (19) was obtained from the German Resource Center for Biologic Material (DSMZ; reference no. ACC-497). Cells were characterized and authenticated by DSMZ through the analysis of cell morphology; the expression of key CD markers by flow cytometry; the presence of minisatellite markers by PCR; karyotype analysis; and tested for the presence of viruses and mycoplasma by PCR. Cells were purchased on April 2014 and used within 6 months. The cell line was authenticated every month by the author by flow cytometry of key CD markers and for mycoplasma by PCR. More details on the cell line can be found at DSMZ website (https://www.dsmz.de/catalogues/details/culture/ACC-497.html?tx_dsmzresources_pi5%5BreturnPid%5D=192).

### DOT cell production

Model-based analysis of ChIP-Seq (MACS)-sorted γδ T cells (obtained as described in Supplementary Methods) were resuspended in serum-free culture medium (OpTmizer-CTS) supplemented with 5% autologous plasma and 2 mmol/L L-glutamine (Thermo Fischer Scientific). Cells were transferred to a closed gas-permeable cell culture plastic bag (Saint-Gobain), at a maximum concentration of $1 \times 10^6$ cells/mL. Animal-free human cytokines (100 ng/mL rIL4, 70 ng/mL rIFNγ, 7 ng/mL rIL21, and 15 ng/mL rIL1β; all from Peprotech) and a soluble antibody (70 ng/mL anti-CD3 mAb, clone OKT-3; BioXcell) were added to the medium. Cells were incubated at 37ºC and 5% $CO_2$. Every 5 to 6 days, old medium was removed and replaced with fresh medium supplemented with cytokines (including 70 ng/mL rIL15 and 30 ng/mL IFNγ), and with 1 μg/mL anti-CD3 mAb. A detailed list of these or functionally equivalent GMP-grade reagents and materials is presented in Supplementary Table S1. Accessory "feeder" cells were not required in this patented method. Live cells were counted using Trypan Blue–positive exclusion in a hemocytometer.

### *In vitro* tumor-killing assays

All tumor cells were cultured in T25 flasks in complete 10% RPMI1640 with 10% FBS, 2 mmol/L L-glutamine, and maintained at $10^5$ up to $10^6$ cells/mL by dilution and splitting in a 1:3 ratio every 3 to 4 days. For cytotoxicity assays, *in vitro* expanded γδ T cells were plated in 96-well round-bottom plates. For receptor blocking, γδ peripheral blood lymphocytes (PBL) were preincubated for 1 hour with blocking antibodies: human anti-TCRγδ (clone B1); human anti-NKG2D (clone 1D11); human anti-CD2 (clone RPA-2.10); human anti-CD3 (clone OKT-3); human anti-NKp30 (clone P30-15); human anti-NKp44 (clone P44-8); mouse IgG1k (clone MOPC-21); mouse IgG2b (clone MPC-11); mouse IgG3k (clone MG3-35), all from Biolegend. Human anti-CD48 (clone TU145) and human anti-CD226 (clone DX11) were from BD Biosciences. Human anti-Vδ1 TCR (clones TCS-1 or TS8.2) was from Fisher Scientific, and human anti-TCRγδ (clone IMMU510) was from BD Biosciences. Alternatively, tumor target cells were preincubated with human anti-HLA,B,C mAb (clone W6/32) and human anti-MICA/MICB mAb (clone 6D4) from Biolegend. The blocking antibodies were maintained in the culture medium during the killing assays. Tumor cell lines or leukemia primary samples were stained with CellTrace Far Red

DDAO-SE (1 μmol/L; Molecular Probes, Invitrogen) and incubated at the indicated target:effector ratio with γδ T cells in RPMI for 3 hours at 37°C and 5% $CO_2$. Cells were then stained with annexin V–FITC (BD Biosciences) and analyzed by flow cytometry.

### Mice and adoptive transfer of DOT cells

Balb/cRag$^{-/-}$γc$^{-/-}$ (20) animals were obtained from Taconic; NOD-SCIDγc$^{-/-}$ (21) mice were obtained from the Jackson Laboratories. Age and sex-matched BRG or NSG mice were injected subcutaneously with MEC-1 cells and treated after 6 and 11 days with two intravenous transfers of $10^7$ or $2 \times 10^7$ DOT cells, and then analyzed (tumor size, histology, flow cytometry of tumor or organ infiltrates, and blood biochemistry) as detailed below or in Supplementary Methods. All animal procedures were performed in accordance to national guidelines from the Direção Geral de Veterinária and approved by the relevant Ethics Committee.

### Flow cytometry analysis

For phenotyping after DOT cell production, cells were stained with anti-CD3-APC (clone UCHT1), anti-TCRVδ1–FITC, and a panel of receptors using the LegendScreen Kit (Biolegend). For phenotyping after *in vivo* DOT-cell transfer, animals were euthanized using Eutasil in order for blood collection via cardiac puncture and quickly perfused with PBS + Heparin. Organs were homogenized and washed in 70-μm cell strainers. Femurs were flushed and then filtered. Cells were then stained with the following antibodies from eBioscience, Biolegend, Miltenyi Biotec, or Beckton Dickinson: anti-mouse CD45 (30-F11), and anti-human: CD45 (HI30), IL17A (BL-168), IFNγ (B27), TNFα (MAb-11), Ki67 (Ki-67). Other antibodies used are common with the *in vitro* studies. Antibodies were coupled to FITC, PE, PerCP, PerCP-Cy5, PE-Cy7, APC, APC-Cy7, Pacific Blue, Brilliant Violet 421, and Brilliant Violet 510 fluorochromes. For intracellular cytokine production analysis, cells were stimulated with PMA + ionomycin + brefeldin A for 4 hours at 37ºC and cells were then stained with eBioscience IC Kit according to manufacturer instructions. For Ki67 staining, cells were stained using Foxp3 staining buffer set from ebioscience. Flow cytometry acquisition was performed on an LSR FortessaII Cell Analyzer (BD Biosciences) and data analyzed with FlowJo 8.8.7 software (TreeStar).

### Statistical analysis

Statistical analysis was performed using GraphPad Prism software. Sample means were compared using the unpaired Student *t* test. In case variances of the two samples were found different using *F* test, the data were log transformed and if variances were found not to be different, the unpaired *t* test was applied to the log-transformed data. For survival data, log-rank (Mantel–Cox) test was used.

## Results

### Clinical-grade two-step method to selectively expand and differentiate DOT cells from healthy donors and CLL patients

We set out to develop a methodology for Vδ1$^+$ γδ T-cell enrichment and expansion compatible with regulatory agency approval. We started with a two-step magnetic bead sorting process, resulting in up to 93% γδ T-cell enrichment but still a minority of Vδ1$^+$ T cells (Supplementary Fig. S1). To optimize the selective expansion of Vδ1$^+$ T cells, isolated γδ T cells were cultured in 96-well plates for 2 to 3 weeks in the presence of 58 different T-/NK-cell–activating molecules (Supplementary Table S2). These included 13 different TCR agonists, 23 different coreceptor agonists, and 22 different cytokines, tested in 2,488 different combinations and concentrations. Highest expansions were obtained by combining anti-CD3 mAb activation in the presence of IL4 and, surprisingly, IFNγ (Fig. 1A; complete set of data available upon request). However, analysis of the final cellular product revealed a general absence of NKp30 or NKp44 and low NKG2D expression, which associated with poor cytotoxic activity against leukemia cells (Fig. 1B). Based on published evidence (22), we reasoned that IL4 could be preventing the differentiation of effector Vδ1$^+$ T cells. We thus devised a two-step method with distinct expansion and differentiation stages: 2 weeks in a first culture medium containing IL4; and then an additional week in a second culture medium containing IL15, a key differentiation factor for type I cytotoxic Vδ1$^+$ T cells (23), instead of IL4. With this combined protocol, we were able to recover high NCR (particularly NKp30) and NKG2D expression on Vδ1$^+$ T cells, which were now highly effective in leukemia cell killing (Fig. 1B). For simplicity, we termed the Vδ1$^+$ T-cell–enriched and NCR-expressing cytotoxic cellular product, derived from our two-step protocol, DOT cells. Complementation experiments with additional stimulation factors revealed synergistic effects of IL21 and IL1β, during the first step, which resulted in markedly increased DOT cell yield (Fig. 1C).

Next, to verify the robustness of our method, we extended its application to a larger number of healthy donors and, critically, also to CLL patients. Instead of plastic plates or flasks, we cultured our cells in closed, large-scale, gas-permeable cell bags developed for clinical applications. Although the composition of the starting cell product was highly variable among donors (Supplementary Tables S3 and S4), Vδ1$^+$ T cells became the dominant cell subset (>60% in all donors) within 11 days of culture and continued to expand until day 21 (Fig. 1D and E). Of note, reproducible expansion was achieved and the composition of the final cellular product was remarkably similar across the multiple donors (Supplementary Tables S5 and S6). Moreover, DOT cells could be efficiently generated from the PBLs of elderly (>65 years old) CLL patients with very high tumor burden (Supplementary Table S6; Fig. 1D and E). Finally, although fold expansions in large-scale plastic bags were, as expected, of lower magnitude than those from plates, they still generated relevant numbers for clinical translation (Supplementary Table S7). Collectively, these experiments established a novel, clinical-grade and robust two-step method to selectively (>65% enrichment) expand (up to 2,500-fold) and differentiate cytotoxic Vδ1$^+$ (DOT) cells from the peripheral blood of healthy donors and CLL patients.

### Characterization of the DOT cell activation and maturation phenotype

Given the novelty of our method and resulting cellular product, we next performed large-spectrum phenotyping of 332 different cell surface markers. Vδ1$^+$ T cells were compared at the beginning (day 0) and the end (day 21) of the DOT culture process (Fig. 2A–C and Supplementary Fig. S2). We observed marked upregulation of the activation markers CD69 and CD25 (Fig. 2A) and HLA-DR (Supplementary Fig. S2), as well as the costimulatory receptors CD27, CD134/OX-40, and CD150/SLAM (Fig. 2A), indicators of

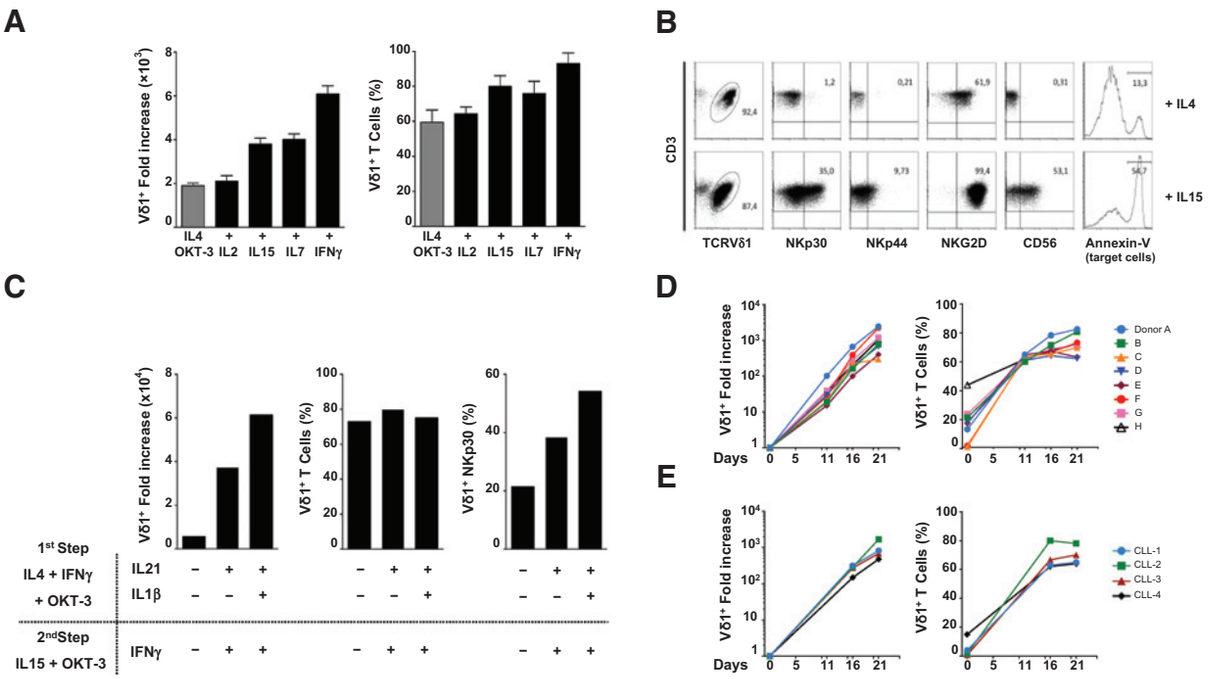


**Figure 1.**

Two-step method to selective expand and differentiate DOT cells from healthy donors or CLL patients. **A**, TCRγδ$^+$ PBLs were MACS sorted from a healthy donor and cultured in the presence of anti-CD3 mAb (clone OKT-3) and IL4 (gray bar). Cells were further supplemented with the indicated cytokines and cultured for 2 weeks in nonclinical grade 96-well plastic plates. Cells were later counted and analyzed by flow cytometry. Shown are Vδ1$^+$ T-cell fold expansion and enrichment percentage (mean + SD, $n = 3$) for the culture conditions producing highest fold expansion with each of the following cytokines: IL2 (2 ng/mL:), IL15 (0.7 ng/mL), IL7 (20 ng/mL), and IFNγ (200 ng/mL). **B**, Cells from the best initial culture condition (+IFNγ in **A**) were split at day 14 and one fraction of cells was cultured in the same conditions (IL4), whereas the other fraction was cultured in presence of OKT-3, IFNγ, and 100 ng/mL IL15 (without IL4). At day 20, cells were counted, their phenotype analyzed by flow cytometry, and their killer function evaluated against leukemia target cells. Percentage of marker-positive cells (Dot plots) or dead leukemia cells (Annexin V histograms) are shown. Data are representative of three independent experiments. **C**, Comparison of the effect of the indicated cytokines (divided in two steps of 6 days + 16 days), in presence anti-CD3 mAb, on Vδ1$^+$ T-cell enrichment, fold expansion, and fraction of NKp30$^+$ cells. **D** and **E**, TCRγδ$^+$ PBLs were MACS sorted from the peripheral blood of healthy donors (**D**) or CLL/SLL patients (**E**), and expanded in clinical grade gas-permeable, 1 L cell culture plastic bags according to our two-step protocol. Depicted are the fold increase (left) and fraction of Vδ1$^+$ T cells (right) at the indicated time points.

enhanced proliferative potential of *in vitro*–generated DOT cells (compared with their baseline Vδ1$^+$ T-cell counterparts). Moreover, DOT cells increased the expression of NK-cell–associated activating/cytotoxicity receptors, namely NKp30, NKp44, NKG2D, DNAM-1, and 2B4 (Fig. 2A and Supplementary Fig. S2), all previously shown to be important players in tumor cell targeting (2, 3). In contrast, key inhibitory and exhaustion-associated molecules such as PD-1, CTLA-4, or CD94 (24) were expressed either at very low levels or not expressed at all (Fig. 2A), demonstrating a striking "fitness" of DOT cells even after 21 days of culture under stimulatory conditions. Notably, the upregulation of multiple molecules involved in cell adhesion (e.g., CD56, CD96, CD172a/SIRPα, integrin-β7, CD31, and ICAM-1) and chemokine receptors (CD183/CXCR3, CD196/CCR6, and CX3CR1) suggested high potential to migrate and recirculate between blood and tissues. Of note, IL18Rα and Notch1, which are known to promote type I (IFNγ-producing) responses (25), were also highly expressed by DOT cells, whereas CD161 (which associates with IL17-producing cell subsets) was downregulated after 21 days of culture (Fig. 2A and B and Supplementary Fig. S2). Importantly, in support of the robustness of our method, we found strikingly similar DOT cell phenotypes across all four tested donors, as illustrated by the heatmap in Fig. 2C. These data collectively characterize DOT cells as a highly reproducible cellular product of activated (nonexhausted) lymphocytes endowed with migratory potential and natural cytotoxicity machinery.

## Potent and selective TCR/NCR-dependent DOT cytotoxicity against leukemic cells

Although the robust expression of activating/cytotoxicity receptors (Fig. 2A) suggested strong cytotoxic potential of DOT cells, we formally tested this function upon short-term (3 hours) coincubation with tumor (CLL) targets *in vitro*. DOT cells generated from either healthy donors (Fig. 3A and B) or CLL patients (Fig. 3C and D) were highly cytotoxic against the CLL cell line, MEC-1 (Fig. 3A and C), as well as autologous (Fig. 3D) and allogeneic (Fig. 3B) CLL primary samples. In contrast, DOT cells did not target healthy autologous leukocytes (Fig. 3A, B, and D). These data demonstrate that DOT cells are potent yet selective (against tumor cells) cytotoxic effector lymphocytes.

To uncover the molecular mechanisms of DOT cell–mediated CLL recognition, we added blocking antibodies against candidate receptors (TCRγδ, TCRVδ1, NKG2D, NKp30, NKp44, DNAM-1, 2B4, CD2), either individually (Expt 1) or in combinations (Expt 2), to MEC-1 killing assays (Fig. 3E). We found that the TCRVδ1 made an important contribution to MEC-1 targeting,

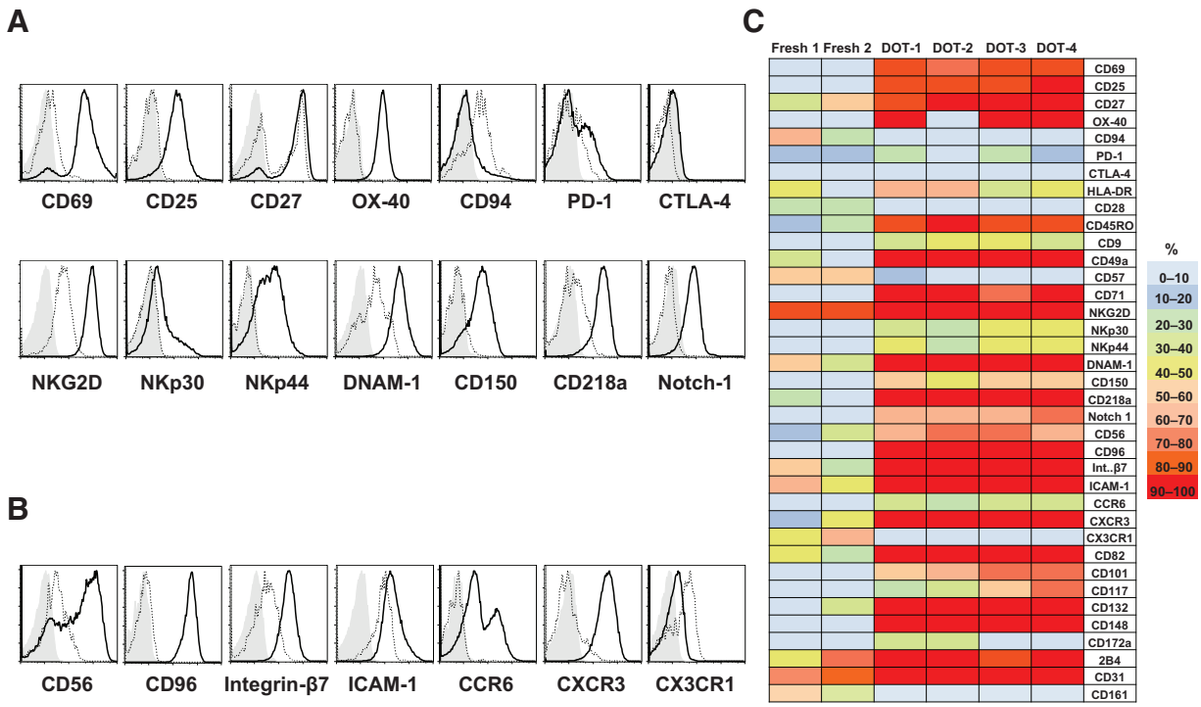


**Figure 2.**

Characterization of the activation and maturation phenotype of DOT cells. Flow cytometry comparison of the cell surface phenotype of DOT cells at day 21 of culture (full lines) with freshly-isolated Vδ1$^+$ T cells (dotted lines), as analyzed using the LEGENDScreen Kit. Shown are histogram overlays for 14 markers related to lymphocyte activation and differentiation (**A**); and seven markers implicated in adhesion and migration (**B**). Cells from one healthy donor are shown. Isotype control staining is shaded grey. **C**, Heatmap representing percentages of positive cells for each surface marker (from **A** and **B** and Supplementary Fig. S2) across DOT cells (at day 21 of culture) produced from four different healthy donors (1–4), compared with freshly isolated Vδ1$^+$ T cells (from donors 1 and 2). The color code is presented on the right.

which also depended on CD2-mediated adhesion, but the combined blockade with NKp30 and NKp44 essentially abolished the DOT-mediated cytotoxic effect (Fig. 3E).

Because DOT cells correspond to yet heterogeneous cell lines with a significant fraction of Vδ1$^{neg}$ cells, we asked whether these distinct subsets were cytotoxic against MEC-1 cells *in vitro*. We reanalyzed DOT cell samples generated from four donors (the same as described in Fig. 2), and identified NKp30, NKp44, and CD2 expression on CD3$^+$Vδ1$^-$Vδ2$^-$ T cells (Supplementary Fig. S3A). To investigate their function, we thawed previously frozen DOT cells from three different donors (viability of thawed cells was 75%–80%), and FACS sorted the Vδ1$^+$ and Vδ1$^-$Vδ2$^-$ T-cell populations, which comprised ≥93% of the final DOT cell product (Supplementary Fig. S3B); both subsets were highly cytotoxic against MEC-1 cell line *in vitro* (Supplementary Fig. S3C). Finally, the simultaneous blockade of TCRVδ1, CD2, NKp30, and NKp44 significantly inhibited DOT cell killing of primary CLL samples *in vitro* (Supplementary Fig. S3D). These data demonstrate that DOT cells combine T-cell–specific (their signature TCRVδ1) and NK-cell–characteristic (NCR) mechanisms to selectively recognize and kill leukemic leukocytes *in vitro*.

### Broad tissue distribution and tumor infiltration of effector DOT cells upon adoptive transfer *in vivo*

To assess the behavior and potential efficacy of DOT cells against CLL *in vivo*, we used a xenograft model of human CLL previously shown to reproduce several aspects of the disease (26, 27). The model relies on the subcutaneous injection of MEC-1 cells into Balb/cRag$^{-/-}$γc$^{-/-}$ (BRG) animals. We detected human Vδ1$^+$ cells early after transfer (e.g., in the liver and lung), and observed a significant reduction in bulk tumor size in DOT cell–treated animals compared with mock (PBS-injected) controls (Supplementary Fig. S4). However, we could not detect human T cells at later time points, and thus modified the model to use more permissive (28) NOD-SCID γc$^{-/-}$ (NSG) mice as hosts (Supplementary Fig. S5). In these hosts, we were able to recover, 25 days after transfer, human T cells in all tissues analyzed by flow cytometry: spleen, liver, lung, bone marrow, and the tumor itself (Fig. 4A). Interestingly, we observed a striking enrichment (98%–100%, up from ∼65% in the inoculated cellular product) of Vδ1$^+$ T cells in all the tissues (Fig. 4A). Histologic analyses confirmed that the transferred T cells infiltrated the tumor and other tissues (liver, kidney, bone marrow; Fig. 4B), and recovered DOT cells expressed NKp30 and NKG2D (Fig. 4C).

To understand whether the *in vivo* enrichment in Vδ1$^+$ T cells reflected initial tissue seeding/homing, or preferential expansion, we performed a similar experiment and analyzed both the seeding (60 hours after transfer; Supplementary Fig. S5) and their maintenance (up to 55 days after inoculation, depending of mouse survival; Fig. 4D). At 60 hours, we found that the Vδ1$^+$ T-cell fraction was mostly identical to that of the transferred inoculum in all tissues analyzed (Supplementary Fig. S5), ruling out preferential seeding. Conversely, at late time points (45–55 days) we recovered substantial numbers of human T cells, strongly

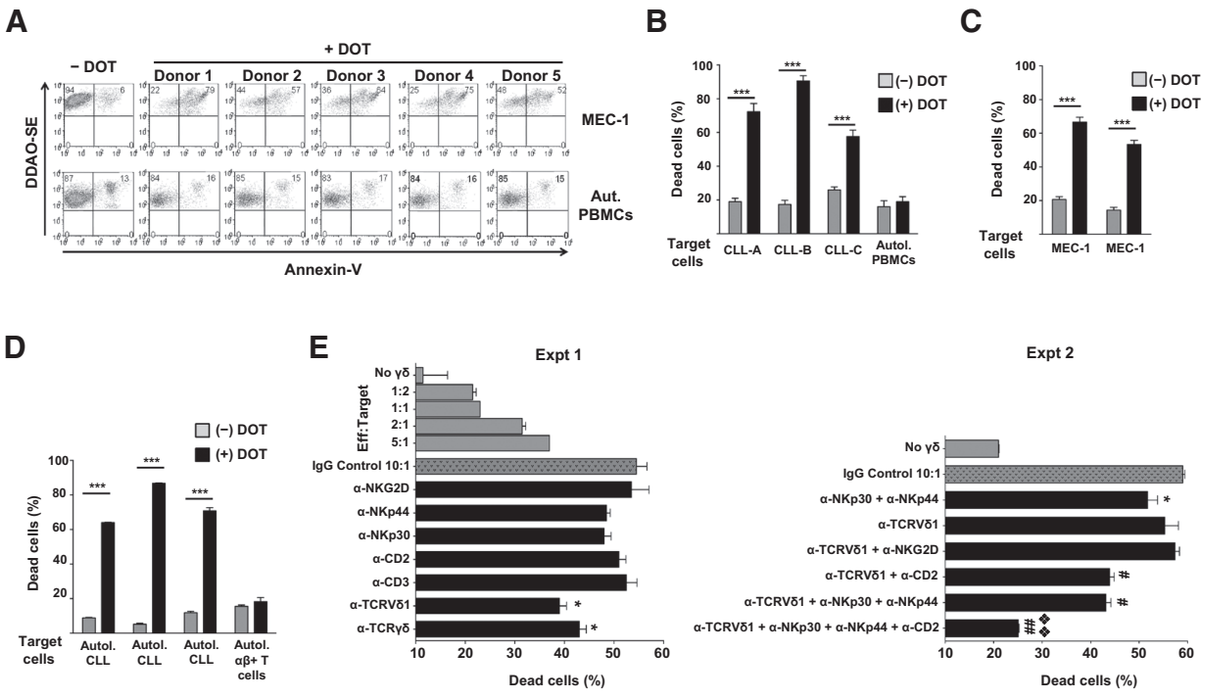


**Figure 3.**

TCR/NCR-dependent DOT cytotoxicity against leukemic (but not healthy) cells. **A**, DOT cells produced from five healthy donors were coincubated with MEC-1 (CLL) cells (top) or autologous healthy PBMCs (bottom). The death of target cells (prelabeled with DDAO-SE dye) was assessed by Annexin-V staining. "-DOT" corresponds to negative control of tumor cells without effector DOT cells. Shown are representative plots of three technical replicates. **B**, DOT cells generated from a healthy donor were coincubated with three CLL primary cell samples (collected from the peripheral blood of CLL patients and enriched for $CD19^+$ cells), or with autologous healthy PBMCs. Graph shows percentages of dead (Annexin $V^+$) target cells (mean + SD) of three technical replicates. **C**, DOT cells generated from two CLL patients were coincubated for 3 hours with MEC-1 (CLL) cells; graph shows percentages of dead (Annexin $V^+$) target cells (mean + SD). **D**, DOT cells generated from three CLL patients were tested against autologous CLL cells and MACS-sorted healthy αβ+ T cells (frozen at day 0 and thawed at day 21, a representative experiment out of three performed is shown). $N = 3$ replicates; mean + SD; ***, $P < 0.001$; Student $t$ test. **E**, DOT cells produced from two healthy donors were tested in different experiments against MEC-1 (CLL) target cells at increasing effector/target ratios (left plot, gray bars) and also in presence of blocking antibodies for (α, anti-) the indicated molecules, either individually (Expt 1) or in combinations (Expt 2). The highest effector/target ratio (10:1) was used in blocking experiments and gray bar at this ratio (with IgG isotype antibody) serves as control. Shown are the percentages of dead (Annexin-$V^+$) MEC-1 target cells. * and # indicate significant differences relative to IgG isotype control or α-TCRVδ1, respectively (mean + SD; *,#, $P < 0.05$; **,##, $P < 0.01$; Student $t$ test).

enriched in $V\delta1^+$ T cells (Fig. 4D). Interestingly, we could not recover DOT cells in animals that had not been preinoculated with tumor (Supplementary Fig. S5).

At the end of the experiment, we analyzed intracellular cytokine expression and Ki67 expression (indicator of proliferation) on DOT cells. In all tissues analyzed, including the tumor itself, we found an activated and proliferative profile associated with potent type I effector phenotype: marked production of IFNγ and TNFα in absence of IL17 (Fig. 4E). These data demonstrate that DOT cells home to multiple tissues, infiltrate the tumor, and persist as fully functional bona fide type I effectors *in vivo*.

### Inhibition of CLL tumor growth and dissemination upon adoptive DOT cell transfer

Finally, we evaluated the *in vivo* efficacy of our clinical product, that is, the impact of DOT-cell ACT on tumor growth (and dissemination). Despite encouraging data in BRG hosts (Supplementary Fig. S4), DOT cells failed to persist in those animals. Because this limitation was solved in NSG mice (Fig. 4), we used them to establish MEC-1 cells ($1 \times 10^7$) and to transfer two doses of $2 \times 10^7$ DOT cells. We measured tumor throughout the experiment, and sacrificed and analyzed animals when tumor reached limit size (1,000 $mm^3$). Data collection and analysis up to 60 days posttumor inoculation showed that DOT cell–treated animals stayed alive for markedly longer (Supplementary Fig. S6A), including one tumor regression, and overall survival data demonstrated the efficacy of DOT cell therapy (Supplementary Fig. S6B).

Importantly, in NSG hosts the tumor disseminates to several organs, providing a unique opportunity to evaluate the capacity of DOT cells to limit tumor spreading. Analysis of histologic samples at the end of the experiment showed a clear reduction in tumor cells recovered from all organs tested (of DOT cell–treated compared with PBS controls), with an emphasis on major tumor dissemination sites such as liver and bone marrow (Fig. 5A and B). Of note, there was a striking difference in the number of animals whose organs displayed tumor infiltrates in the treated and control groups (Fig. 5B). This strong protective effect of DOT cell ACT could be also documented by flow cytometry analysis (Fig. 5C). Analysis of a group of NSG hosts receiving half the dose ($1 \times 10^7$) of DOT cells, showed intermediate results, thus suggesting a dose-dependent therapeutic effect (Fig. 5C, right). Of

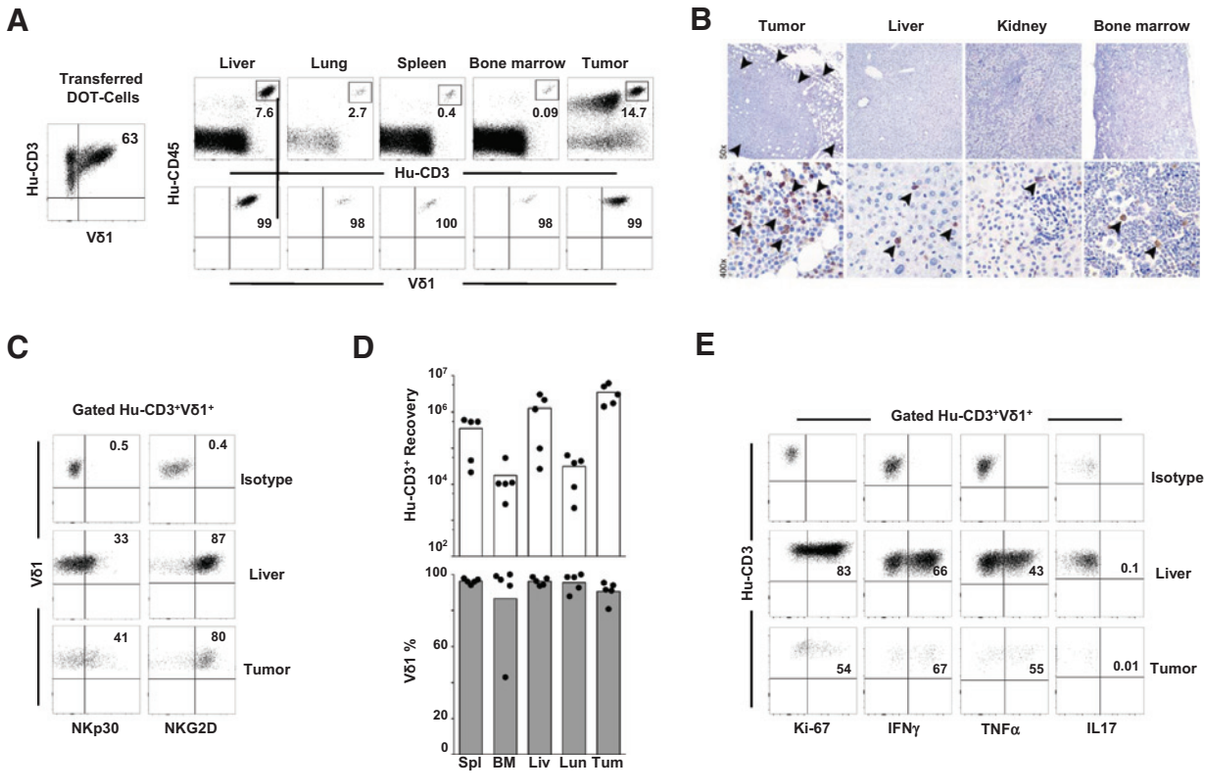


**Figure 4.**

Activated type I effector DOT cells persist, home to peripheral tissues, and infiltrate tumors *in vivo*. **A**, DOT cells ($2 \times 10^7$) were transferred into tumor-bearing NSG hosts and 25 days later organs were collected to detect DOT cells by flow cytometry. Dot plots show human (Hu) $CD3^+$ fraction and $V\delta1^+$ subfraction in transferred and recovered DOT cells (representative of six independent hosts). Top plots were pregated on total (human + mouse) $CD45^+$ cells. **B**, Immunohistochemical analysis of indicated tissues showing staining for Hu-CD3 (arrows) in a representative animal from the same experiment shown in **A**. **C**, phenotype of DOT cells recovered from a representative animal out of three analyzed for NKp30 and NKG2D expression in the indicated tissues. Isotype (and FMO) is also shown. Dot plots are gated on Hu-$CD3^+V\delta1^+$ cells. **D**, DOT cell recovery and $V\delta1^+$ fraction in indicated tissues (spleen, bone marrow, liver, lung, and tumor) from a different experiment, where DOT cells were recovered between day 45 and 55 after first DOT cell ($2 \times 10^7$) transfer. Data from individual animals (dots) and average (bars) is shown. **E**, Expression of the proliferation marker Ki67 and the effector cytokines IFNγ, TNFα, and IL17 in Hu-$CD3^+V\delta1^+$ cells recovered in the indicated organs at the end of the experiment described in **D**. Isotype (and FMO) is also shown. Data are from one animal representative of three analyzed.

note, blood biochemistry analysis (Supplementary Fig. S7), as well as histologic evidence (Fig. 4B and 5A; complete set of data available upon request) did not show signs of treatment-associated toxicity, thus suggesting a favorable safety profile of DOT-cell ACT. Collectively, our data establish the efficacy of adoptive DOT cell immunotherapy at the preclinical level, thus supporting its translation to CLL patient treatment.

## Discussion

The field of ACT for treatment of hematologic malignancies is advancing very rapidly, especially since the advent of CAR-T cell technology. Notwithstanding, various challenges remain to be addressed, including on-target toxicity and high-grade autoimmunity, and concerns of potential long-term undesired effects (29). Here we have explored a different option, based on the large-scale expansion and differentiation of (genetically unmanipulated) type 1 cytotoxic peripheral blood $V\delta1^+$ γδ T cells that selectively recognize and eliminate malignant leukocytes. Although several methods have been described in the past 5 years that can generate substantial numbers of tumor-targeting $V\delta2^{neg}$ T cells *in vitro*, key unresolved problems still excluded a clinical application of these cells: (i) the use of unsafe reagents and materials (such as plant lectins and plastic culture plates) in the manufacturing process; (ii) the high level of variation in the composition of the final cell products, especially between different donors; and/or (iii) the low antitumor activity of the final product (6, 18, 30, 31).

In a previous study, Deniger and colleagues developed tumor-derived artificial antigen presenting cells (aAPC) to propagate high numbers of γδ T cells expressing a polyclonal repertoire of γ and δ TCR chains (32). However, as pointed out by the authors (33), the method could not resolve critical obstacles associated with clinical application of γδ T cells. For example, most ingredients are not currently produced in GMP conditions and further developments still depend on future interest of manufacturers, complex regulatory approvals, while assuming that the same cell product can be obtained with different reagents, and from cancer patients. Furthermore, key details regarding the exact composition (and variability) of the generated cell products were missing in this study, thus hindering the potential application of this method.

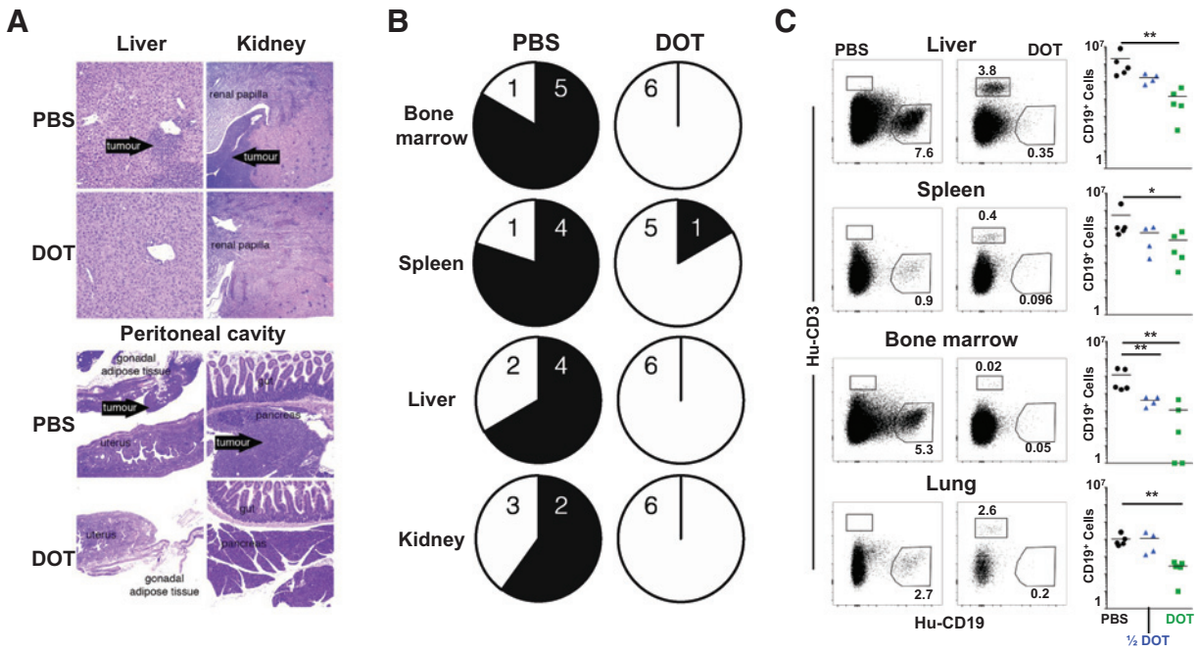


**Figure 5.**

DOT cells inhibit tumor growth and prevent tumor dissemination *in vivo*. **A**, H&E histological analysis of tumor cell infiltration in tissues of DOT-cell–treated or PBS control mice. **B**, Pie charts show animals with non-infiltrated (in white) or infiltrated (in black) tissues from H&E analysis. **C**, Flow cytometry analysis (gated on total $CD45^{\pm}$) showing tumor infiltration (MEC-1, $Hu\text{-}CD19^{+}$) at the end of the experiment in each indicated organ in one representative animal from PBS control group or DOT ($2 \times 10^7$) treated animals. Graphs shown on the right display tumor cell recovery from the same organs calculated for the whole set of data of the experiment, including from a group of animals that received halved dose ($1 \times 10^7$) of DOT cells (*, $P < 0.05$; **, $P < 0.01$; Student *t* test).

In sharp contrast, our study discloses the first protocol with direct clinical adaptability, using reagents and materials currently available in pharmaceutical grade (Supplementary Table S1), facilitating regulatory approval toward phase I clinical trials. Our major breakthrough was to uncouple expansion and differentiation stages, because these were optimally supported by distinct (and antagonistic in their outcome) cytokines, IL4, and IL15, respectively. This new two-step treatment allowed us to obtain the highest expansion of $V\delta1^+$ T cells ever recorded *in vitro* (in plastic culture plates): >60,000-fold increase in 21 days (Fig. 1C), compared to <25,000-fold obtained by Siegers and colleagues (31). Upon transition to a clinical-grade system, we achieved lower fold expansions, but still clinically relevant numbers of DOT cells for infusion in patients. The method is robust enough to enrich (>60%) and expand (up to 2,000-fold) $V\delta1^+$ T cells from highly unpurified samples obtained from CLL patients, differentiating them into NCR-expressing and highly cytotoxic DOT cells.

Few previous studies on $V\delta1^+$ T cells provide preclinical data on their performance *in vivo*. One report showed positive responses and persistence in spleen and bone marrow upon ACT in mice up to 39 days after transfer (30), although it is not clear whether the recovered cells retained effector phenotype and if there was a wide distribution to other organs. Another report provided *in vivo* efficacy data in human colon carcinoma xenografted mice (34). Of note, this interesting study confirmed the greater antitumor efficacy of $V\delta1^+$ cells over $V\delta2^+$ γδ T cells, which correlated with their expression of NCRs, suggesting that colon carcinoma could be an interesting target for DOT cells. Along the same line, Deniger and colleagues showed that, among γδ T-cell subsets, $V\delta1^+$ T cells were the most efficient at targeting ovarian cancer xenografts *in vivo* (32).

We used an *in vivo* xenograft CLL model previously shown to reproduce several aspects of human disease (26, 27). The MEC-1 cell line may not provide information on all poorly immunogenic leukemias, but represents a very aggressive tumor. Interestingly, our experiments in BRG hosts present reduction in tumor size data comparable with the reduction of primary tumor size obtained with the potent CAR-T cell strategy (27). We were particularly struck by the prevention of tumor dissemination upon DOT cell ACT in our modified NSG xenograft model, which was associated with their distribution to the same tissues and organs as circulating tumor cells. This strongly encourages the application of DOT cells to eliminate minimal residual disease (MRD), a major goal in CLL treatment.

Our analysis of DOT cells, almost 2 months after ACT, revealed a fully functional (effector) phenotype in all tissues analyzed, including the tumor itself. Importantly, even after almost 2 months *in vivo*, DOT TILs produced type I cytokines, IFNγ and TNFα, but no IL17, which has been implicated in the promotion of tumor cell growth (3, 10). Also of note, the recovered DOT cells were 98% to 100% $V\delta1^+$ T cells (up from ∼65% in the initial inoculum), demonstrating that these constitute the active principle of our cellular therapy. Finally, *in vivo* data suggested that the antitumor response is required for DOT-cell persistence, although this hypothesis requires further research.

Although our results present a solid case for the efficacy of DOT cells against CLL, we also believe that their persistence as type 1 effectors in multiple organs and tissues, in absence of toxic effects,

is a promising indication of a favorable safety profile for the therapy. However, xenograft murine models have important limitations for safety assessment, because the evaluation of cytokine release syndrome is not accurate in immunodeficient animals; and the absence of autoimmune side effects cannot be translated to human host tissues. These issues will need to be addressed in dose-escalation phase I clinical trials. Encouragingly, the clinical application of $V\delta2^+$ T-cell–based ACT was free of any severe adverse effects (35).

The other limitation of our study is the lack of identification of the relevant CLL antigens recognized by DOT cells. This is not a trivial question given that various receptors, TCRVδ1, NKp30, and NKp44, contributed to MEC-1 cell recognition and targeting *in vitro*; and all these receptors have ill-defined and controversial ligands, particularly in the context of interactions with tumor cells (3, 6, 36). Despite the lack of information on CLL antigens for DOT cell-mediated recognition, we provided strong evidence for a molecular cooperation between the TCR and NCRs on the DOT cell side. Importantly, NKp30 and NKp44 expression is absent in freshly isolated γδ T cells but is induced selectively on $V\delta1^+$ T cells by combined TCR and IL15 stimulation (ref. 9 and Fig. 1B).

When anticipating the clinical application of DOT-ACT, it is encouraging to note the reported persistence of $V\delta1^+$ T cells in leukemia patients that received hematopoietic stem cell transplantation (HSCT; ref. 37). In fact, the period following haploidentical HSCT in leukemia patients is likely a very promising therapeutic setting for DOT cells to prevent leukemia relapse (38, 39). However, our ACT strategy has the potential to synergize with newly approved therapeutic agents (11–13) toward eliminating MRD and thus provide tumor eradication.

Finally, although CLL was chosen as first application of DOT-cell ACT based on our and others' previous supportive preclinical data (9, 17, 18), our preliminary *in vitro* data shows that DOT cells can effectively target various other cancer types, both hematologic (such as multiple myeloma and acute lymphoblastic or myeloid leukemias) and solid tumors (breast, ovarian, prostate, and colon carcinoma cell lines). Thus, besides providing proof of concept for CLL, this study may constitute a platform for wider application of DOT cell ACT in cancer immunotherapy.

## Disclosure of Potential Conflicts of Interest

A.RM. Almeida reports receiving commercial research grants from Lymphact and is listed as a co-inventor on a patent, which is owned by Lymphact SA, on novel methods for isolation and *ex vivo* expansion of Vdelta2 negative gammadelta T cells and their clinical application. D.V. Correia and D.R. Anjos have ownership interest (including patents) in and report receiving commercial research grants from Lymphact. B. Silva-Santos has ownership interest (including patents) in Lymphact S.A. No potential conflicts of interest were disclosed by the other authors.

## Authors' Contributions

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**Development of methodology:** A.R. Almeida, D.V. Correia, A. Fernandes-Platzgummer

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M.G. da Silva, D.R. Anjos

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** A.R. Almeida, D.V. Correia

**Writing, review, and/or revision of the manuscript:** A.R. Almeida, D.V. Correia, M.G. da Silva, D.R. Anjos, B. Silva-Santos

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** D.R. Anjos

**Study supervision:** D.R. Anjos, B. Silva-Santos

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