Delta One T cells for immunotherapy of chronic lymphocytic leukemia: clinical-grade expansion/differentiation and preclinical proof-of-concept

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Vδ1+ T cells, a subset of γδ T lymphocytes with strong tropism for tissues, is endowed with potent anti-tumor 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 pre-clinical 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.
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 GMP-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 (over 2-3 weeks) Vδ1+ T cells from the peripheral blood of healthy donors and chronic lymphocytic leukemia (CLL) patients. 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 (NCRs), 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 IFN-γ and TNF-α, but importantly no IL-17, *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.
Introduction

Among various lymphocyte subsets being considered for cellular immunotherapy of cancer are γδ T cells, which provide robust and durable anti-tumor 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). While 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 to 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 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 interleukin-17 (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 (interferon-γ-producing) Vδ1+ T cells expressing activating/ cytotoxicity receptors using clinical-grade reagents in order to
obtain sufficient numbers for ACT; and to provide robust pre-clinical proof-of-principle of their anti-tumor specificity and efficacy.

Among the various tumor targets of Vδ1+ T cells (6), we focused on B-cell chronic lymphocytic leukemia (CLL) (9). Although CLL treatment has recently seen substantial progress (11-13), most of the newly approved agents fail to induce complete tumor remissions. On the other hand, 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 chronic lymphocytic leukemia 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 Supplemental Methods.

Leukemia cell line

The MEC-1 CLL cell line (19) was obtained from the German Resource Center for Biologic Material (DSMZ; reference number 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

MACS-sorted γδ T cells (obtained as described in Supplemental Methods) were resuspended in serum-free culture medium (OpTmizer-CTS) supplemented with 5% autologous plasma and 2mM L-glutamine (Thermo Fischer Scientific). Cells were transferred to a closed gas-permeable cell culture plastic bag (Saint-Gobain), at a maximum concentration of 1x10^6 cells/ml. Animal-free human cytokines (100ng/ml rIL-4, 70ng/ml rIFN-γ, 7ng/ml rIL-21 and 15ng/ml rIL-1β; all from Peprotech) and a soluble antibody (70ng/ml anti-CD3 mAb, clone OKT-3; BioXcell) were added to the medium. Cells were incubated at 37ºC and 5% CO2. Every 5-6 days, old medium was removed and replaced with fresh medium supplemented with cytokines (including 70ng/ml rIL-15 and 30ng/ml IFN-γ), and with 1μg/ml anti-CD3 mAb. A detailed list of these or functionally equivalent GMP-grade reagents and materials is presented in Table S1. Accessory "feeder" cells were not required in this patented method. Live cells were counted using Trypan Blue-positive exclusion in a haemocytometer.
**In vitro** tumor-killing assays

All tumor cells were cultured in T25 flasks in complete 10% RPMI 1640 with 10% FBS, 2mM L-Glutamine and maintained at $10^5$ up to $10^6$ cells/mL by dilution and splitting in a 1:3 ratio every 3-4 days. For cytotoxicity assays, *in vitro* expanded $\gamma\delta$ T cells were plated in 96-well round-bottom plates. For receptor blocking, $\gamma\delta$ PBLs were pre-incubated for 1 hour with blocking antibodies: human anti-TCR$\gamma\delta$ (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 IgG1,k (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-V$\delta$1 TCR (clones TCS-1 or TS8.2) were from Fisher Scientific, and human anti-TCR$\gamma\delta$ (clone IMM510) was from BD Biosciences. Alternatively, tumor target cells were pre-incubated 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 µM; Molecular Probes, Invitrogen) and incubated at the indicated target:effector ratio with $\gamma\delta$ T cells in RPMI for 3 hours at 37°C and 5% CO2. Cells were then stained with annexin V–FITC (BD Biosciences) and analyzed by flow cytometry.

**Mice and adoptive transfer of DOT cells**

Balb/cRag$^{-/-}\gamma c^{-/-}$ (20) animals were obtained from Taconic (USA); NOD-SCID$\gamma c^{-/-}$ (21) mice were obtained from the Jackson Laboratories (USA). 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 $2\times10^7$ DOT cells, and then analyzed (tumor size, histology, flow cytometry of tumor or organ infiltrates, and blood biochemistry) as detailed below or in Supplemental 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-TCRV$\delta$1–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 70µM cell strainers. Femurs were flushed and then filtered. Cells were then stained with the following antibodies from ebioscience, Biolegend, Myltenyi Biotec or Beckton Dickinson: anti-mouse CD45 (30-F11), and anti-Human: CD45 (HI30), IL-17A (BL-168), IFN-γ (B27), TNF-α (MAb-11), Ki-67 (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 Ki-67 staining, cells were stained using Foxp3 staining buffer set from ebioscience. Flow cytometry acquisition was performed on a LSR FortessaII (BD-Biosciences) and data analyzed with FlowJo 8.8.7 software (Tree Star).

**Statistics.** Statistical analysis was performed using Graphpad-Prism software. Sample means were compared using the unpaired Student’s *t*-test. In case variances of the two samples were found different using F-test, the data was log transformed and if variances were then 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 (Figure S1). In order to optimize the selective expansion of Vδ1+ T cells, isolated γδ T cells were cultured in 96-well plates for 2-3 weeks in the presence of 58 different T/NK cell-activating molecules (Table S2). These included 13 different TCR agonists, 23 different co-receptor 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 IL-4 and, surprisingly, IFN-γ (Figure 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 (Figure 1B). Based on published evidence (22), we reasoned that IL-4 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 IL-4; and then an additional week in a second culture medium containing IL-15, a key differentiation factor for type 1 cytotoxic Vδ1+ T cells (23), instead of IL-4. 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 (Figure 1B). For simplicity, we termed the Vδ1+ T cell-enriched and NCR-expressing cytotoxic cellular product, derived from our two-step protocol, DOT (Delta One T) cells. Complementation experiments with additional stimulation factors revealed synergistic effects of IL-21 and IL-1β, during the first step, which resulted in markedly increased DOT cell yield (Figure 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 (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
(Figure 1D-E). Of note, reproducible expansion was achieved and the composition of the
final cellular product was remarkably similar across the multiple donors (Tables S5 and
S6). Moreover, DOT cells could be efficiently generated from the PBLs of elderly (>65
years old) CLL patients with very high tumour burden (Table S6 and Figure 1D-E).
Finally, while 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 (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 (Figure 2A-C
and Figure S2). We observed marked upregulation of the activation markers CD69 and
CD25 (Figure 2A) and HLA-DR (Figure S2), as well as the costimulatory receptors
CD27, CD134/OX-40 and CD150/SLAM (Figure 2A), indicators of enhanced
proliferative potential of in vitro-generated DOT cells (compared to 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
(Figure 2A and Figure S2), all previously shown to be important players in tumor cell
targeting (2, 3). By 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 (Figure 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, IL-
18Rα and Notch1, which are known to promote type 1 (interferon-γ-producing) responses
(25), were also highly expressed by DOT cells, whereas CD161 (which associates with
IL-17-producing cell subsets), was downregulated after 21 days of culture (Figure 2A-B and Figure S2). Importantly, in support of the robustness of our method, we found strikingly similar DOT cell phenotypes across all 4 tested donors, as illustrated by the heatmap in Figure 2C. These data collectively characterize DOT cells as a highly reproducible cellular product of activated (non-exhausted) lymphocytes endowed with migratory potential and natural cytotoxicity machinery.

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

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

In order 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 (Figure 3E). We found that the TCRVδ1 made an important contribution to MEC-1 targeting, which also depended on CD2-mediated adhesion, but the combined blockade with NKp30 and NKp44 essentially abolished the DOT-mediated cytotoxic effect (Figure 3E).

Since DOT cells correspond to yet heterogeneous cell lines with a significant fraction of Vδ1neg cells, we asked whether these distinct subsets were cytotoxic against MEC-1 cells in vitro. We re-analyzed DOT-cell samples generated from 4 donors (the same as described in Figure 2), and identified NKp30, NKp44 and CD2 expression on CD3+Vδ1- Vδ2- T cells (Figure S3A). To investigate their function, we thawed previously frozen DOT cells from 3 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 (Figure S3B); both subsets were highly cytotoxic against MEC-1 cell line in vitro (Figure S3C). Finally, the simultaneous blockade of TCRVδ1, CD2, NKp30 and NKp44 significantly inhibited DOT-cell killing of primary CLL samples in vitro (Figure S3D). These data demonstrate that DOT cells combine T-cell-specific (their signature TCRVδ1) and NK cell-characteristic (NCRs) 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 sub-cutaneous injection of MEC-1 cells into Balb/cRag-/-γc-/- (BRG) animals. We detected human Vδ1+ cells early after transfer (for example, in the liver and lung), and observed a significant reduction in bulk tumor size in DOT-cell-treated animals compared to mock (PBS-injected) controls (Figure 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 (Figure 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 (Figure 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 (Figure 4A). Histological analyses confirmed that the transferred T cells infiltrated the tumor and other tissues (liver, kidney, bone marrow) (Figure 4B), and recovered DOT cells expressed NKp30 and NKG2D (Figure 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; Figure S5) and their maintenance (up to 55 days after inoculation, depending of mouse survival; Figure 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 (Figure S5), ruling out preferential seeding. Conversely, at late time points (45-55 days) we recovered substantial numbers of human T cells, strongly enriched in Vδ1+ T cells (Figure 4D). Interestingly, we could not recover DOT cells in
animals that had not been pre-inoculated with tumor (Figure 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 1 effector phenotype: marked production of IFN-γ and TNF-α in absence of IL-17 (Figure 4E). These data demonstrate that DOT cells home to multiple tissues, infiltrate the tumor and persist as fully functional bona fide type 1 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, i.e., the impact of DOT-cell ACT on tumor growth (and dissemination). Despite encouraging data in BRG hosts (Figure S4), DOT cells failed to persist in those animals. Since this limitation was solved in NSG mice (Figure 4), we used them to establish MEC-1 cells (10x10^6) and to transfer two doses of 2x10^7 DOT cells. We measured tumor throughout the experiment, and sacrificed and analyzed animals when tumor reached limit size (1000mm^3). Data collection and analysis up to 60 days post-tumor inoculation showed that DOT-cell-treated animals stayed alive for markedly longer (Figure S6A), including one tumor regression, and overall survival data demonstrated the efficacy of DOT cell therapy (Figure 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 histological samples at the end of the experiment showed a clear reduction in tumor cells recovered from all organs tested (of DOT cell-treated compared to PBS-controls), with an emphasis on major tumor dissemination sites such as liver and bone marrow (Figures 5A-B). Of note, there was a striking difference in the number of animals whose organs displayed tumor infiltrates in the treated and control groups (Figure 5B). This strong protective effect of DOT-cell ACT could be also documented by flow cytometry analysis (Figure 5C). Analysis of a group of NSG hosts receiving half the dose (1x10^7) of DOT cells, showed intermediate results, thus suggesting a dose-dependent therapeutic effect (Figure 5C, right panel). Of note, blood biochemistry analysis (Figure S7), as well as histological evidence (Figure 4B, Figure 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 pre-clinical level, thus supporting its translation to CLL patient treatment.
Discussion

The field of ACT for treatment of hematological 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 \( V\delta1^+\gamma\delta \) T cells that selectively recognize and eliminate malignant leukocytes. While several methods have been described in the past 5 years that can generate substantial numbers of tumor-targeting \( V\delta2^{neg} \) T cells \textit{in vitro}, key unresolved problems still excluded a clinical application of these cells: 1) the use of unsafe reagents and materials (such as plant lectins and plastic culture plates) in the manufacturing process; 2) the high level of variation in the composition of the final cell products, especially between different donors; and/ or 3) the low anti-tumor activity of the final product (6, 18, 30, 31).

In a previous study, Deniger et al. developed tumor-derived artificial antigen presenting cells (aAPCs) to propagate high numbers of \( \gamma\delta \) T cells expressing a polyclonal repertoire of \( \gamma \) and \( \delta \) TCR chains (32). However, as pointed out by the authors (33), the method could not resolve critical obstacles associated with clinical application of \( \gamma\delta \) 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.

In sharp contrast, our study discloses the first protocol with direct clinical adaptability, using reagents and materials currently available in pharmaceutical grade (Table S1), facilitating regulatory approval towards phase I clinical trials. Our major breakthrough was to uncouple expansion and differentiation stages, since these were optimally supported by distinct (and antagonistic in their outcome) cytokines, IL-4 and IL-15, respectively. This new two-step treatment allowed us to obtain the highest expansion of \( V\delta1^+ \) T cells ever recorded \textit{in vitro} (in plastic culture plates): >60,000 fold increase in 21 days (Figure 1C), compared to <25,000 fold obtained by Siegers et al. (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) Vδ1+ T cells from highly unpurified samples
obtained from CLL patients, differentiating them into NCR-expressing and highly
cytotoxic DOT cells.

Few previous studies on Vδ1+ T cells provide pre-clinical 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 anti-tumor efficacy of Vδ1+ cells over Vδ2+ 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 et al. showed that, among γδ T cell subsets, Vδ1+ 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 to 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 two months after ACT, revealed a fully functional (effector) phenotype in all tissues analyzed, including the tumor itself. Importantly, even after almost two months in vivo, DOT TILs produced type 1 cytokines, IFN-γ and TNF-α, but no IL-17, which has been implicated in the promotion of tumor cell growth (3, 10). Also of note, the recovered DOT cells were 98-100% Vδ1+ 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 anti-tumor response is required for DOT cell persistence, although this hypothesis requires further research.
While 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, since 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 Vδ2+ 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 Vδ1+ T cells by combined TCR and IL-15 stimulation (9) (Figure 1B).

When anticipating the clinical application of DOT-ACT, it is encouraging to note the reported persistence of Vδ1+ T cells in leukemia patients that received haematopoietic stem cell transplantation (HSCT) (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). On the other hand, our ACT strategy has the potential to synergize with newly approved therapeutic agents (11-13) towards eliminating MRD and thus provide tumor eradication.

Finally, while CLL was chosen as first application of DOT-cell ACT based on our and others’ previous supportive pre-clinical data (9, 17, 18), our preliminary in vitro data shows that DOT cells can effectively target various other cancer types, both hematological (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.
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Author contributions: B.S.-S., D.V.C., A.R.A., C.L.d.S., M.G.d.S. and D.R.A. designed research; A.R.A., D.V.C. and A.F.-P. performed research; B.S.-S. supervised the research; A.R.A., D.V.C. and B.S.-S. wrote the manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1. Two-step method to selective expand and differentiate Delta One T (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 IL-4 (gray bar). Cells were further supplemented with the indicated cytokines and cultured for 2 weeks in non-clinical 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: IL-2 (2ng/ml), IL-15 (0.7ng/ml), IL-7 (20ng/ml) and IFN-γ (200ng/ml). B. Cells from the best initial culture condition (+IFN-γ in panel A) were split at day 14 and one fraction of cells was cultured in the same conditions (IL-4), while the other fraction was cultured in presence of OKT-3, IFN-γ and 100ng/ml IL-15 (without IL-4). 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 3 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-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, 1L 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.

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 LEGENDScren kit. Shown are histogram overlays for 14 markers related to lymphocyte activation and differentiation (A); and 7 markers implicated in adhesion and migration (B). Cells from one healthy donor are shown. C. Heatmap representing percentages of positive cells for each surface marker (from Fig. 2A-B and Fig. S2) across DOT cells (at day 21 of
culture) produced from 4 different healthy donors (1-4), compared to freshly-isolated Vδ1+ T cells (from donors 1 and 2). The color code is presented on the right.

**Figure 3. TCR/ NCR-dependent DOT cytotoxicity against leukemic (but not healthy) cells.**

A. DOT cells produced from 5 healthy donors were co-incubated with MEC-1 (CLL) cells (upper panel) or autologous healthy PBMCs (lower panel). The death of target cells (pre-labeled 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 3 technical replicates. B. DOT cells generated from a healthy donor were co-incubated 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 3 technical replicates. C. DOT cells generated from 2 CLL patients were co-incubated for 3h 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). 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’s t-test).

**Figure 4. Activated type 1 effector DOT cells persist, home to peripheral tissues and infiltrate tumors in vivo.**

A. DOT cells (2x10⁷) 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 Vδ1+ sub-fraction in transferred and recovered DOT cells.
(representative of 6 independent hosts). Top plots were pre-gated on total (human + mouse) CD45\(^+\) cells. **B.** Immunohistochemistry 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\(^+\)Vδ1\(^+\) cells. **D.** DOT cell recovery and Vδ1\(^+\) 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 (2x10\(^7\)) transfer. Data from individual animals (dots) and average (bars) is shown. **E.** Expression of the proliferation marker Ki-67 and the effector cytokines IFN-\(\gamma\), TNF-\(\alpha\) and IL-17 in Hu-CD3\(^+\)Vδ1\(^+\) 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.

**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\(^-\)) showing tumor infiltration (MEC-1, Hu-CD19\(^+\)) at the end of the experiment in each indicated organ in one representative animal from PBS control group or DOT (2x10\(^7\)) 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 (1x10\(^7\)) of DOT cells (*p<0.05; **p<0.01, Student’s t-test).
**Figure 3**

A. Dot blot analysis showing the expression levels of various proteins in PBMCs from different donors. The graphs depict Annexin-V versus DDAO-SE for the donor samples. The presence of (+) DOT and (-) DOT is indicated.

B. Bar graph showing the percentage of dead cells (% dead) for different target cells (CLL-A, CLL-B, CLL-C, Autol. PBMCs) treated with (+) DOT and (-) DOT. The results are statistically significant (***).

C. Similar to B, but for MEC-1.

D. Histogram comparing the percentage of dead cells (%) for different target cells (Autol. CLL, Autol. CLL, Autol. αβ+ T cells) treated with (+) DOT and (-) DOT. The results are statistically significant (***).

E. Graph showing the percentage of dead cells (%) for different conditions (Expt 1 and Expt 2) with varying Eff:Target ratios (1:2, 1:1, 2:1, 5:1). The results are statistically significant (*, #, ##, ***).
Figure 4

A

Transferred DOT-Cells

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Vδ1

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B

Tumor | Liver | Kidney | Bone Marrow

C

Gated Hu-CD3+Vδ1+

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D

Hu-CD3 recovery

Vδ1 %

E

Gated Hu-CD3+Vδ1+

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