Broad Cytotoxic Targeting of Acute Myeloid Leukemia by Polyclonal Delta One T Cells

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Abstract

Acute myeloid leukemia (AML) remains a clinical challenge due to frequent chemotherapy resistance and deadly relapses. We are exploring the immunotherapeutic potential of peripheral blood V61+ T cells, which associate with improved long-term survival of stem-cell transplant recipients but have not yet been applied as adoptive cell therapy. Using our clinical-grade protocol for expansion and differentiation of “Delta One T” (DOT) cells, we found DOT cells to be highly cytotoxic against AML primary samples and cell lines, including cells selected for resistance to standard chemotherapy. Unlike chemotherapy, DOT-cell targeting did not select for outgrowth of specific AML lineages, suggesting a broad recognition domain, an outcome that was consistent with the polyclonality of the DOT-cell T-cell receptor (TCR) repertoire. However, AML reactivity was only slightly impaired upon V61+ TCR antibody blockade, whereas it was strongly dependent on expression of the Nkp30 ligand, B7-H6. In contrast, DOT cells did not show reactivity against normal leukocytes, including CD33+ or CD123+ myeloid cells. Adaptive transfer of DOT cells in vivo reduced AML load in the blood and target organs of multiple human AML xenograft models and significantly prolonged host survival without detectable toxicity, thus providing proof-of-concept for DOT-cell application in AML treatment.

Introduction

Acute myeloid leukemia (AML) has a dismal (10%) survival rate among the elderly (age 65 or older), mostly due to resistance to standard treatment. Available treatment consists of a combination of cytarabine with an anthracyclin drug, which although achieves >1,000-fold expansions of V61+ T cells. During the first stage, the synergistic action of TCR and IL4 stimulation is critical for large expansion yields. The second stage focuses on the IL15-dependent differentiation of antitumor DOT-cell effectors endowed with antitumor cytotoxicity (5, 6). Our 3-week protocol achieves >1,000-fold expansions of V61+ γδ T cells. During the first stage, the synergistic action of TCR and IL4 stimulation is critical for large expansion yields. The second stage focuses on the IL15-dependent differentiation of antitumor DOT-cell effectors endowed with NCR expression (6). Seeing the convergence between the properties of DOT cells with the unmet immunotherapy needs of AML, in this study, we aimed to refine DOT cells for use in treatment of AML.

Materials and Methods

Ethics statement

Primary AML cells were obtained from the peripheral blood of patients at first presentation, after informed consent and institutional review board approval. The study was conducted in accordance with the Declaration of Helsinki.
Mice

NOD SCID $\gamma^{-/-}$ (NSG), NOD SCID $\gamma^{-/-}$ SGM3 (NSGS), and
NOD Rag1$^{-/-}$ $\gamma^{-/-}$ SGM3 (NRGS) mice were obtained from the
Jackson Laboratories. Age and sex-matched mice were randomly
distributed among the different groups. Disease development was
followed through weekly bleedings (in intrabone marrow mod-
el) and disease end-point is achieved upon first indication of
back leg decreased mobility. All animal procedures were per-
formed in accordance to national guidelines from the Direção
Geral de Veterinária and approved by the Animal Ethics Com-
mittee of Instituto de Medicina Molecular João Lobo Antunes
(Lisboa, Portugal).

Figure 1.

DOT cells display higher clonal diversity than ex vivo VSD $^+$ T cells. Graphical representation of TRGV (left) and TRDV (right) repertoires and CDR3 length (number of nucleotides) distribution of FACS-sorted VSD $^+$ T cells from peripheral blood/PB (A); or DOT-cell products (B; see also Supplementary Fig. S2A–S2C). Each square represents a different clonotype (with distinct nucleotide sequence), its area is proportional to the relative abundance in the sample; and the color groups the clonotypes by chains. C, Contribution of the top 20 clones to the overall VSD $^+$ TCR repertoire of each sample (HD1-4, healthy donors 1 to 4). D, Fold expansion of presorted CD27 $^-$ versus CD27 $^+$ VSD $^+$ T cells after 21 days with the DOT-cell protocol (HD5-6, healthy donors 5 and 6). E, Graphical representation of TRGV repertoire and CDR3 length distribution (as in A–B) for CD27 $^-$ versus CD27 $^+$ VSD $^+$ T cells cultured for 21 days with the DOT-cell protocol (see also Supplementary Fig. S3). F, Shannon indices for intrasample variability of TRGV and TRDV repertoires from A–B and E. G, Percentage of CD27 $^+$ cells upon expansion with the
DOT-cell protocol. Data in this Figure are derived from 6 independent healthy donors.
DOT-cell production and TCR repertoire analysis

DOT cells were produced from peripheral blood of healthy donors, cultured for 21 days as previously detailed (6). In brief, MACS-sorted γδ T cells were resuspended in serum-free culture medium (OptiMizer-CTS) supplemented with 5% autologous animal-free human cytokines rIL4 (100 ng/mL), rIFN-γ (70 ng/mL), and rIL1β (7 ng/mL); and a soluble mAb to CD3 (clone OKT-3, 70 ng/mL; BioXcell), were added to the medium. Cells were incubated at 37°C in 5% CO2. Every 5–6 days, old medium was removed and replaced with fresh medium supplemented with cytokines, including rIL15 (70 ng/mL), rIFN-γ (30 ng/mL), and anti-CD3 (1 µg/mL). For TRGV and TRDV repertoire analysis, γδ T cells were FACS-sorted either from the initial blood sample (ex vivo); or from the final (3-week culture) DOT-cell product. Next-generation sequencing was performed as described previously (9, 10). The software for data analysis is described in Supplementary Table S1.

For DOT-cell clone generation, CD3+ TCRV81+ TCRV82+ single cells were FACS-sorted into 96-wells/plates; and cultured for 21 days using the DOT-cell protocol in the presence of (weekly renewed) 103 irradiated autologous peripheral blood mononuclear cells (feeders).

AML cell targeting in vitro and in vivo

AML cell lines (THP-1, HEL, AML-193, MV4-11, HL-60, U-937, OCI-AML3, Kasumi-1, and KG-1) were obtained from and authenticated by the German Resource Center for Biologic Materials (DSMZ); and used at passages p3–p8. Lentiviral barcoding of AML cells was performed and analyzed as detailed previously (11). For in vitro targeting, AML cell lines or primary samples were coincubated with DOT cells for 3 hours; and stained with Annexin V, as detailed previously (6). For in vivo targeting, three xenograft hAML models were established as represented in Supplementary Fig. S5A–S5C. The patient-derived xenograft (intratibial injection) was described previously (12). Tumor burden was assessed by staining with antihuman CD45 (HI30) and CD33 (P67.6). Flow cytometry acquisition was performed on a ISR Fortessa (BD Biosciences) and data was analyzed with FlowJo X software (Tree Star).

CRISPR/Cas9 Knockout

Guide RNAs (gRNA1: CACCGTTCGGGACCCGTATAAC; gRNA2: CACCGGGGCTCTGATCCAAATGAT) were designed to target the genomic sequence of B7-H6 in two areas close to the promoter. gRNAs were inserted into a plasmid containing the sequence codifying for Cas9 enzyme and transacted by electroporation in HEL cells. Successful single-cell knockout clones were confirmed by qRT-PCR (forward primer: CACAGGGAACAGTCGTGTC; reverse primer: TGAATCACACAAATTCCCC). AML cell targeting, AML cell lines or primary samples were coincubated with DOT cells for 3 hours; and stained with Annexin V, as detailed previously (6). For in vivo targeting, three xenograft hAML models were established as represented in Supplementary Fig. S5A–S5C. The patient-derived xenograft (intratibial injection) was described previously (12). Tumor burden was assessed by staining with antihuman CD45 (HI30) and CD33 (P67.6). Flow cytometry acquisition was performed on a ISR Fortessa (BD Biosciences) and data was analyzed with FlowJo X software (Tree Star).

Statistical analysis

Performed using GraphPad Prism software. All data expressed as mean ± SEM. Comparisons of two groups by Student t test; and more than two groups by ANOVA test with Dunnet post test.
Animal survival comparisons performed using log-rank (Mantel–Cox) test.

Data sharing
The TCR repertoire NGS data have been deposited with links to BioProject accession number PRJNA491919 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/); the sample list is available as Supplementary Table S2.

Results and Discussion
We are interested in developing a Vδ1⁺ T cell–based adoptive cell therapy for cancer (5, 6). Here, we initially characterized the DOT-cell product upon expansion of αβ-depleted peripheral blood mononuclear cells with the established DOT-cell protocol (6). We analyzed Vδ1⁺ T-cell percentages and amounts along with NCR expression over time (Supplementary Fig. S1A–S1F).

Because reports described the clonal expansion and focusing of the adult peripheral blood Vδ1⁺ T-cell repertoire (13), likely driven by common pathogens such as CMV (10), we analyzed the effect of the DOT-cell expansion on the TCR repertoire. We performed next-generation sequencing of the CDR3 regions in TRGV and TRDV genes, before and after the cells were 3 weeks in culture. We found DOT-cells to be highly polyclonal and devoid of dominant clones, in contrast to ex vivo Vδ1⁺ T cells from all donors analyzed (Fig. 1A and B; Supplementary Fig. S2A–S2C).
This was illustrated by the contribution of the top 20 expanded clones to the overall Vβ1+ TCR repertoire. Although these 20 clones represented >60% in the peripheral blood, they accounted for less than 10% in the DOT-cell products (Fig. 1C). Moreover, few clonotypes (especially for TRDV) were shared between those identified ex vivo and in DOT cells (Supplementary Table S3). We next aimed to better understand the basis for the diversification of the DOT-cell repertoire. Given the previous association of CD27 downregulation with preexpanded/differentiated Vβ1+ T cells (13), we compared the TCR clonality of DOT cells produced from presorted CD27+ versus CD27– subsets, which displayed distinct proliferation capacities under the DOT-cell protocol (Fig. 1D). We found that the generation of diverse DOT cells was restricted to CD27+ precursors (Fig. 1E and F; Supplementary Fig. S3A). Taking into account that CD27+ cells typically represent only a small fraction of ex vivo Vβ1+ T cells (Supplementary Fig. S3B), these data suggest that the DOT-cell protocol preferentially expands naive-like CD27+ Vβ1+ T cells with a diverse TCR repertoire at the expense of preexpanded and terminally differentiated CD27– Vβ1+ T cells.

In support of this, the DOT-cell population (generated from bulk Vβ1+ T cells) was largely composed of CD27+ cells (Fig. 1G). DOT-cell products originated from presorted CD27+ cells expressed NKp30 and were highly cytotoxic against KG-1 AML cells (Supplementary Fig. S3C and S3D).

To assess the functional relevance of DOT-cell polyclonality, we generated clones from single-cell sorted Vβ1+ T cells, expanded/differentiated using an adapted DOT-cell protocol including the addition of feeder cells. We tested their cytotoxicity against the AML cell line KG-1 (Fig. 2A). We found the majority of clones (from different donors) to be efficient at inducing apoptosis of KG-1 cells upon short (3-hour) coincubation in vitro (Fig. 2A). These results show that DOT-cell products are composed of multiple clones with intrinsic capacity to target AML cells. To functionally test whether the TCR is involved in this reactivity, we performed the killing assay in the presence of a Vβ1+ TCR-specific blocking mAb (or isotype control), and observed only a mild reduction in KG-1 cell targeting across a number of clones from different donors (Fig. 2B). At this stage, we hypothesized that most of the reactivity was mediated by natural cytotoxicity receptors (5–7), particularly Nkp30 (Supplementary Fig. S1F). In fact, DOT-cell cytotoxicity was significantly decreased upon CRISPR/Cas9-mediated knockout (at the population level) of the best established tumor-associated Nkp30 ligand, B7-H6, in target AML cells (Fig. 2C and D).

To further evaluate the anti-AML activity, we tested bulk DOT-cell products (from multiple donors) against various other AML cell lines as well as primary samples obtained from patients at diagnosis. In all cases, DOT cells readily (within 3 hours) killed AML cells in vitro (Fig. 3A and B), in similar fashion to what was reported for CAR-T cells (14–16), and unlike ex vivo Vβ1+ T cells (Supplementary Fig. S4A). DOT-cell cytotoxicity associated with increased degranulation and expression of perforin and granzyme B upon tumor cell recognition (Supplementary Fig. S4B and S4C). DOT cells did not target any normal leukocyte population (myeloid or lymphoid).

Figure 4.

DOT cells (re-)target chemotherapy-resistant AML. Comparison of the in vitro anti-AML activity of DOT cells and standard chemotherapy. A, DOT cells and standard AML chemotherapy (doxorubicin plus cytarabine) protocols were tested against chemotherapy-naïve (wild type, wt) or chemo-relapsed (CR, regrown after >99% HEL cell elimination) AML cells. Shown are the percentages of Annexin V+ HEL cells after 3 hours of treatment. B, Number of AML HEL cells before and after 72 hours of treatment with DOT cells (at 5:1 E:T ratio). Surviving cells (<1%) were resorted and allowed to regrow, thus generating the DOT-treated (DT) samples of (C–E). C, DOT cells were coincubated for 3 hours with nontreated (NT) or previously DOT-treated (DT) AML HEL cells at 5:1 or 10:1 (E:T) ratios. Shown are the percentages of Annexin V+ HEL cells. D, Number of barcoded AML single-cell lineages in non-treated (NT), chemotherapy-treated (CT), or DOT-treated (DOT) AML HEL cells. E, Pearson correlation for distribution of barcoded AML single-cell lineages between different treatments. Red, yellow, and green dashed lines represent low, medium, and high correlations, respectively. Indicated are mean ± SEM (**, P < 0.01; ***, P < 0.001; ****, P < 0.0001).
from the peripheral blood of healthy volunteers (Fig. 3C), including CD33+ and CD123+ myeloid progenitor cells, whose on-target depletion by the respective CAR-T cells is known to be responsible for the unwanted myeloablation (2, 15).

To test DOT cells against AML in vivo, we established various independent xenograft models of AML (Supplementary Fig. S5A–SSC). Both in AML cell line models (Fig. 3D and E; Supplementary Fig. SSC) and in two patient-derived xenografts (Figs. 3F and G; Supplementary Fig. SSD and SSE), DOT-cell treatment reduced tumor burden and increased host survival, without noticeable toxicity. Although CAR-T cells have been reported to produce bigger survival benefits in AML xenografts (14–16), these models were biased to AML cell lines uniformly expressing the target antigens. On the other hand, the toxicity of a strategy predicted to induce myeloablation in patients cannot be evaluated with the use of xenografts. Overall, we believe that the combined safety and efficacy profiles of DOT cells make them candidates for adoptive cell therapy of AML in the future.

Chemoresistance drives deadly relapses in the context of AML therapies. We next asked whether DOT cells could target chemoresistant AML cells. For that purpose, we treated AML cells with cytarabine plus doxorubicin for 72 hours, which led to >99% tumor cell elimination, allowed the surviving cells to regrow, then treated the culture with chemotherapy or DOT cells. Although the cytotoxic efficacy of chemotherapy was reduced, the targeting efficacy of DOT cells was unaffected (Fig. 4A), demonstrating the superior capacity of DOT cells to target chemoresistant AML cells. In light of this, and taking into account the polyclonal and multi-reactive DOT-cell repertoire (Fig. 1A–C), we also questioned the ability of DOT cells to retarget AML cells following a DOT-cell treatment that eliminated >99% tumor cells in 72 hours (Fig. 4B). Thus, we FACs-sorted the remaining approximately 0.1% of AML cells present at 72 hours and allowed the cell culture to regrow before retreated with DOT cells. DOT cells killed DOT-pre-treated AML cells as efficiently as nontreated controls (Fig. 4C), suggesting that DOT-cell treatment did not select for a specific subset of DOT-resistant AML cells. To track the AML clonal dynamics upon therapeutic (DOT cells or chemotherapy) pressure, we tagged single AML cells with cellular barcodes (noncoding DNA sequences that can be tracked by NGS; ref. 11). Although chemotherapy selectively targeted approximately half of all the barcoded AML single-cell lineages, DOT cells preserved the clonal architecture of the AML population (Fig. 4D and E). These data collectively suggest that the breadth of AML targeting by cytotoxic DOT cells avoids the selection of resistant lineages and allows efficient retreatment. Emergence of refractory relapses after chemotherapy needs to be prevented. Our work thus provides preclinical proof-of-concept for clinical application of DOT cells in AML treatment.

Disclosure of Potential Conflicts of Interest

D.V. Correia has ownership interest in GammaDelta Therapeutics; B. Silva-Santos reports receiving commercial research funding from, has ownership interest in, and is a consultant/advisory board member for GammaDelta Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B.D. Lorenzo, A.E. Simões, F. Caiado, D. Vermijlen

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.D. Lorenzo, A.E. Simões

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References

1. Wei AH, Tiong IS. Midostaurin, enasidenib, CPX-351, gemtuzumab ozogamicin, and venetoclax bring new hope to AML. Blood 2017;130: 2469–74.


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