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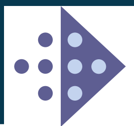

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Brief Communication

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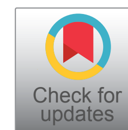
Efficient *Ex Vivo* Expansion of γδ T-Cells from AML Patients Requires Elimination of Circulating Leukemic Blasts



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Abstract

γδ T-cells play an important role in immune surveillance of acute myeloid leukemia (AML). The main circulating subtype expresses a Vγ9Vδ2 T-cell receptor and may be expanded *ex vivo* following aminobisphosphonate activation. While such protocols operate robustly in healthy donors, they are often inefficient using blood samples from patients with advanced malignancy. In keeping with this, we found that when leukemic blasts were present in peripheral blood samples, culture of γδ T-cells from patients with AML was unsuccessful. By contrast, expansion proved much more effective after chemotherapy-mediated clearance of circulating blasts, yielding similar γδ T-cell numbers to healthy donors. This principle was confirmed using serial samples obtained from the same patients. Importantly, expanded γδ T-cells were functional, indicated by bisphosphonate-potentiated anti-leukemic activity and cytokine release *in vitro*. These preliminary findings suggest a therapeutic potential for γδ T-cells, given that they can be readily expanded from AML patients in therapy-induced remission.

Keywords

Leukemia, Adoptive immunotherapy, γδ t-cell, Zoledronic acid

Introduction

γδ T-cells account for up to 3% of peripheral blood mononuclear cells (PBMC). In man, most express the Vγ9Vδ2 receptor, enabling their MHC-independent activation by phosphoantigen (PAg) intermediates of the mevalonate pathway [1]. Dysregulation of mevalonate pathway metabolism is prevalent in AML [2], accounting for the frequent sensitivity of leukemic cells to statins [3] or aminobisphosphonates (e.g. alendronic and zoledronic acid) [4].

Several clinical observations demonstrate a role for γδ T-cells in immune surveillance of AML [5]. First, prolonged survival of AML patients after allogeneic hematopoietic stem cell transplantation (HSCT) correlates with enhanced γδ T-cell recovery. Second, these cells may be expanded *ex vivo* following αβ T-cell-depleted haploidentical HSCT and exhibit potent cytolytic activity against primary AML cells. Third, infusion of haploidentical γδ T-cells followed by administration of zoledronic acid and IL-2 has induced complete remission

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Table 1: Clinical information pertaining to samples tested.

Patient Number	Clinical information	Age	Prior treatment	Interval post CTX (days)	Circulating blasts
001 - sample 1	M1 (FLT3 ITD-)	42	Nil	No CTX	Yes
001 - sample 2			DA × 1	17	No
001 - sample 3			DA × 1 FLAG IDA × 2	42	No*
002 - sample 1	M1 (FLT3 ITD+ NPM+)	19	Nil	No CTX	Yes
002 - sample 2			DA × 1	21	No
003 - sample 1	Transformed from JAK2 mutant myeloproliferative disorder	N/A	FLA × 1	0	No
003 - sample 2			FLA × 1	26	Yes
004 - sample 1	M4 (Inv16)	23	HU	0	No
004 - sample 2			HU + DA × 1	26	No
005 - sample 1	Transformed from refractory anemia with excess blasts with Inv (3)(q21q26)	N/A	DA	46	No
005 - sample 2			DA × 2	15	No
006	M1 FLT3 ITD+	41	DA × 1	28	No
007 - sample 1	M1 FLT3 ITD+	56	DA × 1	25	No
007 - sample 2			DA × 2	28	No
008	M4 t(6;11)(q27;q23)	22	DA × 1	28	No
009	Transformed from JAK2 mutant myeloproliferative disorder; trisomy 8	57	Nil	No CTX	Yes
010	N/A	N/A	Nil	No CTX	Yes

CTX - chemotherapy; DA - daunorubicin + cytarabine; FLA - fludarabine + cytarabine; FLAG-IDA - fludarabine, cytarabine, G-CSF, idarubicin; HU - hydroxyurea; N/A - not available;

*Absolute lymphocyte count 0.2 × 10^9/L with no $CD3^+$ cells detectable. Consequently, no expansion of γδ T-cells was observed from this sample.

in patients with advanced hematologic malignancy, including AML. Fourth, *in vivo* activation of these cells with zoledronic acid and IL-2 has achieved an efficacy signal in AML patients.

These findings provide a rationale to develop γδ T-cell-based immunotherapeutic strategies for AML. In AML patients, circulating γδ T-cells [6,7] which may be expanded *ex vivo* using the synthetic PAg, Phosphostim. Since Phosphostim is no longer undergoing clinical development, we evaluated an alternative system comprising zoledronic acid, IL-2 and IL-15 [8]. We have previously shown that this cocktail can expand γδ T-cells from patients with ovarian cancer [8] and hypothesized that a similar approach could be applied in AML patients.

Methods

Processing of peripheral blood samples

Seventeen blood samples (30 mL each) derived from 10 AML patients (clinical data - Table 1) were provided by the King's College London Haemato-Oncology Tissue Bank (Human Tissue Authority license 12223; National Research Ethics reference 08/H0906/94). All patient-derived peripheral blood samples were independently analyzed for the presence of circulating AML blasts by morphology alone, or accompanied by flow cytometry at the time of banking. Blood samples were also obtained from healthy donors under approval of the South East London Research Ethics Committee 1 (09/H0804/92).

Culture of primary human γδ T-cells

Ficoll-separated PBMC were cultured at 3 × 10^6 cells/ml in RPMI 1640 (Lonza, Basel, Switzerland), 10% human AB serum (Sigma, Poole, UK), GlutaMax (Life Technologies, Paisley, UK) and antibiotic-antimycotic solution (Life Technologies). On day 1, zoledronic acid (1 μg/ml; Zometa, Novartis, Frimley/Camberley, UK), IL-2 (100 U/ml; Proleukin, Novartis) and IL-15 (10 ng/ml; Gentaur, Kampenhout, Belgium) were added. Additional medium and cytokines were added every 2-3 day for 15 days. Numbers of γδ T-cells were determined manually as total cell number multiplied by percentage γδ T-cells in the PBMC gate/100.

Flow cytometry

Flow cytometric analysis was undertaken on freshly isolated PBMC and *ex vivo* expanded γδ T-cell cultures as described [8], using antibodies listed in Supplementary Table 1. Analysis was performed using a FACScalibur cytometer with Cellquest Pro software. Representative forward scatter versus side

scatter gating strategies on freshly isolated PBMC are shown (Supplementary Figure 1).

Cytotoxicity assays

PKH-labeled KG-1 leukemic cells were incubated ± zoledronic acid (5 µg/ml) or alendronic acid (20 µg/ml) overnight before co-culture with γδ T-cells at the indicated effector:target ratio for 4 hours. Apoptotic cells were detected after Annexin V staining by flow cytometry (Supplementary Figure 2) [8].

Enzyme-linked immunosorbent assay

Supernatants were analyzed using a human IFN-γ enzyme-linked immunosorbent assay (ELISA; Ready-set-go kit (eBiosciences, Hatfield, UK)) as described by the manufacturers.

Statistical analysis

Statistical analysis was performed using One-way ANOVA for analysis of groups of 3 or more datasets. Alternatively, an unpaired two-tailed Student's *t* test or Mann-Whitney test was used to compare two groups, where data passed or failed normality testing respectively. All statistical analyses were performed using GraphPad Prism version 5.0, 6.0 or 7.0 (GraphPad software, San Diego, CA, USA).

Results and Discussion

Expansion of γδ T-cells from AML patients

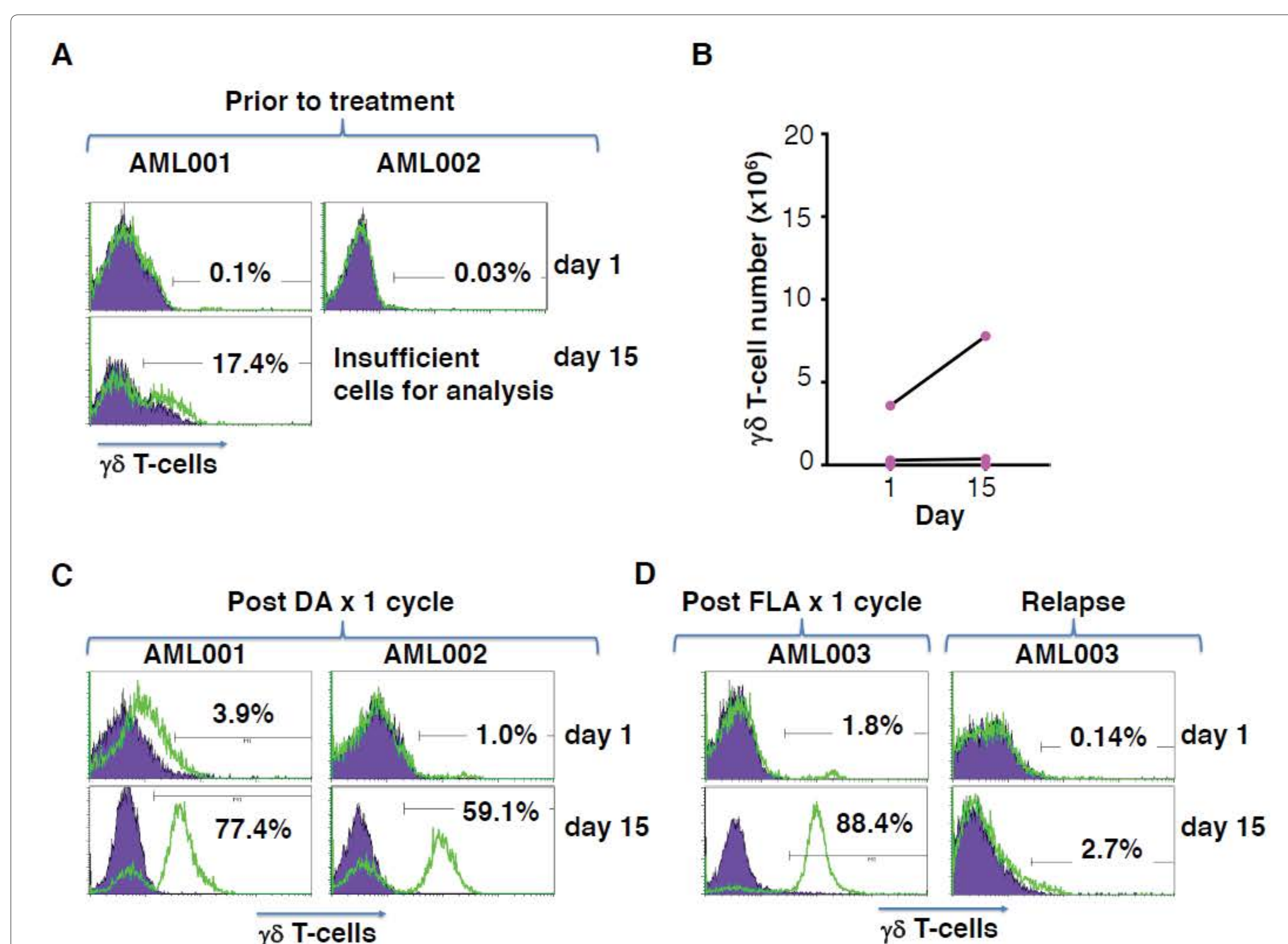

Figure 1: *Ex vivo* expansion of γδ T-cells from AML patients with circulating blast cells is inefficient. (A) PBMC were separated from two patients with newly diagnosed AML (AML001 - M1 subtype without FLT3 internal tandem duplication (ITD); AML002 - M1 with FLT3 ITD and *NPM1* mutation) in whom circulating blast cells were present. Samples were stained with a pan-γδ TCR antibody (open green histogram) and analyzed by flow cytometry, making comparison with an isotype control antibody (filled purple histogram) (day 1). Cultures were then stimulated with ZA followed by IL-2/IL-15 for 15 days prior to re-analysis using similar methodology; (B) Pooled data from 5 AML patients in whom circulating leukemic blasts were present at the time of PBMC collection. In each case, γδ T-cell number was determined manually before (day 1-30mL blood sample) or after culture of derived PBMC for 15 days in zoledronic acid + IL-2/IL-15; (C) Following one cycle of daunorubicin plus cytarabine (DA) induction chemotherapy, PBMC from patients AML001 and AML002 were re-analyzed for γδ T-cells by flow cytometry, both before and after culture in zoledronic acid + IL-2/IL-15; (D) Following 1 cycle of fludarabine plus cytarabine (FLA) chemotherapy, PBMC were separated from AML003 (AML derived from JAK2 mutant myeloproliferative disorder) and were analyzed by flow cytometry before and after culture in zoledronic acid + IL-2/IL-15, as described above. Twenty nine days later, relapse occurred accompanied by circulating blasts and a second sample was analyzed similarly.

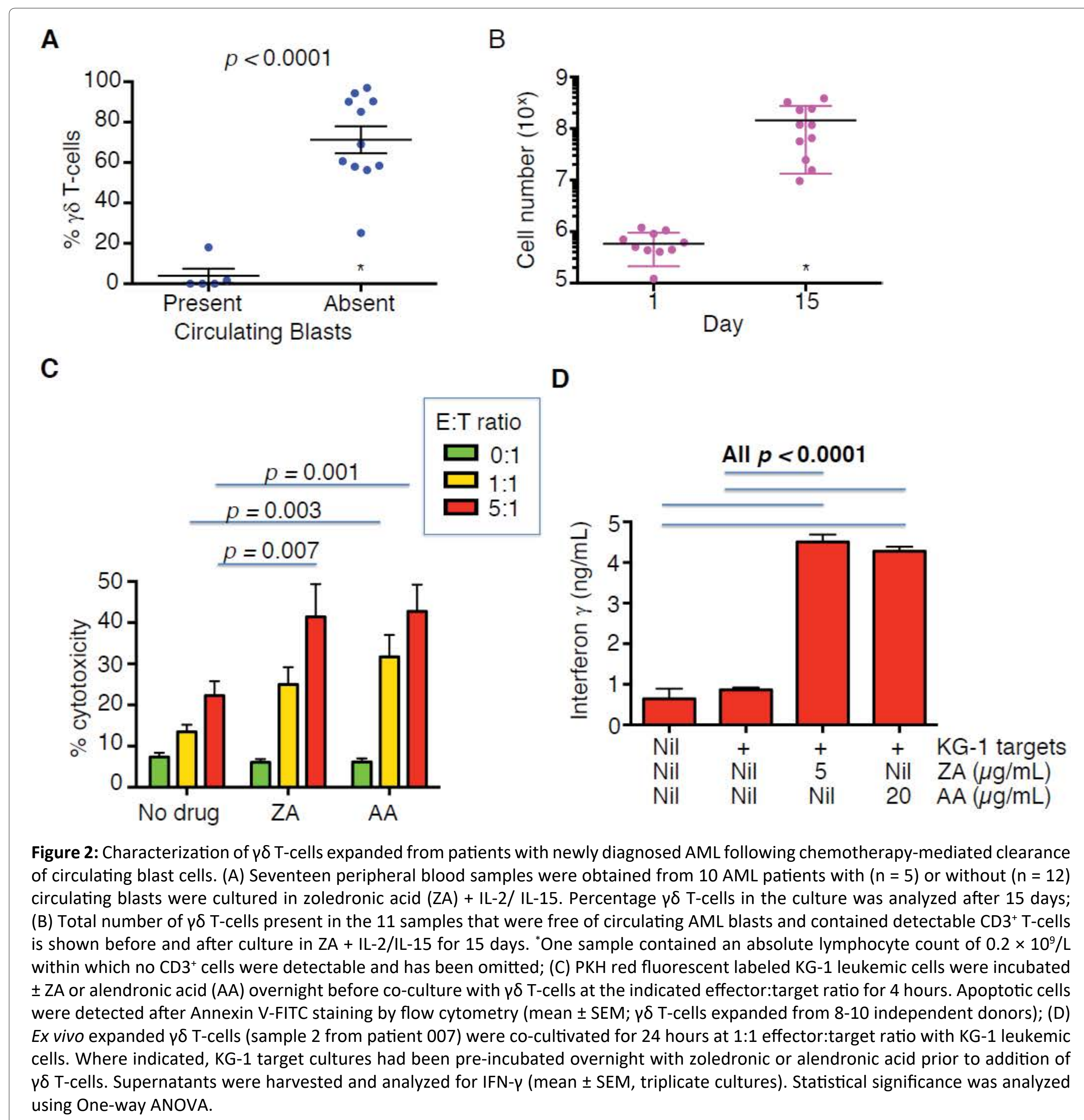

Figure 2: Characterization of γδ T-cells expanded from patients with newly diagnosed AML following chemotherapy-mediated clearance of circulating blast cells. (A) Seventeen peripheral blood samples were obtained from 10 AML patients with (n = 5) or without (n = 12) circulating blasts were cultured in zoledronic acid (ZA) + IL-2/ IL-15. Percentage γδ T-cells in the culture was analyzed after 15 days; (B) Total number of γδ T-cells present in the 11 samples that were free of circulating AML blasts and contained detectable $CD3^+$ T-cells is shown before and after culture in ZA + IL-2/IL-15 for 15 days. *One sample contained an absolute lymphocyte count of 0.2×10^9/L within which no $CD3^+$ cells were detectable and has been omitted; (C) PKH red fluorescent labeled KG-1 leukemic cells were incubated ± ZA or alendronic acid (AA) overnight before co-culture with γδ T-cells at the indicated effector:target ratio for 4 hours. Apoptotic cells were detected after Annexin V-FITC staining by flow cytometry (mean ± SEM; γδ T-cells expanded from 8-10 independent donors); (D) *Ex vivo* expanded γδ T-cells (sample 2 from patient 007) were co-cultivated for 24 hours at 1:1 effector:target ratio with KG-1 leukemic cells. Where indicated, KG-1 target cultures had been pre-incubated overnight with zoledronic or alendronic acid prior to addition of γδ T-cells. Supernatants were harvested and analyzed for IFN-γ (mean ± SEM, triplicate cultures). Statistical significance was analyzed using One-way ANOVA.

We explored the feasibility of zoledronic acid-mediated γδ T-cell expansion from patients with AML. However, only a relatively small percentage of γδ T-cells could be identified in ficoll-separated PBMC derived from newly diagnosed AML patients (representative examples - Figure 1A). Furthermore, zoledronic acid-mediated expansion of these cells was either inefficient or unsuccessful in each of five consecutive attempts (Figure 1B).

Patients with AML who achieve minimal residual disease status following chemotherapy are reported to have increased circulating γδ T-cell numbers. By contrast, these cells are substantially reduced in patients with high disease burden [6]. Consequently, we hypothesized that disease status might be responsible for poor zoledronic acid-mediated γδ T-cell expansion. To test this, we obtained further blood samples from two of the previously described AML patients (AML001 and AML002), following induction chemotherapy with a single cycle of daunorubicin and cytarabine (DA) chemotherapy. In both cases, a discrete population of γδ T-cells was now evident in isolated PBMC (Figure 1C). Moreover, γδ T-cells expanded efficiently following zoledronic acid-mediated activation (Figure 1C), contrasting with the failure of expansion observed prior to chemotherapy. Conversely, sequential samples were also obtained from a third patient (AML003), immediately following FLA chemotherapy (fludarabine/cytarabine), when circulating blasts were undetectable, or following subsequent disease relapse. In this case, a clearly distinct γδ T-cell population was only detected in the leukemia-free sam-

ple and these cells alone were amenable to efficient expansion by ZA (Figure 1D).

Characterization of circulating and *ex vivo* expanded γδ T-cells from AML patients

These preliminary findings suggested that it would be more profitable to expand circulating γδ T-cells from peripheral blood in which circulating leukemic blasts were absent following chemotherapy. In such AML patients, γδ T-cells accounted for 1.55 ± 3.13% of PBMC (19,388 ± 12,302 γδ cells/ml blood; mean ± SD, n = 11), which was not significantly different to healthy donors (19,916 ± 29,887, mean ± SD, n = 21) [8]. In all cases but one, these γδ T-cells were amenable to zoledronic acid-mediated enrichment, unlike those from patients with circulating leukemic cells (Figure 2A). In the single case that expansion failed, there were no $CD3^+$ T-cells detectable (Table 1).

Next, we analyzed the expansion and immunophenotype of zoledronic acid-expanded γδ T-cells following chemotherapy-mediated clearance of circulating blasts. We observed that these cells expanded by an average of 229-fold (Figure 2B), which is similar to healthy donors [8]. Expanded cells exhibited high levels of CD27, CD62L and CCR7 and lower expression of CD70 (Supplementary Figure 3 and Supplementary Figure 4), consistent with the presence of central memory cells [9,10].

Expression of NKG2D also plays an important role in the ability of γδ T-cells to recognize stress ligands on AML blasts [11], although recent data has raised doubts about the expression of such ligands on the stem cell compartment [12]. Cells expanded from AML patients post-chemotherapy expressed comparable high levels of NKG2D to cells derived from healthy donors (Supplementary Figures 3 and Supplementary Figure 4). Furthermore, these cells consistently exhibited anti-leukemic cytotoxic activity in a manner that was potentiated by bisphosphonate sensitization (Figure 2C and Supplementary Figure 1) and accompanied by IFN-γ release (Figure 2D).

Conclusions

The major finding of this short communication is that functional γδ T-cells can only be expanded efficiently from AML patients after chemotherapy-mediated clearance of circulating leukemic cells. Mechanistically, this may reflect the uncoupling of the multiple immunosuppressive mechanisms that are found in newly diagnosed or relapsed AML. Leukemic blasts have been compared to myeloid-derived suppressor cells. In active disease, there is also an increase in regulatory T-cells, immunosuppressive exosomes and inhibitory molecules such as CD200, PD-L1 and indoleamine dioxygenase [13-20]. Future studies should undertaking mixing/depletion experiments with AML cells, analysing effects on γδ T-cell numbers, phenotype and exhaustion markers. Furthermore, in planning for immunotherapy of AML with autologous *ex vivo* expanded γδ T-cells, our data suggest that harvest of starting material should be scheduled when disease is in remission. This is logical since disease burden and circulating blasts are minimal at this time while γδ T-cells are most amenable to expansion, while retaining anti-leukemic activity.

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Declaration of Competing Interests

J.M. is a founding scientist of Leucid Bio. Other authors declare no competing financial interests.

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