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Large scale *ex vivo* expansion of $\gamma\delta$ T cells using artificial antigen presenting cells

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Abstract

Higher $\gamma\delta$ T cell counts in patients with malignancies is associated with better survival. However, $\gamma\delta$ T cells are rare in the blood and functionally impaired in patients with malignancies. Promising results are reported on the treatment of various malignancies with *in vivo* expansion of autologous $\gamma\delta$ T cells using zoledronic acid (zol) and IL-2. Here we demonstrated that zol and IL-2, in combination with a novel genetically engineered K562 CD3scFv/CD137L/CD28scFv/IL15RA quadruplet artificial antigen presenting cell (aAPC), efficiently expand allogeneic donor-derived $\gamma\delta$ T cells using a GMP-compliant protocol sufficient to achieve cell doses for future clinical use. We achieved a 633-fold expansion of $\gamma\delta$ T cells after day 10 of co-culture with aAPC, which exhibited central (47%) and effector (43%) memory phenotypes. Additionally, >90% of the expanded $\gamma\delta$ T cells expressed NKG2D, while they have low cell surface expression of PD1 and LAG2 inhibitory checkpoint receptors. *In vitro* real-time cytotoxicity analysis showed that expanded $\gamma\delta$ T cells were effective in killing target cells. Our results demonstrate that large scale *ex vivo* expansion of donor-derived $\gamma\delta$ T cells in a GMP-like setting can be achieved with the use of quadruplet aAPC and zol/IL-2 for clinical application.

Keywords

Quadruplet artificial antigen presenting cell; Autologous gamma delta T cells; GMP-compliant protocol

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J.C.B. and N.B. wrote the manuscript; M.L.D. and N.B. conceived the study and designed the experiments; B.Y., G.L., B.S., and A.M.L. performed experiments; all authors interpreted the results, edited the manuscript, and approved the final manuscript.

INTRODUCTION

$\gamma\delta$ T cells are innate-like T lymphocytes that have a TCR from rearranged γ and δ genes and can quickly identify pathogens in an MHC-independent manner and act as a first line of defense for the immune system.¹ In contrast to $\alpha\beta$ T cells, $\gamma\delta$ T cells can recognize a broad range of antigens on cancer cells with their innate cytotoxicity receptors which reduces the chance of treatment failure by antigen loss.² The ability to kill tumor cells and their lack of alloreactivity have made $\gamma\delta$ T cells a focus of research for adoptive immunotherapy in recent years.

Intratumoral $\gamma\delta$ T cells are found to be strongly predictive of survival in patients with solid organ malignancies.³ The presence of high levels of $\gamma\delta$ T cells in the bone marrow or peripheral blood of patients is associated with improved survival in patients with leukemia and many other cancer types.^{4–7} The increased recovery of $\gamma\delta$ T cells in patients with acute leukemia who receive $\alpha\beta$ T cell-depleted hematopoietic stem cell transplant (HCT) influences better disease-free survival.⁸ Preemptive or prophylactic immunotherapy with donor lymphocyte infusion (DLI) after HCT can reduce the risk of relapse and prolong survival in patients with acute leukemia at a high risk of relapse after HCT.^{9, 10} However, the $\alpha\beta$ T cell content of donor lymphocytes can result in life-threatening graft versus host disease (GVHD).¹¹ Selective removal of donor $\alpha\beta$ T cells can eliminate the risk of GVHD, while preserving the anti-leukemia effects of $\gamma\delta$ T cells, which have potent cytotoxic activity against neoplastic cells in a major histocompatibility complex (MHC)-independent pathway.¹² The antineoplastic activity of $\gamma\delta$ T cells is mainly through direct cytotoxicity, but also includes NKG2D-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity.^{13–15} Antineoplastic cytotoxic activity of $\gamma\delta$ T cells has also been demonstrated in a xenogenic leukemia model without increased risk of GVHD.¹⁶

The infusion of greater numbers of $\gamma\delta$ T cells from donor lymphocytes may increase the curative outcome of patients with leukemia. However, $\gamma\delta$ T cells are rare in the blood of healthy donors, comprising only 0.5–5% of circulating total T cells, and even lower in numbers and functionally impaired or exhausted in patients with malignancies,¹⁷ resulting in an insufficient dose for effective antineoplastic activity.^{18, 19} Promising early phase clinical trial results have shown that $\gamma\delta$ T cells circulating in the blood can be expanded *in vivo* with zoledronic acid (zol) and interleukin 2 (IL-2).^{5, 6, 16, 20, 21} Zol is an aminobisphosphonate that inhibits farnesyl pyrophosphate synthase, the enzyme acting downstream of isopentenyl pyrophosphate (IPP) in the mevalonate pathway, leading to increased intracellular levels of IPP and subsequent activation of $\gamma\delta$ T cells.²² However, there is little experience with donor-derived allogeneic $\gamma\delta$ T cell adoptive immunotherapy.²³

Ex vivo expansion of $\gamma\delta$ T cells with the use of artificial antigen-presenting cells (aAPC) has previously been reported to be feasible, particularly when zol and IL-2 were used in the initial $\gamma\delta$ T cell enrichment phase.^{24, 25} Previous studies suggest that CD137 ligation induces proliferative signaling^{24, 25} and CD28 mediates co-stimulation, which can lead to activation of $\gamma\delta$ T cells.²⁶ We expanded upon these observations by incorporating a novel genetically engineered K-562 CD3scFv/CD137L/CD28scFv/IL15RA aAPC with zol and IL-2 in an *ex vivo* expansion protocol of donor-derived $\gamma\delta$ T cells for the treatment of

HCT recipients with high risk acute leukemia.²⁷ These quadruple aAPCs were originally developed for the expansion of $\alpha\beta$ T cells.²⁷ Herein, we conducted this study to develop a current Good Manufacturing Practice (cGMP)-like protocol for large scale manufacturing of clinical-grade $\gamma\delta$ T cells with the use of our engineered K-562 aAPC in combination with zol/IL-2.

MATERIALS AND METHODS

Cells

Healthy donor apheresis was purchased from All Cells (Emeryville, CA USA). K-562 and CHO (Chinese hamster ovary) cells were purchased from ATCC (Manassas, VA USA). Cell lines were authenticated by using a cell line authentication kit (ATCC). Prior to co-culture with $\gamma\delta$ T cells CHO target cells and K-562 cells were cultured in their own unique media. CHO cells were grown in F-12K media supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. RPMI media supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin was used to culture K-562 cells. Certified bovine spongiform encephalitis free fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA USA), and all other media and reagents were obtained from ThermoFisher (Waltham, MA USA).

Genetic constructs and cell-based aAPCs

All constructs used the SFG retroviral backbone and were cloned by GENEWIZ (South Plainfield, NJ USA). The SFG plasmid was modified to include an anti-human CD3 scFv, a P2A self-cleaving sequence, and human CD137L. The second SFG-based construct included an anti-human CD28 scFv, a P2A self-cleaving sequence, and human IL15RA. Full sequences can be found in Table S1. Both SFG constructs were transfected into H29 packaging cells using a Calcium Phosphate Transfection Kit (Prometa, Madison, WI USA) as previously described.^{28, 29} K-562 cells were transduced with H29 retroviral supernatant expressing CD3scFv/CD137L, and cultured in RPMI complete media for 4–5 days. K-562 CD137L positive cells were flow-sorted with a 5-laser FACSria (BD Biosciences, San Jose, CA USA) and expanded in RPMI media. K-562 CD3scFv/137L cells were then transduced with H29 retroviral supernatant expressing CD28scFv/IL15RA. CD137L and IL15RA double-positive cells were flow-sorted with a 5-laser FACSria. K-562 CD3scFv/CD137L/CD28scFv/IL15RA cells were expanded, collected, and cryopreserved.

Enrichment and expansion of $\gamma\delta$ T Cells

1×10^9 PBMC were obtained from apheresis products from each donor and used to initiate the expansion process in AIM-V supplemented with 10% human AB serum, $5 \mu\text{M}$ zol, and 300IU/mL IL-2. On day 0, 1×10^9 PBMC were seeded into GREX 100 flasks at a concentration of 1×10^6 cells/ml in a total of 1000mL per flask. On day 7, cells were harvested and subjected to $\alpha\beta$ T cell depletion. Seeding densities of $\gamma\delta$ T cells post $\alpha\beta$ T cell depletion and total viable counts of $\gamma\delta$ T cells at harvest are listed in the supplementary Table S2. Media changes occurred if the Glucose level was $< 250 \text{ dL}$ or the lactate level was $> 7 \text{ mmol/L}$. Total viable cell count was not held constant. The AIM-V supplemented media

was used at all times with $\gamma\delta$ T cells including when they were co-cultured with K-562 aAPCs and CHO target cells.

Flow cytometry

$\gamma\delta$ T cells were defined by gating on live CD45⁺ CD3⁺ TCR $\gamma\delta$ ⁺ CD20⁻ TCR $\alpha\beta$ ⁻ cells. The percentage natural killer (NK) cells (live CD45⁺ CD16⁺ CD56⁺ CD3⁻) was also assessed. All the other biomarkers were gated on $\gamma\delta$ T cells including $\gamma\delta$ T cell memory subtypes: central memory (CM) defined as CD45RO⁺ CD45RA⁻ CCR7⁺, effector memory (EM) as CD45RO⁺ CD45RA⁻ CCR7⁻, terminally differentiated effector memory RA (EMRA) cells as CD45RO⁻ CD45RA⁻, and naïve cells as CD45RO⁻ CD45RA⁺.

$\gamma\delta$ T Cell Cytotoxicity

Cytotoxicity assays were performed on an xCelligence RTCA (real-time cell analysis) instrument (ACEA Biosciences, San Diego, CA USA) according to the manufacturer's instructions. Briefly, $\gamma\delta$ T cells were stimulated with CD3/CD28 Dynabeads (ThermoFisher) for 7 days. Target CHO cells were plated at 1×10^4 per well on an E-Plate 96. The next day $\gamma\delta$ T cells were resuspended in fresh complete medium without IL-2 and added onto target cells at various E/T ratios, and growth was monitored.

RESULTS

K-562 aAPCs enhance $\gamma\delta$ T cell expansion

Cell-based aAPCs can be an economical way to generate a large number of antineoplastic T cells.³⁰⁻³³ We had previously generated aAPCs expressing CD3scFv, CD137L, and CD28scFv but these constructs included fluorescent proteins which are not suitable for clinical applications.²⁷ To expand $\gamma\delta$ T cells, we created a novel quadruple aAPC, K-562 CD3scFv/CD137L/CD28scFv/IL15RA, by transducing 2 vectors into K-562 cells (Figure 1A). The first vector contained anti-human CD3 scFv, a P2A self-cleaving sequence, and human CD137L. The second encoded for an anti-human CD28 scFv, a P2A self-cleaving sequence, and human IL15RA. After transduction, K-562 cells were FACS sorted, and only cells that were positive for both CD137L and IL15RA were collected and used for subsequent experiments (Figure 1B).

To investigate the ability of aAPCs to support $\gamma\delta$ T cell expansion, we isolated $\gamma\delta$ T cells from healthy donor PBMCs by $\alpha\beta$ T cell depletion followed by CD3 positive selection. aAPCs were cultured with the enriched $\gamma\delta$ T cells at a 100:1 aAPC: $\gamma\delta$ T cell ratio for up to 14 days (Figure 2A). At days 7, 10, and 14 after aAPC addition, cells were counted, and $\gamma\delta$ T cell percentage was determined by flow cytometry (Figure 2B). We found a 156-fold $\gamma\delta$ T cell expansion at day 7 but by days 10 and 14, there was a 2612- and a 2429-fold increase in $\gamma\delta$ T cells respectively from day 0 (Figure 2C). We also observed an 820-fold expansion of CD16⁺ (Figure 2D) and 1461-fold increase in CD56⁺ (Figure 2E) $\gamma\delta$ T cells after day 10 of aAPC co-culture. The fold increase of these fell by day 14. These data are representative of 4 independent donors and demonstrate that $\gamma\delta$ T cells can rapidly and significantly increase in numbers after aAPC co-culture.

To examine these $\gamma\delta$ T cell phenotypes, we used flow cytometry and found that at all days examined the CM $\gamma\delta$ T cells constituted the most abundant phenotype (Figure 2F). At day 10, there were 86% CM cells, while at day 14, there was a reduction of CM cells to 47%, and effector $\gamma\delta$ T cells had increased to 36%. Additionally, we found less than 0.05% aAPC in the final expanded $\gamma\delta$ T cell product which is below our release criteria of 0.05%. Based on $\gamma\delta$ T cell fold increase and memory phenotype, we determined 10 days was the optimal co-culture period.

$\gamma\delta$ T cell enrichment and expansion by zoledronic acid and IL-2

Our data demonstrate that $\gamma\delta$ T cell co-culture with aAPCs enhances $\gamma\delta$ T cell expansion and memory phenotypes. To further enhance $\gamma\delta$ T cell expansion, we incorporated a pre-culture of PBMCs with 5 μ M zol and 300IU/mL IL-2, as previously reported,²⁵ prior to $\alpha\beta$ T cell depletion and co-culture with K-562 quadruplet aAPCs. By using this method, we were able to achieve an enrichment of $\gamma\delta$ T cells from 1.98% to 54.58% while reducing the $\alpha\beta$ T cell component from 67.40% to 26.83% after 7 days of culture. $\gamma\delta$ T cells were further enriched by $\alpha\beta$ T cell depletion, which increased the average percentage of $\gamma\delta$ T cells to 74.80% and decreased the $\alpha\beta$ T cells to 0.05% (Table 1).

10:1 is the optimal aAPC: $\gamma\delta$ T cell ratio for expansion

Our previous experiments (Figure 2) were performed at aAPC: $\gamma\delta$ T cell ratios of 100:1. To determine if the total number of aAPCs could be reduced, thus reducing the culture volume and facilitating scale-up for clinical use, aAPC: $\gamma\delta$ T cell ratios were examined. Enriched $\gamma\delta$ T cells were co-cultured with various numbers of aAPCs in fresh media containing the same concentration of zol and IL-2 that had been used from day -7 to day 0 (Figure 3A). We observed no substantial differences between 100:1, 50:1, and 10:1 aAPC: $\gamma\delta$ T cell ratios in $\gamma\delta$ T cell fold change or absolute count (Figure S1A). Lower aAPC: $\gamma\delta$ T cell ratios (0:1, 1:1, and 5:1) were evaluated in subsequent experiments and it was determined that $\gamma\delta$ T cells had the greatest fold change and increased in absolute count at a 10:1 ratio at both days 7 and 10 (Figure 3B and Table 2). CD16⁺ $\gamma\delta$ T cells (Figure 3C and Figure S1B) and CD56⁺ $\gamma\delta$ T cells (Figure 3D and Figure S1C) were also optimally expanded at ratios of 10:1 and 50:1. Therefore, all subsequent experiments were performed at 10:1 aAPC: $\gamma\delta$ T cell. Contamination of $\alpha\beta$ T cells in post-expansion $\gamma\delta$ T cell product was reproducibly <1%.

Zol/IL-2 enriched $\gamma\delta$ T cells have increased expansion after aAPC co-culture

We performed flow cytometry to determine whether pre-culture with zol affects subsequent $\gamma\delta$ T cell expansion with aAPC and their memory phenotype (Figure 4A). We found $\gamma\delta$ T cells co-cultured with aAPCs resulted in a 730-fold increase for donor 1, a 132-fold increase for donor 2, and a 633-fold increase for donor 3 by day 10 (Figure 4B). Absolute numbers of $\gamma\delta$ T cells for donor 1 increased from 4.4 \times 10⁶ at day 0 to 3.2 \times 10⁹ at day 10. Donor 2 $\gamma\delta$ T cells increased from 1.9 \times 10⁶ to 2.5 \times 10⁸ from day 0 to 10. $\gamma\delta$ T cells also increased from 4.0 \times 10⁶ at day 0 to 2.5 \times 10⁹ by day 10 for donor 3 (Figure 4C). We also observed a fold increase of 244, 153, and 259 in CD16⁺ (Figure 4D) and 397, 136, and 2578 in CD56⁺ (Figure 4E) $\gamma\delta$ T cells on day 10 in the 3 donors respectively.

The expression of inhibitory or cytotoxic markers on $\gamma\delta$ T cells can affect function. After aAPC co-culture, we found variability in PD1 expression between the donors (Figure 4F), but a consistent decrease in the $\gamma\delta$ T cell LAG3 expression level in all three donors (Figure 4G). After aAPC co-culture for 10 days we found increased TIM3 expression in donor 2, but stable levels in donors 1 and 3 (Figure 4H). We also observed an increase in the percentage, fold change, and total count of $\gamma\delta$ T cells expressing NKG2D for donors 1 and 3. In contrast, there was a decreased percentage and only a small increase in fold change and total count of NKG2D+ $\gamma\delta$ T cells in donor 2 on day 10 (Figure 4I).

To assess $\gamma\delta$ T cell differentiation we analyzed naïve, CM, EM, and EMRA $\gamma\delta$ T cells. We found 32.3% \pm 14.7 had a CM phenotype and 58.3% \pm 13.1 expressed an EM phenotype at day 10 (Figure 4J). We also observed a low percentage of 5.3% \pm 2.9 of $\gamma\delta$ T cells consistent with the EMRA cell phenotype at day10.

$\gamma\delta$ T cells are cytotoxic after aAPC expansion

To demonstrate that culture with zol/IL-2 and quadruple aAPCs results in functional $\gamma\delta$ T cells we examined their cytotoxicity *in vitro* using a real-time cell killing assay. To better approximate use in a clinical setting we used $\gamma\delta$ T cells that were previously cryopreserved. When we examined the cytotoxic ability of these cells from 2 healthy donors we found they were able to effectively kill the target cells (Figure 5). These results demonstrate that zol enriched $\gamma\delta$ T cells after 10 days of aAPC co-culture retain their cytotoxic abilities.

DISCUSSION

Healthy donor $\gamma\delta$ T cell infusion have antineoplastic therapeutic potential. However, low numbers of circulating peripheral blood $\gamma\delta$ T cells limit their clinical use. Here we demonstrate that $\gamma\delta$ T cells can significantly expand *ex vivo* in co-culture with genetically engineered K-562 CD3scFv/CD137L/CD28scFv/IL15RA aAPC using a scaled-up production system suitable for clinical-grade cells. Thus, this methodology provides an opportunity to use *ex vivo* expanded healthy donor-derived $\gamma\delta$ T cells for clinical application as antineoplastic immunotherapy.

Although our process builds upon the $\gamma\delta$ T cell expansion protocol reported by Xiao and colleagues, there are several critical differences between the methodologies used.²⁵ Following the initial step of zol and IL-2 treatment and subsequent $\alpha\beta$ T cell depletion, we proceed with co-culture using K-562 quadruplet aAPC and zol/IL-2 without need to use human anti-CD3 monoclonal antibody OKT3 since our aAPCs already express anti-CD3. We originally generated aAPCs expressing anti-CD3, anti-CD28, and CD137L to use for expansion of $\alpha\beta$ T cells.²⁷ The original genes published in Shrestha et al included fluorescent proteins, which could not be used for GMP clinical production of cells. Therefore, we replaced the fluorescent reporters with CD137L and 41BBL to allow us to use them as surrogate markers for the scFv and to potentially provide benefit to *ex vivo* T cells in co-culture. We did not examine how CD3scFv, CD137L, CD28scFv, or IL15RA individually effect $\gamma\delta$ T cell expansion or which may be the most important. However, CD137L is shown to be the dominant co-stimulatory proliferative signal on aAPCs for the expansion of $\gamma\delta$ T cells.²⁴ Xiao et al. also reported using an aAPCs expressing CD137L

in addition to CD64 and CD86 to expand V γ 9V δ 2 T cells in their study.²⁵ Additionally, CD28-mediated costimulation is necessary for the activation of $\gamma\delta$ T cells,²⁶ and IL-15 is important for *in vivo* expansion of $\gamma\delta$ T cells in the absence of exogenous IL-2.³⁴ This suggests that the expression of both anti-CD28scFv and IL-15RA on aAPCs could further optimize our protocol for the clinical application.

We determined that a 10:1 aAPC to $\gamma\delta$ T cell ratio was optimal for expansion. This ratio is markedly less than the 100:1 ratio used by Xiao et al. which also included zol and IL-2 in their aAPC and $\gamma\delta$ T cell co-culture.²⁵ The reduced ratio in our system can decrease costs by needing fewer aAPCs for a sufficient number of $\gamma\delta$ T cell expansion to be used in a clinical trial setting.

While several studies report the effective expansion of $\gamma\delta$ T cells with *in vivo* use of zol in patients with malignancies,^{5, 6, 16, 21, 23} the experience of *ex vivo* $\gamma\delta$ T cell expansion is still limited.^{25, 35, 36} We identified that initial treatment of PBMCs with zol and IL-2 is an important phase that yields >90% $\gamma\delta$ T cell enrichment. These $\gamma\delta$ T cells preferentially express NKG2D that can further enhance the cytotoxicity of $\gamma\delta$ T cells as previously reported.^{37, 38} NKG2D is an activating receptor expressed on $\gamma\delta$ T cells, CD8 T cells, and natural killer cells that can provide potent co-stimulatory and activation signals^{39, 40} and mediate antineoplastic cytotoxicity.^{24, 38, 41} NKG2D expression with the use of quadruplet aAPCs in our protocol further increased to >90% after day 10 of expansion. These cells were found to exhibit potent cytotoxic activity against target cells. These findings suggest that our aAPC expanded $\gamma\delta$ T cells can enhance tumor killing by NKG2D expression in addition to $\gamma\delta$ T cell expansion. This is particularly important in AML therapy since NKG2D ligand expression in leukemic blasts is a determinant of susceptibility to $\gamma\delta$ T cell cytotoxicity.¹⁵ We also observed significant fold increase in CD16+ and CD56+ expressing $\gamma\delta$ T cells following aAPC co-culture, which can further enhance $\gamma\delta$ T cell cytotoxicity by mechanisms that also includes antibody-dependent cell-mediated cytotoxicity via CD16.⁴²⁻⁴⁵

Ex vivo stimulation and expansion of T cells can cause a transition through progressive stages of differentiation, which is characterized by a loss of effector function and therapeutic potential.^{46, 47} We found 10 days of co-culture with aAPCs resulted in optimal expansion of $\gamma\delta$ T cell with less terminal differentiation as compared to expansion results at day 14. We also found an increase in EM but not in EMRA phenotype with the addition of zol into the co-culture compared to our initial experiments without the use of zol. Thus, our observations demonstrate that incorporating zol in *ex vivo* expansion of $\gamma\delta$ T cells with the use of aAPCs maintains their antineoplastic efficacy. This is an informative observation since T cells that maintain a less differentiated state are critical for therapeutic efficacy.^{25, 47} Downregulation of immune checkpoint receptors can potentially promote effective antineoplastic activity.⁴⁸ We could not determine the trend for checkpoint markers PD1, LAG3, and TIM3 because of high expression level variability between donors. This suggests that screening of donors for optimal phenotype after expansion may be beneficial.

Although the scarcity of $\gamma\delta$ T cells circulating in patients with malignancies is a significant obstacle for $\gamma\delta$ T cell adoptive transfer,¹⁷ our robust production system results in >600-

fold increase in $\gamma\delta$ T cells for donors 1 and 3, making *ex vivo* expanded $\gamma\delta$ T cell immunotherapy feasible in patients with malignancies. Donor 2 had a modest 132-fold expansion that would likely not support use in the clinic. We hope to mitigate these occurrences in future clinical applications by screening healthy donors prior to expansion and only using high responders for allogeneic therapies. The effective reduction of $\alpha\beta$ T cells to <1% in a final expansion product makes $\gamma\delta$ T cells an attractive allogeneic donor-derived immunotherapy that is not associated with increased risk of GVHD.^{11, 25} Such therapy can potentially benefit patients with various cancer.^{49, 50} Moreover, T cells in patients with malignancies can exhibit increased exhaustion phenotype,⁵¹ thus using allogeneic donor-derived $\gamma\delta$ T cells can provide an additional advantage over the use of autologous cells as an anticancer immunotherapy. We currently have an ongoing clinical trial that studies the safety and effectiveness of *ex vivo* aAPC expanded donor-derived $\gamma\delta$ T cells for the treatment of patients with high-risk acute leukemia ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT05015426) Identifier: [NCT05015426](https://clinicaltrials.gov/ct2/show/study/NCT05015426)).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Patents:

A provisional patent has been filed over the CD3/CD137L/CD28/IL15RA aAPC and the production process.

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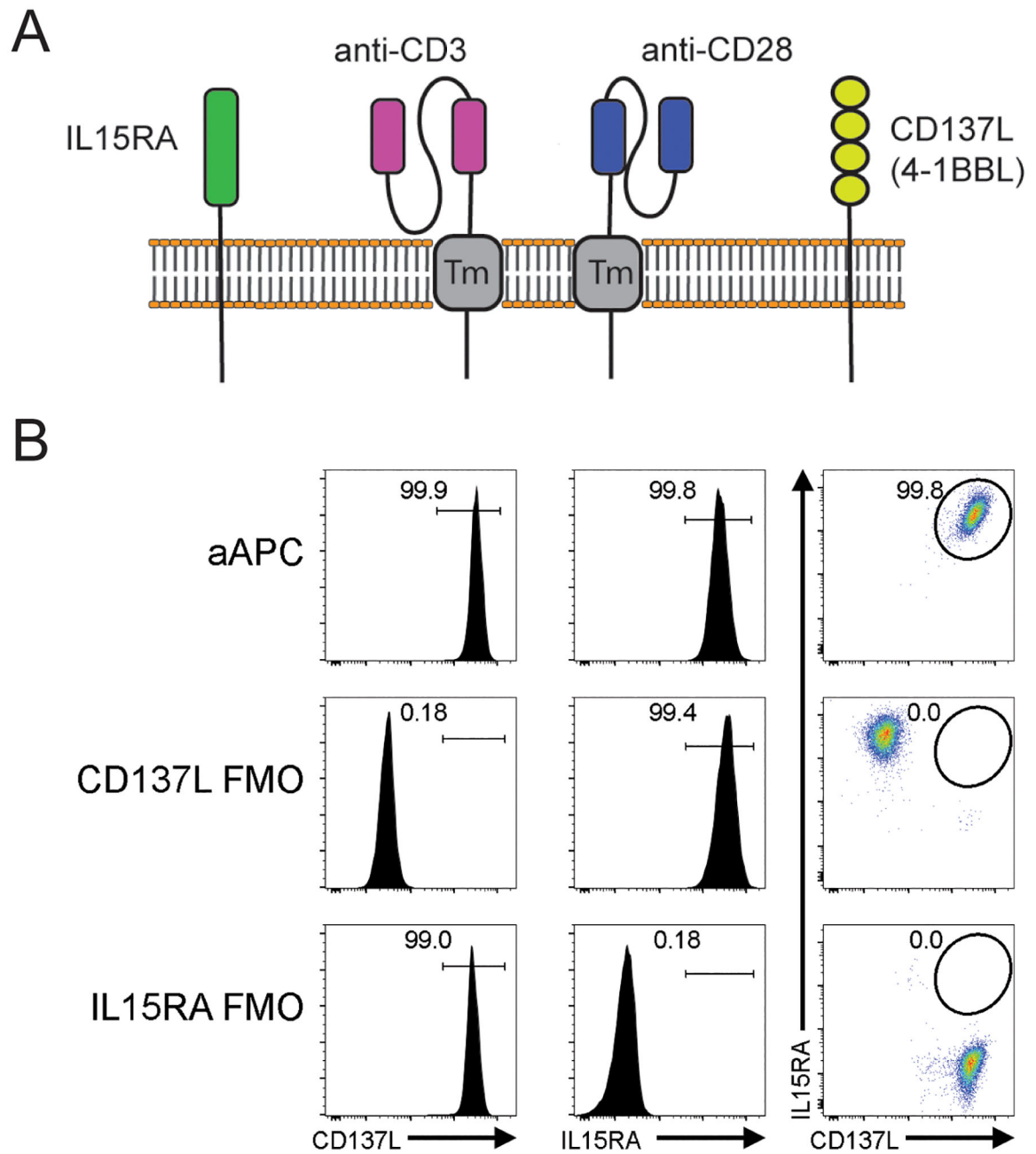


Figure 1. K-562 CD3scFv/CD137L/CD28scFv/IL15RA aAPC characterization.

(A) Schema of K-562 CD3scFv/CD137L/CD28scFv/IL15RA aAPC. (B) Post-sort analysis of aAPC. Flow cytometry plots and histograms of aAPCs and FMO controls.

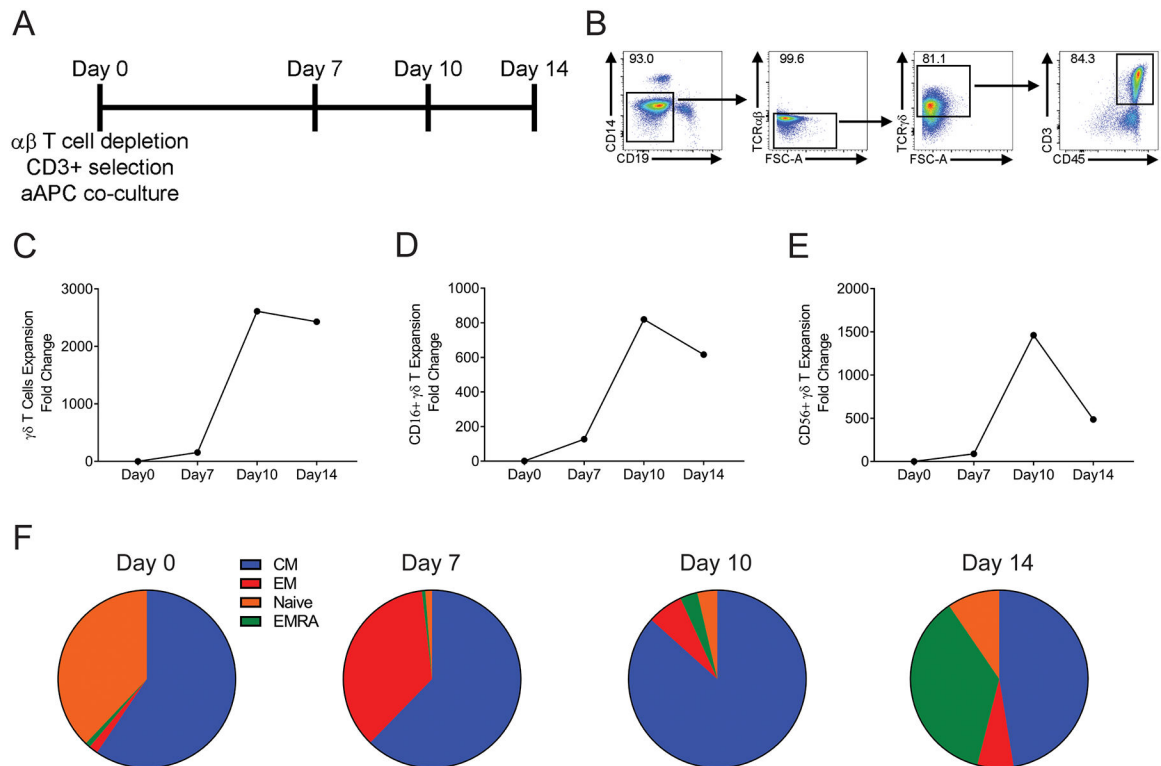


Figure 2. The co-culture of K-562 aAPC enhances $\gamma\delta$ T cell expansion and memory phenotype. (A) Experimental timeline. At day 0, 1×10^6 $\gamma\delta$ T cells were added to 1×10^8 irradiated aAPCs. At days 7, 10, and 14, a portion of cells were removed, counted, and phenotypic markers analyzed by flow cytometry. (B) Flow cytometry gating strategy for $\gamma\delta$ T cells. (C) Co-culture of $\gamma\delta$ T cells with aAPCs results in 2429-fold expansion. (D) CD16+ $\gamma\delta$ T cell counts expand between days 0 and 10. (E) CD56+ $\gamma\delta$ T cells counts increase between days 7 and 10. (F) Percentages of $\gamma\delta$ T cell memory phenotypes at indicated days. Data representative of 4 independent, healthy donors.

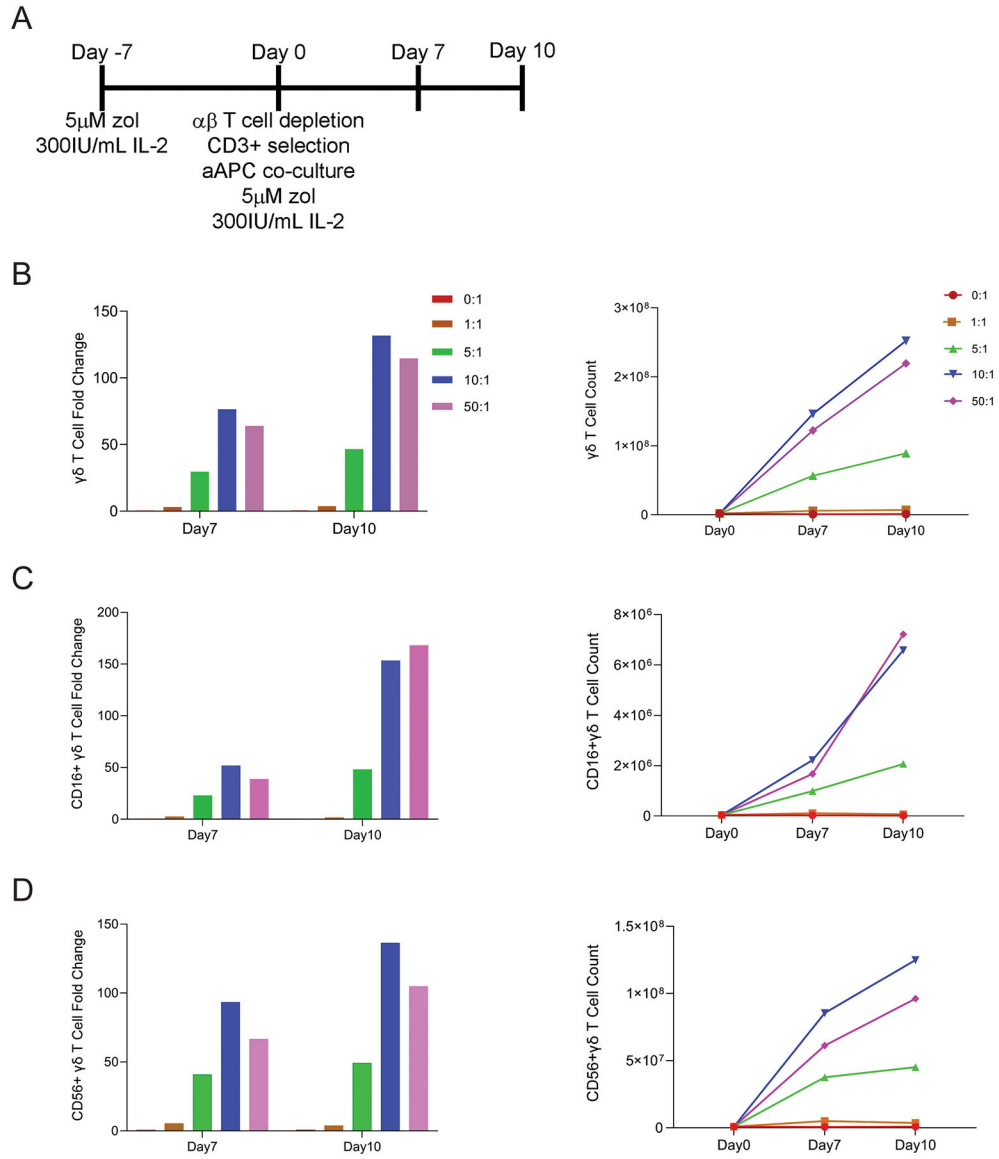


Figure 3. 10:1 aAPC: $\gamma\delta$ T cell ratio is optimal for expansion.

(A) Experimental timeline. PBMCs were cultured with zol and IL-2 at day -7. Zol enriched $\gamma\delta$ T cells were co-cultured with irradiated aAPCs at 1:0, 1:1, 5:1, 10:1, and 50:1 aAPC: $\gamma\delta$ T cell ratios. A portion of cells were collected at days 7 and 10 for enumeration and phenotyping by flow cytometry. (B) $\gamma\delta$ T cell fold change and cell counts are highest at a 10:1 aAPC: $\gamma\delta$ T cell ratio at days 7 and 10. (C) CD16+ $\gamma\delta$ T cell fold change and counts are similar at either a 10:1 or 50:1 aAPC: $\gamma\delta$ T cell ratio. (D) CD56+ $\gamma\delta$ T cells have the highest fold change and count at a 10:1 aAPC: $\gamma\delta$ T cell ratio at days 7 and 10. Data is from one healthy donor.

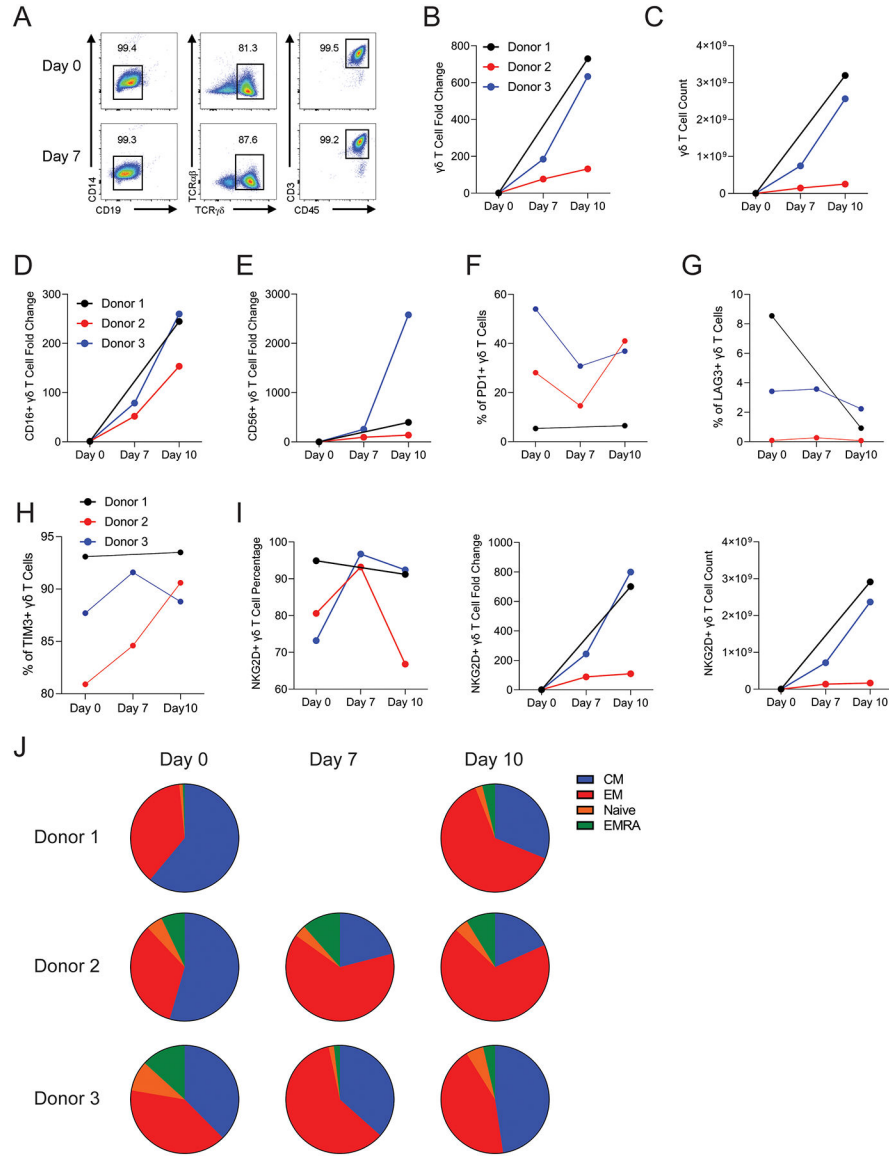
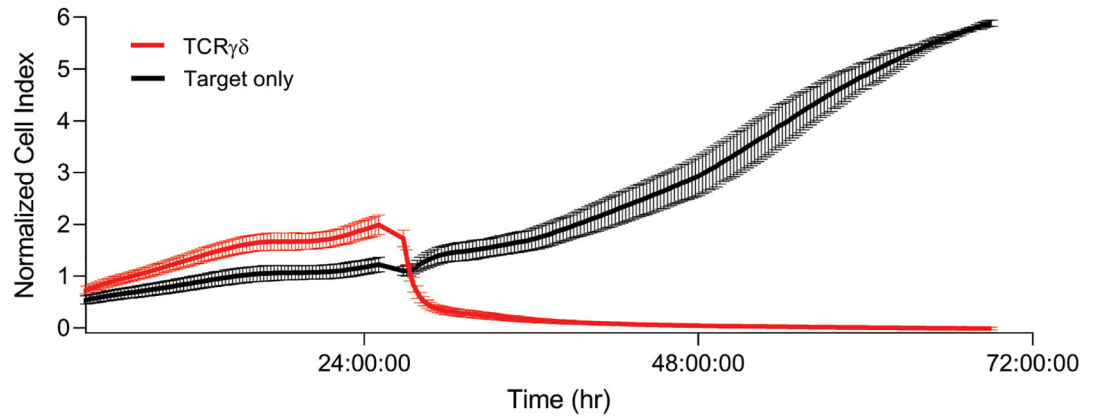


Figure 4. The co-culture of zol enriched $\gamma\delta$ T cells with K-562 aAPCs enhances expansion and memory phenotype.

(A) Flow cytometry gating strategy for $\gamma\delta$ T cells. At days 7 and 10, a portion of cells were removed, counted, and phenotypic markers analyzed by flow cytometry. (B) Co-culture of $\gamma\delta$ T cells with aAPCs results expansion. (C) $\gamma\delta$ T cell absolute numbers increase with aAPC co-culture. (D) CD16+ $\gamma\delta$ T fold change increases after 10 days of aAPC co-culture. (E) CD56+ $\gamma\delta$ T fold change expands with aAPC co-culture. Percentage of $\gamma\delta$ T cells that are PD1+ (F), LAG3+ (G), or TIM3+ (H). (I) NKG2D percentage, fold change, and number of $\gamma\delta$ T cells expressing NKG2D increases after 10 days of aAPC co-culture. (J) Percentages of $\gamma\delta$ T cell memory phenotypes at indicated days. At day 0 $\gamma\delta$ T cells were added to irradiated aAPCs at a 10:1 aAPC: $\gamma\delta$ T cell ratio. Data shows 3 independent, healthy donors.

A



B

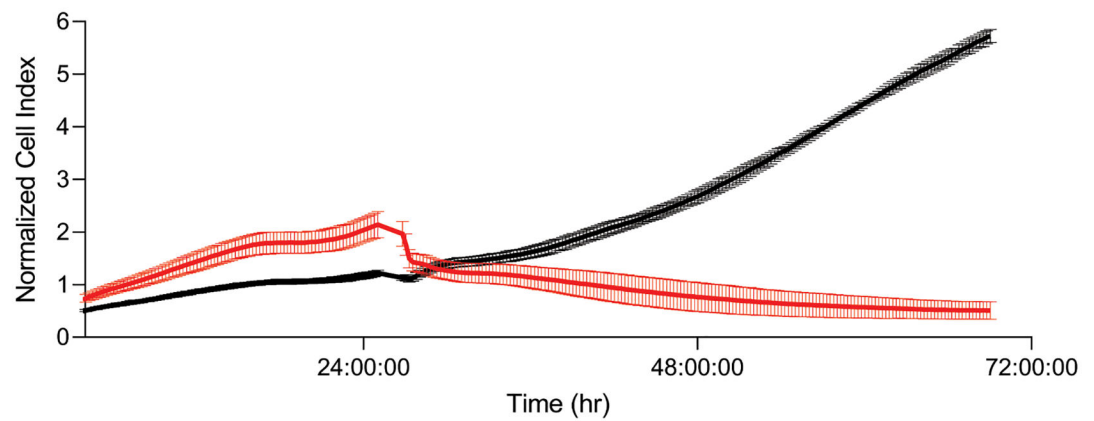


Figure 5. $\gamma\delta$ T cells maintain cytotoxic function after expansion with aAPCs.

(A) Donor 1 $\gamma\delta$ T cell cytotoxicity. (B) Donor 2 $\gamma\delta$ T cell cytotoxicity. Target CHO cells were co-cultured with $\gamma\delta$ T cells at a 10:1 E:T ratio in triplicate. Cytotoxicity was measured by an xCelligence RTCA assay.

Table 1.
 $\alpha\beta$ T cell depletion enhances $\gamma\delta$ T cell purity.

Average $\gamma\delta$ T cell percentage during GMP-like enrichment of 3 independent healthy donors.

	$\gamma\delta$ T cell Average (%)	Standard Deviation
PBMC Isolation (Day -7)	1.98	0.54
Pre $\alpha\beta$ depletion (Day 0)	54.58	58.80
Post $\alpha\beta$ depletion (Day 0)	74.80	26.80
Harvest (Day 10)	75.23	32.43

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Table 2.
 $\gamma\delta$ T cell expansion with different aAPC ratios.

Fold change and standard deviation of $\gamma\delta$ T cells at indicated aAPC: $\gamma\delta$ T cell ratios.

aAPC: $\gamma\delta$ T cell ratio	Total Donors (N=)	$\gamma\delta$ T cell fold change	Standard Deviation
0:1	2	5.73	6.73
1:1	1	4.9	
5:1	1	53.5	
10:1	3	491.3	296.0
50:1	2	451.0	409.1
100:1	1	778.7	

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