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Activating and propagating polyclonal gamma delta T cells with broad specificity for malignancies

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Abstract

Purpose—To activate and propagate populations of $\gamma\delta$ T cells expressing polyclonal repertoire of γ and δ TCR chains for adoptive immunotherapy for cancer, which has yet to be achieved.

Experimental Design—Clinical-grade artificial antigen presenting cells (aAPC) derived from K562 tumor cells were used as irradiated feeders to activate and expand human $\gamma\delta$ T cells to clinical scale. These cells were tested for proliferation, TCR expression, memory phenotype, cytokine secretion, and tumor killing.

Results— $\gamma\delta$ T cell proliferation was dependent upon CD137L expression on aAPC and addition of exogenous IL-2 and IL-21. Propagated $\gamma\delta$ T cells were polyclonal as they expressed V δ 1, V δ 2, V δ 3, V δ 5, V δ 7, and V δ 8 with V γ 2, V γ 3, V γ 7, V γ 8, V γ 9, V γ 10, and V γ 11 TCR chains. Interferon- γ production by V δ 1, V δ 2, and V δ 1^{neg}V δ 2^{neg} subsets was inhibited by pan-TCR $\gamma\delta$ antibody when added to co-cultures of polyclonal $\gamma\delta$ T cells and tumor cell lines. Polyclonal $\gamma\delta$ T cells killed acute and chronic leukemia, colon, pancreatic, and ovarian cancer cell lines, but not healthy autologous or allogeneic normal B cells. Blocking antibodies demonstrated that polyclonal $\gamma\delta$ T cells mediated tumor cell lysis through combination of DNAM1, NKG2D, and TCR $\gamma\delta$. The adoptive transfer of activated and propagated $\gamma\delta$ T cells expressing polyclonal versus defined V δ TCR chains imparted a hierarchy (polyclonal>V δ 1>V δ 1^{neg}V δ 2^{neg}>V δ 2) of survival of mice with ovarian cancer xenografts.

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Conclusions—Polyclonal $\gamma\delta$ T cells can be activated and propagated with clinical-grade aAPC and demonstrate broad anti-tumor activities, which will facilitate the implementation of $\gamma\delta$ T cell cancer immunotherapies in humans.

INTRODUCTION

Human $\gamma\delta$ T cells exhibit an endogenous ability to specifically kill tumors and hold promise for adoptive immunotherapy. They have innate and adaptive qualities exhibiting a range of effector functions, including cytotoxicity upon cell contact (1, 2). Recognition and subsequent killing of tumor is achieved upon ligation of antigens to heterodimers of γ and δ T-cell receptor (TCR) chains. The human TCR variable (V) region defines 14 unique $V\gamma$ alleles, 3 unique $V\delta$ alleles ($V\delta 1$, $V\delta 2$, and $V\delta 3$), and 5 $V\delta$ alleles that share a common nomenclature with $V\alpha$ alleles ($V\delta 4/V\alpha 14$, $V\delta 5/V\alpha 29$, $V\delta 6/V\alpha 23$, $V\delta 7/V\alpha 36$, and $V\delta 8/V\alpha 38-2$) (3). T cells expressing $TCR\alpha/TCR\beta$ heterodimers compose approximately 95% of peripheral blood (PB) T cells and recognize peptides in the context of major histocompatibility complex (MHC) (4). In contrast, $TCR\gamma\delta$ ligands are recognized independent of MHC and these cells are infrequent (1-5% of T cells) in PB (1, 5, 6). Many conserved ligands for $TCR\gamma\delta$ are present on cancer cells, thus an approach to propagating these T cells from small starting numbers while maintaining a polyclonal repertoire of $\gamma\delta$ TCRs has appeal for human application.

Clinical trials highlight the therapeutic potential of $\gamma\delta$ T cells, but numeric expansion is needed for adoptive immunotherapy because they circulate at low frequencies in PB. Methods to propagate $\alpha\beta$ T cells, *e.g.*, using interleukin-2 (IL-2) and/or antibody cross-linking CD3, cannot sustain proliferation of $\gamma\delta$ T cells (7, 8). Aminobisphosphonates, *e.g.*, Zoledronic acid (Zol), have been used to initiate a proliferative signal in $\gamma\delta$ T cells (5, 9), but only one lineage of $\gamma\delta$ T cells, expressing $V\gamma 9V\delta 2$ TCR, can be reliably expanded by Zol. The adoptive transfer of $V\gamma 9V\delta 2$ T cells has yielded clinical responses for investigational treatment of solid and hematological cancers (10-14). Furthermore, long-term remission of leukemia among recipients of haploidentical $\alpha\beta$ T cell-depleted hematopoietic stem-cell transplantation (HSCT) correlated with increased engraftment frequency of donor-derived $V\delta 1$ cells (8, 15-17). However, direct administration of $V\delta 1$ cells or other non- $V\gamma 9V\delta 2$ cell lineages has yet to be performed. In addition, no reports to date have described the therapeutic impact of $V\delta 1^{neg}V\delta 2^{neg}$ cells in cancer immunotherapy and this subset has not been directly compared to T cells expressing $V\delta 1$ and $V\delta 2$ TCRs. Thus, there are significant gaps in the knowledge and human application of non- $V\gamma 9V\delta 2$ lineages.

Given that $\gamma\delta$ T cells have endogenous anti-cancer activity, such as against K562 cells (8, 18), we hypothesized that malignant cells would serve as a cellular substrate to propagate polyclonal $\gamma\delta$ T cells. K562 cells have been genetically modified to function as artificial antigen presenting cells (aAPC) to *ex vivo* activate and numerically expand $\alpha\beta$ T cells and NK cells (19-23). We determined that interleukin-2 (IL-2), IL-21, and γ -irradiated K562-derived aAPC (designated clone #4, genetically modified to co-express CD19, CD64, CD86, CD137L, and a membrane-bound mutein of IL-15 (mIL15); used in selected clinical trials at MD Anderson Cancer Center) can sustain the proliferation of $\gamma\delta$ T cells with polyclonal TCR repertoire. Polyclonal $\gamma\delta$ T cells exhibited broad tumor reactivity and displayed a multivalent

response to tumors as evidenced by the ability of separated V δ sub-populations to kill and secrete cytokine against the same tumor target. Further, killing by polyclonal populations was multifactorial being mediated through DNAM1, NKG2D, and TCR $\gamma\delta$. Tumor xenografts were eliminated by both polyclonal and distinct $\gamma\delta$ T-cell subsets, and mice treated with polyclonal $\gamma\delta$ T cells had superior survival. Given the availability of aAPC as a clinical reagent, trials can for the first time, evaluate polyclonal populations of $\gamma\delta$ T cells as a cancer immunotherapy.

MATERIALS AND METHODS

Cell lines

HCT-116, Kasumi-3, and K562 were acquired from American Type Culture Collection (ATCC; Manassas, VA). Jurkat was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Germany). cALL-2 and RCH-ACV were gifts from Dr. Jeff Tyner (Oregon Health & Science University). BxPC-3, MiaPaCa-2, and Su8686 (pancreatic cancer) were donated by Dr. Vijaya Ramachandran (MD Anderson Cancer Center). A2780, CAOV3, EFO21, EFO27, Hey, IGROV1, OAW42, OC314, OVCAR3, and UPN251 (ovarian cancer) were provided by Dr. Robert C. Bast, Jr. (MD Anderson Cancer Center). Identities of all cell lines were confirmed by STR DNA Fingerprinting at MD Anderson Cancer Center's "Characterized Cell Line Core" and cells were used within 6 months of authentication.

Propagation of $\gamma\delta$ T cells

Peripheral blood mononuclear cells (PBMC) and umbilical cord blood (UCB) were isolated from healthy volunteers by Ficoll-Hypaque (GE Healthcare) after informed consent (24). Thawed PBMC (10^8) were initially treated with CD56 microbeads (cat# 130-050-401, Miltenyi Biotec, Auburn, CA) and separated on LS columns (cat# 130-042-401, Miltenyi Biotec) to deplete NK cells from cultures because they proliferate on aAPC (23) and would contaminate the purity of the $\gamma\delta$ T-cell product. Unlabeled cells from CD56 depletion sorting were then labeled with TCR γ/δ + T-cell isolation kit (cat# 130-092-892, Miltenyi Biotec) and placed on LS columns to separate $\gamma\delta$ T cells in the unlabeled fraction from other cells attached to magnet. $\gamma\delta$ T cells were co-cultured at a ratio of one T cell to two γ -irradiated (100 Gy) aAPC (clone #4) in presence of exogenous IL-2 (Aldesleukin; Novartis, Switzerland; 50 IU/mL), and IL-21 (cat# AF20021; Peprotech, Rocky Hill, NJ; 30 ng/mL) in complete media (CM; RPMI, 10% FBS, 1% Glutamax). Cells were serially re-stimulated with addition of γ -irradiated aAPC every 7 days for 2 to 5 weeks in presence of soluble cytokines, which were added three times per week beginning the day of aAPC addition. K562 were genetically modified to function as aAPC (clone #4) as previously described (25, 26). Validation of co-expression of CD19, CD64, CD86, CD137L, and eGFP (IL-15 peptide fused in frame to IgG4 Fc stalk and co-expressed with eGFP) on aAPC clone #4 was performed before addition to T-cell cultures (25). Fluorescence activated cell sorting (FACS) was used to isolate V δ 1 (TCR δ 1⁺TCR δ 2^{neg}), V δ 2 (TCR δ 1^{neg}TCR δ 2⁺), and V δ 1^{neg}V δ 2^{neg} (TCR δ 1^{neg}TCR δ 2^{neg}) populations, which were stimulated twice as above with aAPC clone #4, phenotyped, and used for functional assays. $\gamma\delta$ T cells from UCB were isolated by FACS from thawed mononuclear cells using anti-TCR $\gamma\delta$ and anti-CD3

monoclonal antibodies (mAbs) and were stimulated for five weeks on aAPC/cytokines as per PBMC.

Abundance and identity of mRNA molecules by DTEA

At designated times after co-culture on aAPC, T cells were lysed at a ratio of 160 μ L RLT Buffer (Qiagen) per 10^6 cells and frozen at -80°C . RNA lysates were thawed and immediately analyzed using nCounter Analysis System (NanoString Technologies, Seattle, WA) with “designer TCR expression array” (DTEA), as previously described (27, 28). DTEA data was normalized to both spike positive control RNA and housekeeping genes (ACTB, G6PD, OAZ1, POLR1B, POLR2A, RPL27, Rps13, and TBP). Spiked positive control normalization factor was calculated from the average of sums for all samples divided by the sum of counts for an individual sample. Spiked positive control normalization factor was calculated from the average of geometric means for all samples divided by the geometric mean for an individual sample. Normalized counts were reported.

Flow cytometry

Cells were phenotyped with antibodies detailed in Supplemental Table 1. Gating strategy is displayed in Supplemental Figure 1. Samples were acquired on FACS Calibur (BD Biosciences, San Jose, CA) and analyzed with FlowJo software (version 7.6.3).

Cytokine production and cytotoxicity assays

Expression of cytokines was assessed by intracellular staining and secretion of cytokines into tissue culture supernatants was evaluated by Luminex multiplex analysis. *In vitro* specific lysis was assessed using a standard 4-hour CRA, as previously described (25). Additional information can be found in the supplemental materials and methods.

Mouse experiments

In vivo anti-tumor efficacy was assessed in NSG mice (NOD.Cg-Prkdc^{scid}Il2r γ ^{tm1Wjl}/SzJ; Jackson Laboratories). CAOV3 ovarian cancer cell line was transduced with recombinant lentivirus (Supplemental Figure 2) encoding mKate red fluorescence protein (29) to identify transduced cells and enhanced *firefly luciferase* (*effLuc*) for non-invasive bioluminescence imaging (30). CAOV3-*effLuc*-mKate (clone 1C2; 3×10^6 cells/mouse) tumors were established by intraperitoneal (i.p.) injection and mice were randomly distributed into treatment groups. Eight days later (designated Day 0), a dose escalation regimen was initiated with $\gamma\delta$ T cells administered i.p. and PBS administered i.p. as a negative control. T-cell doses were 3×10^6 , 6×10^6 , 10^7 , and 1.5×10^7 on days 0, 7, 14, and 21, respectively. Non-invasive BLI was performed during the course of the experiments to serially measure tumor burden of CAOV3-*effLuc*-mKate following subcutaneous administration of D-Luciferin (cat#122796, Caliper, Hopkinton, MA) as detected with IVIS-100 Imager (Caliper). BLI was analyzed using Living Image software (version 2.50, Xenogen, Caliper).

RESULTS

***Ex vivo* numeric expansion of $\gamma\delta$ T cells on aAPC depends on co-stimulation and cytokines**

The adoptive transfer of $\gamma\delta$ T cells requires *ex vivo* propagation as starting numbers from PBMC are limiting (gating on lymphocyte pool: $3.2\% \pm 1.2\%$; mean $\pm$ standard deviation (SD); $n=4$; Figure 1A). $\gamma\delta$ T cells from PBMC were isolated by “negative” paramagnetic bead selection and co-cultured for 22 days with weekly addition of γ -irradiated K562-derived aAPC (clone #4) in the presence of soluble recombinant IL-2 and IL-21 in alignment with protocols at MD Anderson for propagation of clinical-grade $\alpha\beta$ T cells. This resulted in the outgrowth of a population of T cells homogeneously co-expressing CD3 and TCR $\gamma\delta$ ($97.9\% \pm 0.6\%$). NK cells (CD3^{neg}CD56⁺) and $\alpha\beta$ T cells (TCR $\alpha\beta$ ⁺) were absent from these cultures supporting the purity of the $\gamma\delta$ T-cell product. Populations of TCR δ 1⁺TCR δ 2^{neg}, TCR δ 1^{neg}TCR δ 2⁺, and TCR δ 1^{neg}TCR δ 2^{neg} were detected indicating that aAPC, IL-2, and IL-21 supported polyclonal $\gamma\delta$ T cell proliferation (Figure 1A far right). Cells were activated as marked by expression of CD38 ($93.5\% \pm 3.5\%$) and CD95 ($99.7\% \pm 0.1\%$) (Supplemental Figure 3). This approach to propagation yielded $>10^9$ $\gamma\delta$ T cells from $<10^6$ total initiating cells (Figure 1B), which represented a $4.9 \times 10^3 \pm 1.7 \times 10^3$ (mean $\pm$ SD; $n=4$) fold increase. Thus, aAPC with recombinant human cytokines supported the robust numeric expansion of polyclonal $\gamma\delta$ T cells from small starting numbers of $\gamma\delta$ T cells derived from PBMC.

The addition of exogenous cytokines and presence of mIL15, CD86, and CD137L on clinical-grade aAPC were assessed for their ability to support the outgrowth of $\gamma\delta$ T cells. Parental K562 cells were stably transfected with *Sleeping Beauty* (SB) transposons to introduce individual stimulatory molecules, cloned to achieve homogeneous expression (Supplemental Figure 4), and then used to assess their impact on $\gamma\delta$ T-cell proliferation. Co-cultures with exogenous IL-2 and IL-21 were initiated with paramagnetic bead-purified $\gamma\delta$ T cells and five sets of γ -irradiated K562: (i) parental, (ii) mIL15⁺, (iii) mIL15⁺CD86⁺, (iv) mIL15⁺CD137L⁺, and (v) mIL15⁺CD86⁺CD137L⁺ (clone #4). $\gamma\delta$ T cells cultured in parallel without APC demonstrated that soluble IL-2 and IL-21 sustained only limited numeric expansion of $\gamma\delta$ T cells (Figure 1C). Propagation improved upon addition of parental K562 cells, indicating that endogenous molecules on these cells can activate $\gamma\delta$ T cells for proliferation. The expression of mIL15 with or without CD86 did not further improve the ability of $\gamma\delta$ T cells to propagate compared with parental K562. In contrast, improved rates of propagation of $\gamma\delta$ T cells were observed upon co-culture with mIL15⁺CD137L⁺ and mIL15⁺CD86⁺CD137L⁺ aAPC. Thus, it appears that CD137L on aAPC clone#4 provides a dominant co-stimulatory proliferative signal for $\gamma\delta$ T cells. In the absence of IL-2 and IL-21 the proliferation of $\gamma\delta$ T cells ceased on aAPC clone#4, and together these cytokines exhibited an additive benefit to the rate of $\gamma\delta$ T-cell propagation (Figure 1D). This validated our approach to combining aAPC clone #4 with cytokines to sustain the proliferation of polyclonal $\gamma\delta$ T cells *ex vivo*, and demonstrated that CD137L on aAPC, IL-2, and IL-21 were driving factors for proliferation of polyclonal $\gamma\delta$ T cells to clinical scale.

***Ex vivo* numeric expansion of neonatal $\gamma\delta$ T cells on aAPC in presence of IL-2 and IL-21**

Allogeneic UCB is an important source of $\gamma\delta$ T cells for adoptive transfer, because it contains younger cells and a more diverse TCR $\gamma\delta$ repertoire relative to PBMC, which could increase the number of ligands targeted by the engrafted cells and result in long-term engraftment in the recipient (31). However, the limited number of mononuclear cells within a banked UCB unit curtails the number of neonatal $\gamma\delta$ T cells directly available for adoptive transfer. Thus, we evaluated whether aAPC could sustain proliferation from small starting numbers of neonatal $\gamma\delta$ T cells. Fluorescence activated cell sorting (FACS) was used to isolate 10^4 UCB-derived $\gamma\delta$ T cells ($\sim 0.01\%$ of a typical UCB unit) which were co-cultured on aAPC clone #4 with IL-2 and IL-21. After 35 days, there was a 10^7 -fold increase in cell number, as an average of 10^{11} UCB-derived $\gamma\delta$ T cells (Range: $6 \times 10^9 - 3 \times 10^{11}$; $n=5$) were propagated from the 10^4 initiating $\gamma\delta$ T cells (Supplemental Figure 5A). Two additional stimulations were performed for $\gamma\delta$ T cells derived from UCB compared to PBMC highlighting their potential for proliferating to clinically-appealing numbers. The propagated $\gamma\delta$ T-cell populations exhibited uniform co-expression of CD3 and TCR $\gamma\delta$ and lacked TCR $\alpha\beta^+$ T cells or presence of CD3^{neg}CD56⁺ NK cells (Supplemental Figure 5B-D). Collectively, these data demonstrate that aAPC clone #4 with IL-2 and IL-21 could sustain the *ex vivo* proliferation of $\gamma\delta$ T cells from a small starting population of neonatal UCB.

***Ex vivo* activated and propagated $\gamma\delta$ T cells express polyclonal and defined TCR $\gamma\delta$ repertoire**

Upon establishing that $\gamma\delta$ T cells could numerically expand on aAPC and selected cytokines, we sought to determine the TCR repertoire of the propagated cells. Prior to numeric expansion, resting $\gamma\delta$ T cell repertoire followed TCR $\delta 2 > \text{TCR}\delta 1^{\text{neg}} \text{TCR}\delta 2^{\text{neg}} > \text{TCR}\delta 1$ by flow cytometry (Supplemental Figure 6). However, the $\gamma\delta$ T-cell repertoire followed TCR $\delta 1 > \text{TCR}\delta 1^{\text{neg}} \text{TCR}\delta 2^{\text{neg}} > \text{TCR}\delta 2$ following expansion, suggesting that there was a proliferative advantage for V $\delta 1$ cells within polyclonal $\gamma\delta$ T-cell cultures. To look more in-depth at TCR $\gamma\delta$ diversity in aAPC-expanded $\gamma\delta$ T cells, we adapted a non-enzymatic digital multiplex assay used to quantify the TCR diversity in $\gamma\delta$ T cells expressing a CD19-specific chimeric antigen receptor (CAR) (27) termed “direct TCR expression array” (DTEA). Following expansion (Day 22), four of eight V δ alleles (V $\delta 1$, V $\delta 2$, V $\delta 3$, and V $\delta 8$) were detected in PBMC-derived $\gamma\delta$ T cells (Figure 2A) and were co-expressed with V $\gamma 2$, V $\gamma 7$, V $\gamma 8$ (two alleles), V $\gamma 9$, V $\gamma 10$, and V $\gamma 11$ (Figure 2B). Similarly, a polyclonal assembly of V δ and V γ chains was observed in $\gamma\delta$ T cells from UCB following expansion (Day 34-35), albeit with reduced abundance of V $\delta 2$ cells, more V $\gamma 2$, and presence of V $\gamma 3$, V $\delta 5$, and V $\delta 7$ cells not seen from PBMC (Figure 2C-D). Similar patterns of V δ and V γ mRNA usage were detected in PBMC and UCB before and after expansion (Supplemental Figure 7) although overall mRNA counts were fewer in the resting cells (Day 0) relative to the activated $\gamma\delta$ T cells. Thus, aAPC-expanded $\gamma\delta$ T cells maintain a polyclonal TCR repertoire from both PBMC and UCB.

We sought to validate these mRNA data by sorting polyclonal populations with TCR δ -specific antibodies and repeating DTEA on isolated cultures. There are only two TCR δ -specific mAbs commercially available and they identified 3 discrete V δ populations (V $\delta 1$: TCR $\delta 1^+$ TCR $\delta 2^{\text{neg}}$, V $\delta 2$: TCR $\delta 1^{\text{neg}}$ TCR $\delta 2^+$, and V $\delta 1^{\text{neg}}$ V δ^{neg} : TCR $\delta 1^{\text{neg}}$ TCR $\delta 2^{\text{neg}}$) within

aAPC-expanded $\gamma\delta$ T cells from PBMC (Figure 1A) and UCB (Supplemental Figure 8) with abundance following $V\delta 1 > V\delta 1^{\text{neg}}V\delta^{\text{neg}} > V\delta 2$. FACS isolated subsets from PBMC-derived $\gamma\delta$ T-cell pools were propagated with clone #4 as discrete populations and maintained their identity as assessed by expression of TCR δ isotypes (Figure 3A). Each of the separated subsets could be identified by a pan-specific TCR $\gamma\delta$ antibody confirming that these cells were indeed $\gamma\delta$ T cells (Figure 3B). Furthermore, each population could be differentiated based on pan-TCR $\gamma\delta$ antibody mean fluorescence intensity (MFI) where $V\delta 2$, $V\delta 1^{\text{neg}}V\delta^{\text{neg}}$, and $V\delta 1$ T cells corresponded to the TCR $\gamma\delta^{\text{low}}$ (43 ± 9 ; mean $\pm$ SD; $n=4$), TCR $\gamma\delta^{\text{intermediate}}$ (168 ± 40), and TCR $\gamma\delta^{\text{hi}}$ (236 ± 56) groupings, respectively. No differences in proliferation kinetics on aAPC were observed between isolated $V\delta$ -sorted subsets (Figure 3C) indicating that the observed inversion of $V\delta 1$ and $V\delta 2$ frequencies in polyclonal cultures before versus after expansion was not due to a proliferative defect in one of the subsets. DTEA demonstrated that isolated and propagated $V\delta 1$, $V\delta 2$, and $V\delta 1^{\text{neg}}V\delta^{\text{neg}}$ sub-populations were homogeneous populations as they predominantly expressed $V\delta 1^*01$, $V\delta 2^*02$, and $V\delta 3^*01$ mRNA species at 261 ± 35 , 3910 ± 611 , and 5559 ± 1119 absolute counts, respectively (Figure 3D). Therefore, there were fewer $V\delta 1^*01$ mRNA species expressed by $V\delta 1$ cells relative to the $V\delta 2^*02$ expressed by $V\delta 2$ cells and $V\delta 3^*01$ expressed by $V\delta 1^{\text{neg}}V\delta^{\text{neg}}$ cells. Moreover, these data indicated that the relatively low counts observed for $V\delta 1^*01$ in polyclonal populations with a preponderance of TCR $\delta 1^+$ cells was not a defect in DTEA detection but rather a product of fewer total mRNA transcripts relative to other $V\delta$ species. Given the wide range of mRNA transcript quantities for each allele, DTEA was not useful for calculation of relative frequencies of $V\delta$ subsets in polyclonal populations but rather was indicative of presence or absence of a particular $\gamma\delta$ T cell subset. Expression of other $V\delta 2$ alleles ($V\delta 1^*01_07$ and $V\delta 1^*01_75$) was absent from polyclonal $\gamma\delta$ T cells (Figure 2A) and each of the sorted subsets (data not shown). Small amounts of $V\delta 4$, $V\delta 5$, $V\delta 6$, and $V\delta 7$ mRNA species were detected in the three subsets of T cells sorted for $V\delta$ expression (Supplemental Figure 9). $V\delta 8$ mRNA was exclusively present in sorted $V\delta 1^{\text{neg}}V\delta^{\text{neg}}$ cells and these T cells are likely the main contributors of $V\delta 8$ in bulk $\gamma\delta$ T cells. The same $V\gamma$ mRNA present in polyclonal cultures was detected in $V\delta$ -sorted cultures (Supplemental Figure 10). Furthermore, $V\delta 1$ and $V\delta 1^{\text{neg}}V\delta^{\text{neg}}$ were not different ($p=0.419$; Two-way ANOVA) but $V\delta 2$ was different to both $V\delta 1$ ($p<0.0001$) and $V\delta 1^{\text{neg}}V\delta^{\text{neg}}$ ($p<0.0001$) in $V\gamma$ usage. Collectively, these results confirmed DTEA from unsorted cultures and strongly supported the polyclonal TCR $\gamma\delta$ expression on $\gamma\delta$ T cells activated to proliferate by aAPC and cytokines.

Interferon- γ produced in response to tumors is dependent on TCR $\gamma\delta$

A multiplex analysis of cytokines and chemokines was performed to determine whether aAPC-propagated $\gamma\delta$ T cells might foster a pro-inflammatory response in a tumor micro-environment (Figure 4A). The T_H1 -associated cytokines interferon- γ (IFN γ) and tumor necrosis factor- α (TNF α) were secreted in abundance by $\gamma\delta$ T cells upon exposure to leukocyte activated cocktail (LAC; PMA and Ionomycin for non-specific mitogenic stimulation), in addition to small amounts of IL-2 and IL-12 p70. In contrast, no significant production of the T_H2 -associated cytokines IL-4, IL-5, and IL-13 was observed from LAC-treated $\gamma\delta$ T cells, but there was a small increase in IL-10 production over baseline. Similarly, Th17-associated cytokines IL-1RA, IL-6, and IL-17 were secreted at low levels

by LAC-treated $\gamma\delta$ T cells. The chemokines CCL3, CCL4, and CCL5 were detected in abundance. Minor contributions of non- $\gamma\delta$ T cells in the culture that could have been activated by LAC to secrete cytokines could not be ruled out, but given that the cells tested were $97.9\% \pm 0.6\%$ $CD3^+TCR\gamma\delta^+$ these data indicate that it was activation of $\gamma\delta$ T cells that led to a largely pro-inflammatory response. $IFN\gamma$ was the most responsive of all the assessed cytokines and was chosen to measure responses of V δ subsets to tumor cells (Figure 4B). Co-culture of polyclonal aAPC-propagated/activated $\gamma\delta$ T cells with cancer cells resulted in a hierarchy of $IFN\gamma$ production following $V\delta2 > V\delta1 > V\delta1^{neg}V\delta2^{neg}$ as shown by MFI of 855 ± 475 , 242 ± 178 , and 194 ± 182 (mean $\pm$ SD; n=4), respectively. $IFN\gamma$ production by V $\delta1$, V $\delta2$, and $V\delta1^{neg}V\delta2^{neg}$ subsets was inhibited by pan-TCR $\gamma\delta$ antibody when added to of $\gamma\delta$ T cell/tumor co-cultures indicating that response to the tumor in each subset was dependent upon activation through TCR $\gamma\delta$ (Figure 4C). This observation supported the premise that a single cancer cell could be targeted by discrete $\gamma\delta$ TCRs. Thus, a multivalent pro-inflammatory response to the tumor cell was achieved by polyclonal $\gamma\delta$ T cells.

Polyclonal $\gamma\delta$ T cells lyse a broad range of tumor cells through combination of DNAM1, NKG2D, and TCR $\gamma\delta$

After establishing that propagated $\gamma\delta$ T cells could be activated to produce pro-inflammatory cytokines, we examined their ability to specifically lyse a panel of tumor cell lines. Polyclonal $\gamma\delta$ T cells demonstrated a range of cytolysis against solid and hematological cancer-cell lines without a clear preference towards a particular tumor histology or grade (Figure 5 and Supplemental Figure 11). We previously established that B-cell acute lymphoblastic leukemia (ALL) cell line NALM-6 was largely resistant to lysis by $\gamma\delta$ T cells, which required a CD19-specific CAR to acquire significant killing capability (27). In this study it was also observed that autologous and allogeneic normal B cells were spared from cytolysis (Figure 5A), and that B-ALL cell line cALL-2 and murine T cell lymphoma cell line EL4 were lysed poorly by polyclonal $\gamma\delta$ T cells, which indicated that some cells were resistant and/or not recognized by polyclonal $\gamma\delta$ T cells. In contrast, T-ALL cell line Jurkat and B-ALL cell lines RCH-ACV were both killed efficiently by polyclonal $\gamma\delta$ T cells (Figure 5B), indicating that $\gamma\delta$ T cells could be used to target some B-cell and T-cell malignancies. Kasumi-3 is a $CD33^+CD34^+$ undifferentiated leukemia cell line that was lysed at intermediate levels by $\gamma\delta$ T cells. Chronic myelogenous leukemia (CML) cell line K562 and K562-derived clone#4 aAPC were killed by polyclonal $\gamma\delta$ T cells, which corroborated the notion that these cells could serve as a proliferative substrate. Pancreatic cancer cell lines BxPc-3, MiaPaCa-2, and Su8686, were lysed by $\gamma\delta$ T cells, as was the colon carcinoma cell line HCT-116 (Figure 5C). Ovarian cell lines were killed by polyclonal $\gamma\delta$ T cells in the following order of decreasing sensitivity: CAOV3 > EFO21 > UPN251 > IGROV1 > OC314 > Hey > A2780 > OVCAR3 > OAW42 > EFO27. Each of the separated V δ subsets lysed hematological (Jurkat and K562) and solid (OC314 and CAOV3) tumor cell lines, which showed that polyclonal $\gamma\delta$ T cells could direct a multivalent response against common targets (Supplemental Figure 12). The strength of cytolysis followed the hierarchy of TCR usage ($V\delta2 > V\delta1^{neg}V\delta2^{neg} > V\delta1$) that was consistent with the premise that a propensity to be triggered for effector function would increase with T-cell differentiation (Supplemental Figure 13). Lysis by polyclonal populations was apparently not due to one specific V δ subtype but rather from contributions of multiple $\gamma\delta$ T-cell subsets, because it was observed

that (1) a number of tumor cell lines were equivalently killed by polyclonal $\gamma\delta$ T cells containing different frequencies of V δ 1, V δ 2, and V δ 1^{neg}V δ 2^{neg} cells and (2) a polyclonal population was not identified with dominant cytolysis. We also sought to determine which surface molecules were responsible for cytolysis by blocking immunoreceptors with antibodies (Figure 5D). Our experimental approach also took into account that $\gamma\delta$ T cells co-express DNAM1 (97.7% $\pm$ 0.9%; mean $\pm$ SD; n=4) and NKG2D (40.1% $\pm$ 16.5%) which can activate both T cells and NK cells for killing (32, 33). Addition of individual antibodies did not reduce lysis, except for TCR $\gamma\delta$ in 2 of 3 cell lines tested. In contrast, a pool of antibodies binding NKG2D, DNAM1, TCR $\gamma\delta$ resulted in significant inhibition, in a dose-dependent manner, of $\gamma\delta$ T-cell mediated cytolysis against all 3 targets. Collectively, these data established that *ex vivo*-propagated $\gamma\delta$ T cells have broad anti-tumor capabilities likely mediated by activation through DNAM1, NKG2D, and TCR $\gamma\delta$.

Established ovarian cancer xenografts are eliminated by adoptive transfer of $\gamma\delta$ T cells

To test whether polyclonal $\gamma\delta$ T cells were effective in targeting and killing tumors *in vivo*, we created a xenograft model for ovarian cancer in immunocompromised mice. NSG mice were injected intraperitoneally with CAOV3-*effLuc*-mKate ovarian cancer cells and then randomized into five treatment groups. Following eight days of tumor engraftment, either PBS (vehicle/mock), V δ 1, V δ 2, V δ 1^{neg}V δ 2^{neg}, or polyclonal $\gamma\delta$ T cells were administered in escalating doses (Figure 6). Tumor burden and biodistribution were serially measured by non-invasive bioluminescence imaging. Established tumors continued to grow in vehicle-treated mice, but tumor bioburden was significantly reduced (p = 0.001) in mice receiving $\gamma\delta$ T-cell treatments at day 72, relative to their initial tumor burden (Figure 6A-B). Adoptive transfer of polyclonal $\gamma\delta$ T cells, V δ 1, and V δ 1^{neg}V δ 2^{neg} T cells significantly (p = 0.01), and V δ 2 almost significantly (p=0.055), increased long-term survival compared to mock-treated mice. This corresponded to overall survival following polyclonal > V δ 1 > V δ 1^{neg}V δ 2^{neg} > V δ 2 (Figure 6C). This is the first time that three V δ subsets have been compared for their ability to target tumor *in vivo* and is the first display of *in vivo* anti-tumor activity by V δ 1^{neg}V δ 2^{neg} cells. In sum, activated and propagated $\gamma\delta$ T cells were effective in treating cancer *in vivo* and thus represent an attractive approach to adoptive immunotherapy.

DISCUSSION

This study establishes our aAPC clone #4 as a cellular platform for the sustained proliferation of multiple $\gamma\delta$ T cell populations that demonstrate extensive reactivity against hematologic and solid malignancies. T cells expressing defined V δ TCRs have been associated with clinical responses against cancer. For example, the V δ 1 subset correlated with complete responses observed in patients with ALL and acute myelogenous leukemia (AML) after $\alpha\beta$ T cell-depleted haploidentical HSCT (15-17). V δ 1 cells were also shown to kill glioblastoma independent of cytomegalovirus (CMV) status (34). However, V δ 1 cells have not been directly administered. Our data establish that such cells could mediate anti-tumor immunity and supports the adoptive transfer V δ 1 T cells for cancer therapy. In contrast to V δ 1 and V δ 1^{neg}V δ 2^{neg} cells, T cells expressing V δ 2 TCR have been directly infused and elicited responses against solid and hematological tumors (9, 35). Little is known about V δ 1^{neg}V δ 2^{neg} T cells, but these lymphocytes have displayed recognition of the

non-classical MHC molecule CD1d with corresponding NKT-like functions and have also been correlated with immunity to human immunodeficiency virus (HIV) and CMV (36-39). Our results are the first to directly show that V δ 1^{neg}V δ neg cells exhibit anti-tumor activities, and given their propensity to engage both viruses and cancer the add-back of this subset could especially benefit immunocompromised cancer patients. Because aAPC with IL-2 and IL-21 can propagate polyclonal $\gamma\delta$ T cells, mAbs can now be raised against V δ 3, V δ 5, V δ 7, and V δ 8 isotypes to help elucidate their potential roles in clearance of pathogens and cancer. In aggregate, our data support the adoptive transfer of $\gamma\delta$ T cells that maintain expression of multiple V δ TCR types as investigational treatment for cancer.

The molecules on aAPC that activate $\gamma\delta$ T cells for numeric expansion are not well known. K562-derived aAPC express endogenous MHC Class-I chain-related protein A and B (MICA/B) which are ligands for both V δ 1 and NKG2D (6, 40). Indeed, NKG2D was observed on polyclonal $\gamma\delta$ T cells that also predominantly expressed V δ 1 TCR (Figure 1A). Polyclonal $\gamma\delta$ T cells also demonstrate expression for activating receptors typically found on NK cells (NKp30, NKp44, and NKp46; collectively expressed at 26% $\pm$ 7%), and future studies will examine their contribution to $\gamma\delta$ T-cell effector function. Some malignant cells were recognized poorly by $\gamma\delta$ T cells, *e.g.*, EL4, EFO27, OAW42, cALL-2, and NALM-6, which provides an opportunity to further interrogate the mechanism by which $\gamma\delta$ T cells recognize and kill tumor cells. Given that inhibition of cytolysis was maximized by neutralizing DNAM1, NKG2D, and TCR $\gamma\delta$ receptors simultaneously, it may be that sensitivity of a tumor cell resides on the expression of ligand combinations that can bind these receptors. Two ligands recognized by V δ 2 TCR are surface mitochondrial F₁-ATPase and phospho-antigens, both of which are found in K562 cells (41, 42). Enhanced responses of T cells expressing V γ 9V δ 2 were observed when K562 cells were treated with aminobisphosphonates (41) and a similar strategy could be employed upon co-culture with aAPC clone #4 to increase the abundance of T cells bearing V δ 2 TCR (18). Future studies will evaluate additional TCR $\gamma\delta$ ligands that naturally occur in these aAPC.

We enforced expression of co-stimulatory molecules to ascertain and improve the capability of K562-derived aAPC to propagate $\gamma\delta$ T cells expressing a diversity of TCR. Indeed, CD137L was the dominant co-stimulatory proliferative signal on aAPC for expansion of $\gamma\delta$ T cells with broad tumor-reactivity (Figure 1C), and its receptor, CD137, has been used to enrich tumor-reactive $\alpha\beta$ T cells following antigen exposure and presumably TCR stimulation (43-45). CD137 was not expressed on resting $\gamma\delta$ T cells prior to expansion, suggesting that the importance of CD137L co-stimulation by aAPC followed TCR stimulation by the aAPC and expression of CD137 on the $\gamma\delta$ T cell surface. CD27⁺ and CD27^{neg} $\gamma\delta$ T cells have been shown to produce IFN γ and IL-17 (46), respectively; therefore, CD27 could be used as a marker for isolating $\gamma\delta$ T cells with a preferred cytokine output. ICOS-ligand in absence of CD86 was shown to polarize CD4⁺ $\alpha\beta$ T cells to produce IL-17 instead of IFN γ (47), and current studies are investigating whether combinations of co-stimulatory molecules can selectively propagate cytokine-producing sub-populations of $\gamma\delta$ T cells. Thus, the aAPC co-culture system in the context of desired cytokines provides a clinically-relevant methodology to tailor the type of therapeutic $\gamma\delta$ T cell produced for adoptive immunotherapy.

Our data have implications for the design and interpretation of clinical trials. Expression of IL-15 was important for the maintenance of transferred $\gamma\delta$ T cells *in vivo* (48), supporting the use of IL-15 on aAPC, and future studies could inform on other molecules that could be introduced to maximize the cell therapy product. Correlative studies are enhanced by our observation that TCR $\gamma\delta$ mAb can be used to readily distinguish the three (V δ 1, V δ 2, and V δ 1^{neg}V δ 2^{neg}) T-cell subsets based on MFI of TCR $\gamma\delta$ expression (Figure 3B). Given that $\gamma\delta$ T cells are not thought to recognize ligands in the context of MHC (17), there is potential to infuse allogeneic, including 3rd party, $\gamma\delta$ T cells in lymphodepleted hosts to achieve an anti-tumor effect while mitigating the risk of graft-versus-host disease. Restoration of lymphopoiesis may result in graft rejection, but a therapeutic window could be established whereby tumors are directly killed by infused $\gamma\delta$ T cells, which may result in desired bystander effects as conserved or neo-antigens are presented to other lymphocytes. Indeed, $\gamma\delta$ T cells have been shown to lyse cancer cells, cross-present tumor-specific antigens to $\alpha\beta$ T cells, and license them to kill tumors (49, 50). The aAPC clone #4 has been produced as a master cell bank in compliance with current good manufacturing practice and provides a clear path to generating clinical-grade $\gamma\delta$ T cells for human application. Human trials can now, for the first time, test the efficacy of adoptive transfer of T cells with polyclonal TCR $\gamma\delta$ repertoire for treatment of solid and hematological tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TRANSLATIONAL RELEVANCE

$\gamma\delta$ T cells have anti-cancer activity, but only one subset, V γ 9V δ 2, has been harnessed for immunotherapy. Our study establishes that artificial antigen presenting cells (aAPC), IL-2, and IL-21 can activate and propagate $\gamma\delta$ T cells with polyclonal TCR repertoire to clinical scale. The heterogeneous population of $\gamma\delta$ T cells produced from *ex vivo* culture secreted pro-inflammatory cytokines, lysed a broad range of malignancies, and improved survival in an ovarian cancer xenograft model. Given that $\gamma\delta$ T cells are not thought to recognize ligands in the context of MHC, there is limited risk of graft-versus host disease in an allogeneic setting. Thus, 3rd party $\gamma\delta$ T cells from an unrelated (healthy) donor could be produced in bulk and be administered as an off-the-shelf investigational therapy for hematologic and solid tumors. The aAPC are already available as a clinical reagent, which will facilitate the human application of polyclonal $\gamma\delta$ T cells.

Figure 1. Sustained proliferation of polyclonal PBMC-derived $\gamma\delta$ T cells on γ -irradiated aAPC in presence of soluble IL-2 and IL-21

(A) Frequency of $\gamma\delta$ T cells before (Day 0) and after (Day 22) co-culture on γ -irradiated aAPC, IL-2, and IL-21 where expression of CD3, CD56, TCR $\alpha\beta$, TCR $\gamma\delta$, TCR $\delta 1$, and TCR $\delta 2$ is shown at Day 22 of co-culture. One of 7 representative donors is shown. Quadrant frequencies (percentage) within flow plots are displayed in upper right corners. (B) Inferred cell counts of polyclonal $\gamma\delta$ T cells are displayed calculated based on weekly yields and relative fold changes, where three arrows represent addition of aAPC. Black line is mean $\pm$ SD (n=4) pooled from 2 independent experiments and each gray line is an individual donor. (C) Fold increase over 9 days of $\gamma\delta$ T cells co-cultured with IL-2 and IL-21 along with aAPC expressing membrane-bound IL-15 (mIL15), CD86, and/or CD137L. Data are mean $\pm$ SD (n=3) pooled from 2 independent experiments and each shape represents an individual donor. Two-way ANOVA with Bonferroni's post-tests was used for statistical analysis. *p<0.05 and **p<0.01 (D) Fold increase over 9 days of $\gamma\delta$ T cells co-cultured with aAPC (clone #4) in the presence of either soluble recombinant IL-2 and/or IL-21. Data are mean $\pm$ SD (n=3) pooled from 2 independent experiments where each shape represents an individual donor. Two-way ANOVA with Bonferroni's post-tests was used for statistical analysis. *p<0.05

Figure 2. Abundance of Vδ and Vγ mRNA species in γδT cells propagated and activated *ex vivo*
 Quantification of mRNA species coding for (A) Vδ and (B) Vγ alleles in PBMC-derived γδT cells by DTEA at day 22 of co-culture on aAPC/IL-2/IL-21. Quantification of mRNA species coding for (C) Vδ and (D) Vγ alleles in UCB-derived γδT cells by DTEA at day 34-35 of co-culture on aAPC/IL-2/IL-21. Box-and-whiskers plots display 25% and 75% percentiles where lines represent maximum, mean, and minimum from top to bottom (n=4). Solid lines at bottom of graphs represent limit-of-detection (LOD) calculated from mean $\pm$ 2xSD of DTEA negative controls. Student's paired one-tailed t-tests were performed for each allele relative to the sample LOD. *p<0.05 and **p<0.01

Figure 3. Sustained proliferation of PBMC-derived Vδ T-cell subsets expanded on γ-irradiated aAPC/IL-2/IL-21

After two 7-day stimulations with aAPC (clone #4) and IL-2/IL-21 the bulk population of γδT cells were separated into Vδ1, Vδ2, and Vδ1^{neg}Vδ2^{neg} subsets by FACS based on staining of T cells defined as TCRδ1⁺TCRδ2^{neg}, TCRδ1^{neg}TCRδ2⁺, and TCRδ1^{neg}TCRδ2^{neg}, respectively. **(A)** Expression of TCRδ1 and TCRδ2 chains on Vδ1, Vδ2, and Vδ1^{neg}Vδ2^{neg} subsets of γδT cells (from left to right) after 15 days of numeric expansion on aAPC and cytokines as isolated groups. One of 4 representative donors is shown pooled from 2 independent experiments. Quadrant frequencies (percentage) within flow plots are displayed in upper right corners. Frequency of TCRδ1⁺TCRδ2^{neg} (open bars), TCRδ1^{neg}TCRδ2⁺ (black bars), and TCRδ1^{neg}TCRδ2^{neg} (gray bars) cell surface protein expression in subsets of γδT cells after 15 days numeric expansion on aAPC and cytokines as isolated groups. Data are mean ± SD (n=4) pooled from 2 independent experiments. **(B)** Flow cytometry plots of CD3 and TCRγδ expression in Vδ1, Vδ2, and Vδ1^{neg}Vδ2^{neg} subsets (from left to right). Mean fluorescence intensity (MFI) of TCRγδ staining in Vδ1, Vδ2, and Vδ1^{neg}Vδ2^{neg} T-cell subsets where each shape represents a different donor and data are mean ± SD (n=4) pooled from 2 independent experiments. **(C)** Proliferation of each isolated Vδ subset stimulated twice with aAPC clone #4 (arrows) in presence of cytokines and total cell counts are displayed. Data are mean ± SD (n=4) pooled from 2 independent experiments. **(D)** DTEA was used to identify and measure abundance of mRNA species coding for Vδ1*01, Vδ2*02, and Vδ3*01 (from left to right) in γδT-cell sub-populations after 15 days of proliferation on aAPC and cytokines as separated subsets. Box-and-whiskers plots display 25% and 75% percentiles where lines represent maximum, mean, and minimum from top to bottom (n=4). Student's paired, two-tailed t-tests were undertaken for statistical analyses between groups. **p<0.01 and ***p<0.001

Figure 4. Dependence on TCRγδ for IFNγ secretion in response to tumor cells

At Day 22 of co-culture on γ-irradiated aAPC (clone #4) with IL-2 and IL-21, T cells were incubated with CM (mock) or leukocyte activation cocktail (LAC; PMA/Ionomycin) for 6 hours at 37 °C. Tissue culture supernatants were interrogated using 27-Plex Luminex array to detect presence of (A) T_H1, T_H2, and T_H17 cytokines and selected chemokines (from left to right). Data are mean ± SD pooled from 4 donors in 2 independent experiments where each donor had triplicate experimental wells pooled prior to multiplex analysis. Student's one-tailed t-test performed for statistical analysis between mock and LAC groups. *p<0.05, **p<0.01, and ***p<0.001 (B) Polyclonal γδT cells were incubated for 1 hour prior to and during 6 hour tumor cell co-culture with normal mouse serum or neutralizing TCRγδ antibody (clone IM). Cells were stained for TCRδ1, TCRδ2, CD3, and IFNγ to gate T-cell subsets and assess IFNγ production. Comparisons of histograms detailing Vδ1, Vδ2, and Vδ1^{neg}Vδ2^{neg} gates (from left to right) co-cultured with CAOV3 ovarian cancer cells and treated with serum (open) or TCRγδ (shaded). Numbers next to histograms are MFI. Flow plots are representative of 1 of 3 PB donors co-cultured with CAOV3 cells in 2 independent experiments. (C) Percent inhibition of IFNγ secretion in response to CAOV3 cells was calculated for each Vδ T-cell subset based on the following equation: Inhibition (%) = 100 – 100 × [(MFI_{TUMOR} + T_{CELL} – MFI_{T CELL ONLY})_{TCRγδ} / (MFI_{TUMOR} + T_{CELL} – MFI_{T CELL ONLY})_{Serum}]. Data are mean ± SD (n=3) pooled from 2 independent experiments.

Figure 5. Specific lysis of tumor-cell panel by polyclonal $\gamma\delta$ T cells

(A-C) Standard 4-hour CRA was performed with increasing effector (polyclonal $\gamma\delta$ T cells; each shape represents a different donor) to target (E:T) ratios against (A) healthy B cells from an allogeneic donor (one of four representative donors), (B) hematological tumor cell lines derived from B-ALL: RCH-ACV, T-ALL: Jurkat, and CML: K562, (C) solid tumor cell lines derived from pancreatic cancer: BxPc-3, colon cancer: HCT-116, and ovarian cancer: OC314 and CAOV3. Data are mean $\pm$ SD (n=3 wells per assay) from 2 independent experiments. (D) Neutralizing antibodies to NKG2D (squares), DNAM1 (triangles), TCR $\gamma\delta$ (inverted triangles), or a pool (diamonds) of all three antibodies were used to block killing of Jurkat (left), IGROV1 (middle), or OC314 (right) tumor targets antibodies at 0.3, 1, and 3 μ g/mL and an E:T ratio of 12:1 in standard 4-hour CRA. Normal mouse serum (circles) served as control for addition of antibody and wells without antibody were used for normalization purposes. Specific lysis was normalized to wells without antibody to yield relative cytotoxicity as defined by: $\text{Relative cytotoxicity (\%)} = \frac{(\text{Specific Lysis})_{\text{With Antibody}}}{(\text{Specific Lysis})_{\text{Without Antibody}}} \times 100$. Data are mean $\pm$ SD (n=4 donors) from triplicates pooled and normalized from 2 independent experiments. Repeated-measures Two-way ANOVA was used for statistical analysis between antibody treatments. **p<0.01 and ***p<0.001

Figure 6. *In vivo* clearance of ovarian cancer upon adoptive transfer of polyclonal $\gamma\delta$ T cells and $\gamma\delta$ T-cell subsets propagated/activated on aAPC with IL-2 and IL-21

CAOV3-*effLuc*-mKate tumor cells were injected into NSG mice at Day -8 and engrafted until Day 0 when treatment was started with either PBS (vehicle/mock) or $\gamma\delta$ T cells. Four T-cell doses were administered in weekly escalating doses. (A) BLI images at Day 0 (top panels) or Day 72 (bottom panels) in PBS, Vδ1, Vδ2, Vδ1^{neg}Vδ2^{neg}, and polyclonal $\gamma\delta$ T-cell treatment groups. Images are representative of 6-14 mice from 2 independent experiments. (B) BLI measurements of mice at Day 0 (white) and Day 72 (gray) pooled from 2 independent experiments. Box-and-whiskers plots display 25% and 75% percentiles where lines represent maximum, mean, and minimum from top to bottom (n = 6-14). Student's paired, two-tailed t-tests were used for statistical analysis between time points. (C) Overall survival of mice treated with PBS (dashed), polyclonal (black), Vδ1 (red), Vδ2 (blue), or Vδ1^{neg}Vδ2^{neg} (green) $\gamma\delta$ T cells. Log-rank (Mantel-Cox) test was used to calculate p values. *p<0.05, **p<0.01, and ***p 0.001