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 γδ T cells expressing polyclonal repertoire of γ and δ T-cell receptor (TCR) chains for adoptive immunotherapy of 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 γδ T cells to clinical scale. These cells were tested for proliferation, TCR expression, memory phenotype, cytokine secretion, and tumor killing.

Results: γδ T-cell proliferation was dependent upon CD137L expression on aAPC and addition of exogenous IL2 and IL21. Propagated γδ T cells were polyclonal as they expressed TRDV1, TRDV2-2, TRDV3, TRDV5, TRDV7, and TRDV8 with TRGV2, TRGV3F, TRGV7, TRGV8, TRGV9A1, TRGV10A1, and TRGV11 TCR chains. IFNγ production by Vδ1, Vδ2, and Vδ1negVδ2neg subsets was inhibited by pan-TCRγδ antibody when added to cocultures of polyclonal γδ T cells and tumor cell lines. Polyclonal γδ 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 γδ T cells mediated tumor cell lysis through combination of DNAM1, NKG2D, and TCRγδ. The adoptive transfer of activated and propagated γδ T cells expressing polyclonal versus defined Vδ TCR chains imparted a hierarchy (polyclonal>Vδ1>Vδ1negVδ2neg>Vδ2) of survival of mice with ovarian cancer xenografts.

Conclusions: Polyclonal γδ T cells can be activated and propagated with clinical-grade aAPCs and demonstrate broad antitumor activities, which will facilitate the implementation of γδ T-cell cancer immunotherapies in humans. Clin Cancer Res; 20(22); 1–12. ©2014 AACR.

Introduction

Human γδ 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 cytolsis 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 Vγ alleles (TRGV), 3 unique Vδ alleles (TRDV1, TRDV2, and TRDV3), and 5 Vδ alleles that share a common nomenclature with Vγ alleles (TRDV4/TRA14, TRDV5/TRA29, TRDV6/TRA23, TRDV7/TRA36, and TRDV8/TRA38-2; ref. 3). T cells expressing TCRγ/TCRδ heterodimers compose approximately 95% of peripheral blood T cells and recognize peptides in the context of MHC (4). In contrast, TCRγδ ligands are recognized independent of MHC and these cells are infrequent (1%–5% of T cells) in peripheral blood (1, 5, 6). Many conserved ligands for TCRγδ are present on cancer cells, thus an approach to propagating these T cells from small starting numbers while maintaining a polyclonal repertoire of γδ TCRs has appeal for human application.

Clinical trials highlight the therapeutic potential of γδ T cells, but numeric expansion is needed for adoptive immunotherapy because they circulate at low frequencies in peripheral blood. Methods to propagate γδ T cells, for e.g., using interleukin-2 (IL2) and/or antibody cross-linking CD3, cannot sustain proliferation of γδ T cells (7, 8). Aminobisphosphonates, for e.g., zoledronic acid, have been used to initiate a proliferative signal in γδ T cells (5, 9), but only one lineage of γδ T cells, expressing Vγ9Vδ2 TCR, can be reliably expanded by zoledronic acid. The adoptive transfer of Vγ9Vδ2 T cells has yielded clinical responses for investigational treatment of solid and hematologic cancers (10–14). Furthermore, long-term remission of leukemia among recipients of haploididentical αβ T-cell-depleted
Translational Relevance

γδT cells have anticancer activity, but only one subset, Vγ9Vδ2, has been harnessed for immunotherapy. Our study establishes that artificial antigen-presenting cells (aAPC), IL2, and IL21 can activate and propagate γδ T cells with polyclonal T-cell receptor repertoire to clinical scale. The heterogeneous population of γδ T cells produced from ex vivo culture secreted proinflammatory cytokines, lysed a broad range of malignancies, and improved survival in an ovarian cancer xenograft model. Given that γδ 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, third party γδ 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 aAPCs are already available as a clinical reagent, which will facilitate the human application of polyclonal γδ T cells.

Materials and Methods

Cell lines

HCT-116, Kasumi-3, and K562 were acquired from ATCC. Jurkat was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen. CALL-2 and RCH-ACV were gifts from Dr. Jeff Tyner (Oregon Health & Science University, Portland, OR). 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, Houston, TX). 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 γδ 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 PBMCs (10⁶) were initially treated with CD56 microbeads (cat# 130-050-401, Miltenyi Biotec) and separated on LS columns (cat# 130-042-401, Miltenyi Biotec) to deplete NK cells from cultures because they proliferate on aAPCs (23) and would contaminate the purity of the γδ T-cell product. Unlabeled cells from CD56 depletion sorting were then labeled with TCRγδ T-cell isolation kit (cat# 130-092-892, Miltenyi Biotec) and placed on LS columns to separate γδ T cells in the unlabeled fraction from other cells attached to magnet. γδ T cells were cocultured at a ratio of one T cell to two γ-irradiated (100 Gy) aAPCs (clone #4) in presence of exogenous IL2 (Aldesleukin; Novartis; 50 IU/mL), and IL21 (cat# AF20021; Peprotech; 30 ng/mL) in complete media (CM; RPMI, 10% FBS, 1% Glutamax). Cells were serially restimulated with addition of γ-irradiated aAPCs 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 aAPCs (clone #4) as previously described (25, 26). Validation of coexpression of CD19, CD64, CD86, CD137L, and eGFP (IL15 peptide fused in frame to IgG4 Fc stalk and coexpressed with eGFP) on aAPC clone #4 was performed before addition to T-cell cultures (25). Fluorescence-activated cell sorting (FACS) was used to isolate Vδ1 (TCRδ1⁺TCRδ2⁻neg), Vδ2 (TCRδ1negTCRδ2⁺), and Vδ1negVδ2neg (TCRδ1negTCRδ2neg) populations, which were stimulated twice as above with aAPC clone #4, phenotyped, and used for functional assays. 

Abundance and identity of mRNA molecules by DTEA

At designated times after coculture on aAPCs, T cells were lysed at a ratio of 160 μL RLT buffer (Qiagen) per 10⁶ cells and frozen at −80°C. RNA lysates were thawed and
immediately analyzed using nCounter Analysis System (NanoString Technologies) with "designer TCR expression array" (DTEA), as previously described (27, 28). DTEA data were normalized to both spiked 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 Supplementary Table S1. Gating strategy is displayed in Supplementary Fig. S1. Samples were acquired on FACS Calibur (BD Biosciences) and analyzed with FlowJo software (version 7.6.3).

Cytokine production and cytolyis 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 Supplementary Materials and Methods.

Mouse experiments
In vivo antitumor efficacy was assessed in NSG mice (NOD.Cg-PrkdcscidIl2rg−/−Il2rgtm1Wjl/Sjl; Jackson Laboratories). CAOV3 ovarian cancer cell line was transduced with recombinant lentivirus (Supplementary Fig. S2) encoding mKate red fluorescence protein (29) to identify transduced cells and enhanced firefly luciferase (effLuc) for noninvasive bioluminescence imaging (30). CAOV3-effLuc-mKate (clone 1C2; 3 × 106 cells/mouse) tumors were established by intraperitoneal injection and mice were randomly distributed into treatment groups. Eight days later (designated day 0), a dose escalation regimen was initiated with γ T cells and PBS (negative control) administered intraperitoneally. T-cell doses were 3 × 106, 6 × 106, 107, and 1.5 × 107 on days 0, 7, 14, and 21, respectively. Noninvasive bioluminescence images (BLI) was performed during the course of the experiments to serially measure tumor burden of CAOV3-effLuc-mKate following subcutaneous administration of α-Luciferin (cat#122796, Caliper) 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 γ T cells on aAPCs depends on costimulation and cytokines
The adoptive transfer of γ T cells requires ex vivo propagation as starting numbers from PBMCs are limiting (gating on lymphocyte pool: 3.2% ± 1.2%; mean ± SD; n = 4; Fig. 1A). γ T cells from PBMCs were isolated by "negative" paramagnetic bead selection and cocultured for 22 days with weekly addition of γ-irradiated K562-derived aAPCs (clone #4) in the presence of soluble recombinant IL2 and IL21 in alignment with protocols at MD Anderson Cancer Center (Houston, TX) for propagation of clinical-grade γ T cells. This resulted in the outgrowth of a population of T cells homogeneously coexpressing CD3 and TCRγδ (97.9% ± 0.6%). NK cells (CD3negCD56+), and γ T cells (TCRγδ−) were absent from these cultures supporting the purity of the γ T-cell product. Populations of TCRγδ1/TCRδ2neg, TCRδ1negTCRδ2+, and TCRδ1negTCRδ2neg were detected indicating that aAPCs, IL2, and IL21 supported polyclonal γ T-cell proliferation (Fig. 1A far right). Cells were activated as marked by expression of CD38 (93.5% ± 3.5%) and CD95 (99.7% ± 0.1%; Supplementary Fig. S3). This approach to propagation yielded >104 γ T cells from <106 total initiating cells (Fig. 1B), which represented a 4.9 × 105 ± 1.7 × 105 (mean ± SD; n = 4) fold increase. Thus, aAPCs with recombinant human cytokines supported the robust numeric expansion of polyclonal γ T cells from small starting numbers of γ T cells derived from PBMCs.

The addition of exogenous cytokines and presence of mIL15, CD86, and CD137L on clinical-grade aAPCs were assessed for their ability to support the outgrowth of γ T cells. Parental K562 cells were stably transfected with Sleeping Beauty (SB) transposons to introduce individual stimulatory molecules, cloned to achieve homogeneous expression (Supplementary Fig. S4), and then used to assess their impact on γ T-cell proliferation. Cocultures with exogenous IL2 and IL21 were initiated with paramagnetic bead-purified γ 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). γ T cells cultured in parallel without APC demonstrated that soluble IL2 and IL21 sustained only limited numeric expansion of γ T cells (Fig. 1C). Propagation improved upon addition of parental K562 cells, indicating that endogenous molecules on these cells can activate γ T cells for proliferation. The expression of mIL15 with or without CD86 did not further improve the ability of γ T cells to propagate compared with parental K562. In contrast, improved rates of propagation of γ T cells were observed upon coculture with mIL15/CD137L+ and mIL15/CD86+CD137L+ aAPCs. Thus, it appears that CD137L on aAPC clone #4 provides a dominant costimulatory proliferative signal for γ T cells. In the absence of IL2 and IL21, the proliferation of γ T cells ceased on aAPC clone #4, and together these cytokines exhibited an additive benefit to the rate of γ T-cell propagation (Fig. 1D). This validated our approach to combining aAPC clone #4 with cytokines to sustain the proliferation of polyclonal γ T cells ex vivo, and demonstrated that CD137L on aAPCs, IL2, and IL21 were driving factors for proliferation of polyclonal γ T cells to clinical scale.

Ex vivo numeric expansion of neonatal γ T cells on aAPCs in presence of IL2 and IL21
Allogeneic UCB is an important source of γ T cells for adoptive transfer, because it contains younger cells and a more diverse TCRγδ repertoire relative to PBMCs, 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 monoclonal T cells within a banked UCB unit curtails the number of neonatal γδ T cells directly available for adoptive transfer. Thus, we evaluated whether aAPCs could sustain proliferation from small starting numbers of neonatal γδ T cells. Fluorescence-activated cell sorting (FACS) was used to isolate $10^4$ UCB-derived γδ T cells (~0.01% of a typical UCB unit) which were cocultured on aAPC clone #4 with IL2 and IL21 along with aAPC expressing membrane-bound IL15 (mIL15), CD86, and/or CD137L.

The propagated γδ T-cell populations exhibited uniform coexpression of CD3 and TCRγδ and lacked TCRβ+ T cells or presence of CD56−NK cells (Supplementary Fig. S5B–S5D). Collectively, these data demonstrate that aAPC clone #4 with IL2 and IL21 could sustain the ex vivo proliferation of γδ T cells from a small starting population of neonatal UCB.

**Ex vivo activated and propagated γδ T cells express polyclonal and defined TCRγδ repertoire**

Upon establishing that γδ T cells could numerically expand on aAPCs and selected cytokines, we sought to determine the TCR repertoire of the propagated cells. Before numeric expansion, resting γδ T-cell repertoire followed TCRδ2>TCRδ1>TCRδ2>γδ TCRδ1 by flow cytometry (Supplementary Fig. S6). However, the γδ T-cell repertoire followed TCRδ1>TCRδ2>γδ TCRδ2 following expansion, suggesting that there was a proliferative advantage for Vδ1 cells within polyclonal γδ T-cell cultures. To look more in-depth at TCRδ diversity in aAPC-expanded γδ T cells, we adapted a nonenzymatic digital multiplex assay used to quantify the TCR diversity in γδ T cells expressing a CD19-specific chimeric antigen receptor (CAR; ref. 27) termed DTEA. After expansion (day 22), 4 of 8 Vδ alleles (TRDV1, TRDV2-2, TRDV3, and TRDV8) were detected in PBMC-derived γδ T cells (Fig. 2A) and were coexpressed with Vγ alleles TRGV2, TRGV7, TRGV8 (two probes), TRGV9-A1, TRGV10-A1, and TRGV11-A2 (Fig. 2B). Similarly, a polyclonal assembly of Vδ and Vγ chains was observed in γδ T cells from UCB following expansion (days 34–35), albeit with reduced abundance of TRDV2-2, more TRGV2, and presence of TRGV3F, TRVD5, and TRDV7 cells not seen from PBMCs (Fig. 2C and D). Similar patterns of Vδ and Vγ mRNA usage were detected in PBMCs and UCB before and after expansion (Supplementary Fig. S7) although overall mRNA counts were fewer in the resting cells (day 0) relative to the activated γδ T cells. Thus, aAPC-expanded γδ T cells maintain a polyclonal TCR repertoire from both PBMCs and UCB.

We sought to validate these mRNA data by sorting polyclonal populations with TCRδ-specific antibodies and

**Figure 1.** Sustained proliferation of polyclonal PBMC-derived γδ T cells on γ-irradiated aAPCs in the presence of soluble IL2 and IL21. A, frequency of γδ T cells before (day 0) and after (day 22) coculture on γ-irradiated aAPCs, IL2, and IL21 where expression of CD3, CD56, TCRγδ, TCRβ, TCRδ1, and TCRδ2 is shown at day 22 of coculture. One of 7 representative donors is shown. Quadrant frequencies (percentage) within flow plots are displayed in top right corners. B, inferred cell counts of polyclonal γδ T cells are displayed calculated on the basis of weekly yields and relative fold changes, where three arrows represent additional addition of aAPCs. Black line, mean ± SD (n = 4) pooled from 2 independent experiments and each gray line is an individual donor. C, fold increase over 9 days of γδ T cells cocultured with IL2 and IL21 along with aAPC expressing membrane-bound IL15 (mIL15), CD86, and/or CD137L. Data, mean ± SD (n = 3) from two independent experiments and each shape represents an individual donor. Two-way ANOVA with Bonferroni posttests was used for statistical analysis. *, $P < 0.05$; **, $P < 0.01$. D, fold increase over 9 days of γδ T cells cocultured with aAPCs (clone #4) in the presence of either soluble recombinant IL2 and/or IL21. Data are mean ± SD (n = 3) pooled from two independent experiments where each shape represents an individual donor. Two-way ANOVA with Bonferroni post tests was used for statistical analysis. *, $P < 0.05$. 

**Table 1.** Analysis of γδ T-cell repertoire. Ex vivo and in vitro γδ T cell populations were isolated from PBMCs and banked UCB units, respectively. The γδ T-cell repertoire was determined by a nonenzymatic digital multiplex assay in the presence or absence of CD19-specific CAR expressing aAPCs. The TCR repertoire was displayed as a Vγ/Vδ clustergram.
repeating DTEA on isolated cultures. There are only two TCRδ-specific mAbs commercially available and they identified three discrete Vδ populations (Vδ1: TCRδ1+TCRδ2neg, Vδ2: TCRδ1negTCRδ2+, and Vδ1negVδ2neg). TCRδ1negTCRδ2neg) within aAPC-expanded γδ T cells from PBMCs (Fig. 1A) and UCB (Supplementary Fig. S8) with abundance following Vδ1>Vδ1negVδ2neg>Vδ2. FACS-isolated subsets from PBMC-derived γδ T-cell pools were propagated with clone #4 as discrete populations and maintained their identity as assessed by expression of TCRδ isotypes (Fig. 3A). Each of the separated subsets could be identified by a pan-specific TCRδ antibody confirming that these cells were indeed γδ T cells (Fig. 3B). Furthermore, each population could be differentiated based on pan-TCRδ antibody mean fluorescence intensity (MFI) where Vδ2, Vδ1negVδ2neg, and Vδ1 T cells corresponded to the TCRδlow (43 ± 9; mean ± SD; n = 4), TCRδintermediate (168 ± 40), and TCRδhigh (236 ± 56) groupings, respectively. No differences in proliferation kinetics on aAPCs were observed between isolated Vδ-sorted subsets (Fig. 3C) indicating that the observed inversion of Vδ1 and Vδ2 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 Vδ1, Vδ2, and Vδ1negVδ2neg subpopulations were homogeneous populations as they predominantly expressed TRDV1, TRDV2-2, and TRDV3 mRNA species at 261 ± 35, 3,910 ± 611, and 5,559 ± 1119 absolute counts, respectively (Fig. 3D). Therefore, there were fewer TRDV1 mRNA species expressed by Vδ1 cells relative to the TRDV2-2 expressed by Vδ2 cells and TRDV3 expressed by Vδ1negVδ2neg cells. Moreover, these data indicated that the relatively low counts observed for TRDV1 in polyclonal populations with a preponderance of TCRδ1+ cells was not a defect in DTEA detection but rather a product of fewer total mRNA transcripts relative to other Vδ species. Given the wide range of mRNA

Figure 2. Abundance of Vδ and Vγ mRNA species in γδ T cells propagated and activated ex vivo. Quantification of mRNA species coding for (A) Vδ and (B) Vγ alleles in PBMC-derived γδ T cells by DTEA at day 22 of coculture on aAPCs/IL2/IL21. Quantification of mRNA species coding for (C) Vδ and (D) Vγ alleles in UCB-derived γδ T cells by DTEA at day 34 to 35 of coculture on aAPCs/IL2/IL21. 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 ± 2 × SD of DTEA-negative controls. Student paired one-tailed t tests were performed for each allele relative to the sample LOD. *, P < 0.05; **, P < 0.01.
transcript quantities for each allele, DTEA was not useful for calculation of relative frequencies of Vδ subsets in polyclonal populations but rather was indicative of presence or absence of a particular Vγ T-cell subset. Expression of other Vδ2 alleles (TRDV2-1 and TRDV2-1F) was absent from polyclonal Vγ T cells (Fig. 2A) and each of the sorted subsets (data not shown). Small amounts of TRDV4, TRDV5, TRDV6, and TRDV7 mRNA species were detected in the three subsets of T cells sorted for Vδ expression (Supplementary Fig. S9). TRDV8 mRNA was exclusively present in sorted Vδ1negδ2neg cells and these T cells are likely the main contributors of TRDV8 in bulk Vγ T cells. The same Vγ mRNA present in polyclonal cultures was detected in Vδ-sorted cultures (Supplementary Fig. S10). Furthermore, Vδ1 and Vδ1negδ2neg were not different (P = 0.419; two-way ANOVA) but Vδ2 was different to both Vδ1 (P < 0.00001) and Vδ1negδ2neg (P < 0.00001) in Vγ usage. Collectively, these results confirmed DTEA from unsorted cultures and strongly supported the polyclonal TCRγδ expression on γδ T cells activated to proliferate by aAPCs and cytokines.

IFNγ produced in response to tumors is dependent on TCRγδ

A multiplex analysis of cytokines and chemokines was performed to determine whether aAPC-propagated γδ T cells might foster a proinflammatory response in a tumor microenvironment (Fig. 4A). The Th1-associated cytokines IFNγ and TNFα were secreted in abundance by γδ T cells upon exposure to leukocyte-activated cocktail (LAC; PMA and ionomycin for nonspecific mitogenic stimulation), in addition to small amounts of IL2 and IL12 p70. In contrast, no significant production of the Th2-associated cytokines IL4, IL5, and IL13 was observed from LAC-treated γδ 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% CD3+ TCRγδ these data indicate that it was activation of γδ T cells that led to a largely proinflammatory response. IFNγ was the most responsive
of all the assessed cytokines and was chosen to measure responses of Vδ8 subsets to tumor cells (Fig. 4B). Coculture of polyclonal αAPC-propagated/activated γδ T cells with cancer cells resulted in a hierarchy of IFNγ production following Vδ2>Vδ1>Vδ1expδ2mut as shown by MFI of 855 ± 475, 242 ± 178, and 194 ± 182 (mean ± SD; n = 4), respectively. IFNγ production by Vδ1, Vδ2, and Vδ1expδ2mut subsets was inhibited by pan-TCRδγ antibody when added to γδ T-cell/tumor cocultures indicating that response to the tumor in each subset was dependent upon activation through TCRδγ (Fig. 4C). This observation supported the premise that a single cancer cell could be targeted by discrete γδ TCRs. Thus, a multivalent proinflammatory response to the tumor cell was achieved by polyclonal γδ T cells.

**Polyclonal γδ T cells lyse a broad range of tumor cells through combination of DNAM1, NKG2D, and TCRγδ**

After establishing that propagated γδ T cells could be activated to produce proinflammatory cytokines, we examined their ability to specifically lyse a panel of tumor cell lines. Polyclonal γδ T cells demonstrated a range of cytotoxicity against solid and hematologic cancer cell lines without a clear preference towards a particular tumor histology or grade (Fig. 5 and Supplementary Fig. S11). We previously established that B-cell acute lymphoblastic leukemia (ALL) cell line NALM-6 was largely resistant to lysis by γδ T cells, which required a CD19-specific CAR to acquire significant killing capability (27). In this study, we also observed that autologous and allogeneic normal B cells were spared from cytolysis (Fig. 5A), and that B-ALL cell line cALL-2 and murine T-cell lymphoma cell line EL4 were lysed poorly by polyclonal γδ T cells, which indicated that some cells were resistant and/or not recognized by polyclonal γδ T cells. In contrast, T-ALL cell line Jurkat and B-ALL cell lines RCH-ACV were both killed efficiently by polyclonal γδ T cells (Fig. 5B), indicating that γδ 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 γδ T cells. Chronic myelogenous leukemia (CML) cell line K562 and K562-derived clone#4 αAPCs were killed by polyclonal γδ 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 γδ T cells, as was the colon carcinoma cell line HCT-116 (Fig. 5C). Ovarian cell lines were killed by polyclonal γδ T cells in the following order of decreasing sensitivity: CAOV3 > EFO21 > UPN251 > IGROV1 > OC314 > Hey > A2780 > OVCAR3 > OAW42 > EFO27. Each of the separated Vδ subsets lysed hematologic (Jurkat and K562) and solid (OC314 and CAOV3) tumor cell lines, which showed that polyclonal γδ T cells could direct a multivalent response against common targets (Supplementary Fig. S12). The strength of cytoly sis followed the
hierarchy of TCR usage (V\textsubscript{\textdagger}2\textgreater V\textsubscript{\textdagger}1\textsuperscript{\textdagger}negV\textsubscript{\textdagger}2neg\textgreater V\textsubscript{\textdagger}1) that was consistent with the premise that a propensity to be triggered for effector function would increase with T-cell differentiation (Supplementary Fig. S13). Lysis by polyclonal populations was apparently not due to one specific V\textsubscript{\textdagger} subtype but rather from contributions of multiple \textgamma T-cell subsets, because it was observed that (i) a number of tumor cell lines were equivalently killed by polyclonal \textgamma T cells containing different frequencies of V\textsubscript{\textdagger}1, V\textsubscript{\textdagger}2, and V\textsubscript{\textdagger}1\textsuperscript{\textdagger}negV\textsubscript{\textdagger}2neg cells and (ii) 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 (Fig. 5D). Our experimental approach also took into account that \textgamma T cells coexpress DNAM1 (97.7% ± 0.9%; mean ± SD; n = 4) and NKG2D (40.1% ± 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\textgamma in 2 of 3 cell lines tested. In contrast, a pool of antibodies binding NKG2D, DNAM1, TCR\textgamma resulted in significant inhibition, in a dose-dependent manner, of \textgamma T-cell–mediated cytolysis against all 3 targets. Collectively, these data established that ex vivo-propagated \textgamma T cells have broad antitumor capabilities likely mediated by activation though DNAM1, NKG2D, and TCR\textgamma.

Figure 5. Specific lysis of tumor cell panel by polyclonal \textgamma T cells. A–C, standard 4-hour CRA was performed with increasing effector (polyclonal \textgamma 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, hematologic 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 ± SD (n = 3 wells/assay) from two independent experiments. D, neutralizing antibodies to NKG2D (squares), DNAM1 (triangles), TCR\textgamma (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 μg/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 cytolysis as defined by: Relative cytolysis (%) = (Specific Lysis)\textsubscript{With Antibody} / (Specific Lysis)\textsubscript{Without Antibody} × 100. Data are mean ± SD (n = 4 donors) from triplicates pooled and normalized from two independent experiments. Repeated-measures two-way ANOVA was used for statistical analysis between antibody treatments. **, P < 0.01; ***, P < 0.001.
Established ovarian cancer xenografts are eliminated by adoptive transfer of γδ T cells

To test whether polyclonal γδ 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. After 8 days of tumor engraftment, either PBS (vehicle/mock), Vδ1, Vδ2, Vδ1negVδ2neg, or polyclonal γδ T-cell treatments at day 72, relative to their initial tumor burden (Fig. 6A and B). Tumor burden and biodistribution were serially measured by noninvasive BLI. Established tumors continued to grow in vehicle-treated mice, but tumor burden was significantly reduced (P ≤ 0.001) in mice receiving γδ T-cell treatments at day 72, relative to their initial tumor burden (Fig. 6A and B). Adoptive transfer of polyclonal γδ T cells, Vδ1, and Vδ1negVδ2neg T cells significantly (P ≤ 0.01), and Vδ2 almost significantly (P = 0.055), increased long-term survival compared with mock-treated mice. This corresponded to overall survival following polyclonal>Vδ1>Vδ1negVδ2neg>Vδ2 (Fig. 6C). This is the first time that three Vδ subsets have been used for their ability to target tumor in vivo and is the first display of in vivo antitumor activity by Vδ1negVδ2neg cells. In sum, activated and propagated γδ 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 γδ T-cell populations that demonstrate extensive reactivity against hematologic and solid malignancies. T cells expressing defined Vδ TCRs have been associated with clinical responses against cancer. For example, the Vδ1 subset correlated with complete responses observed in patients with ALL and acute myelogenous leukemia (AML) after αβ
T-cell–depleted haploidentical HSCT (15–17). V81 cells were also shown to kill glioblastoma independent of cytomegalovirus (CMV) status (34). However, V81 cells have not been directly administered. Our data establish that such cells could mediate antitumor immunity and supports the adoptive transfer of V81 T cells for cancer therapy. In contrast to V81 and V81\textsuperscript{neg}V82\textsuperscript{neg} cells, T cells expressing V82 TCR have been directly infused and elicited responses against solid and hematologic tumors (9, 35). Little is known about V81\textsuperscript{neg}V82\textsuperscript{neg} T cells, but these lymphocytes have displayed recognition of the nonclassical MHC molecule CD1d with corresponding NKT-like functions and have also been correlated with immunity to HIV and CMV (36–39). Our results are the first to directly show that V81\textsuperscript{neg}V82\textsuperscript{neg} cells exhibit antitumor activities, and given their propensity to engage both viruses and cancer the add-back of this subset could especially benefit immunocompromised cancer patients. Because aAPCs with IL2 and IL21 can propagate polyclonal γδ T cells, mAbs can be used to raise against V83, V85, V87, and V88 isotypes to help elucidate their potential roles in clearance of pathogens and cancer. In aggregate, our data support the adoptive transfer of γδ T cells that maintain expression of multiple V6 TCR types as investigational treatment for cancer.

The molecules on aAPCs that activate γδ T cells for numeric expansion are not well known. K562-derived aAPCs express endogenous MHC class-I chain-related protein A and B (MICA/B) which are ligands for both V81 and NKG2D (6, 40). Indeed, NKG2D was observed on polyclonal γδ T cells that also predominantly expressed V81 TCR (Fig. 1A). Polyclonal γδ T cells also demonstrate expression for activating receptors typically found on NK cells (Nkp30, Nkp44, and Nkp46; collectively expressed at 26% ± 7%), and future studies will examine their contribution to γδ T-cell effector function. Some malignant cells were recognized poorly by γδ T cells, for e.g., EL4, EFO27, OAW42, CALL-2, and NALM-6, which provides an opportunity to further interrogate the mechanism by which γδ T cells recognize and kill tumor cells. Given that inhibition of cytosis was maximized by neutralizing DNAM1, NKG2D, and TCRγδ 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 V82 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 Vγ9V82 were observed when K562 cells were treated with aminobisphosphonates (41) and a similar strategy could be employed upon coculture with aAPC clone #4 to increase the abundance of T cells bearing V82 TCR (18). Future studies will evaluate additional TCRγδ ligands that naturally occur in these aAPCs.

We enforced expression of costimulatory molecules to ascertain and improve the capability of K562-derived aAPCs to propagate γδ T cells expressing a diversity of TCR. Indeed, CD137L was the dominant costimulatory proliferative signal on aAPCs for expansion of γδ T cells with broad tumor reactivity (Fig. 1C), and its receptor, CD137, has been used to enrich tumor-reactive αβ T cells following antigen exposure and presumably TCR stimulation (43–45). CD137 was not expressed on resting γδ T cells before expansion, suggesting that the importance of CD137L co-stimulation by aAPCs followed TCR stimulation by the aAPCs and expression of CD137 on the γδT-cell surface. CD27\textsuperscript{+} and CD27\textsuperscript{neg} γδ T cells have been shown to produce IFNγ and IL17 (46), respectively; therefore, CD27 could be used as a marker for isolating γδ T cells with a preferred cytokine output. ICOS-ligand in absence of CD86 was shown to polarize CD4\textsuperscript{+} αβ T cells to produce IL17 instead of IFNγ (47), and current studies are investigating whether combinations of costimulatory molecules can selectively propagate cytokine-producing subpopulations of γδ T cells. Thus, the aAPC coculture system in the context of desired cytokines provides a clinically relevant methodology to tailor the type of therapeutic γδ T cell produced for adoptive immunotherapy.

Our data have implications for the design and interpretation of clinical trials. Expression of IL15 was important for the maintenance of transferred γδ T cells in vivo (48), supporting the use of IL15 on aAPCs, 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 TCRγδ mAb can be used to readily distinguish the three (V81, V82, and V81\textsuperscript{neg}V82\textsuperscript{neg}) T-cell subsets based on MFI of TCRγδ expression (Fig. 3B). Given that γδ T cells are not thought to recognize ligands in the context of MHC (17), there is potential to infuse allogeneic, including third party, γδ T cells in lymphodepleted hosts to achieve an antitumor 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 γδ T cells, which may result in desired bystander effects as conserved or neoantigens are presented to other lymphocytes. Indeed, γδ T cells have been shown to lyse cancer cells, cross-present tumor-specific antigens to αβ 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 γδ T cells for human application. Human trials can now, for the first time, test the efficacy of adoptive transfer of T cells with polyclonal TCRγδ repertoire for treatment of solid and hematologic tumors.

Disclosure of Potential Conflicts of Interest

D. Deniger has, along with L. Cooper, submitted a patent application, through his institution, to the US patent office for the methods described in this paper. L. Cooper reports receiving speakers’ bureau honoraria from Milennyi Biotec; has ownership interest (including patents) in American Stem Cell and Sangamo Bioscience; and is a consultant/advisory board member for Ferring pharmaceuticals and GE Healthcare. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Dr. Carl June and colleagues (University of Pennsylvania, Pittsburgh, PA) for help generating K562-derived aAPC clone #4 and Dr. Perry Hackett (University of Minnesota, Minneapolis, MN) for his assistance with the SB system. The authors also thank Drs. George McNamara (MD Anderson Cancer Center) and Eric Tran (National Cancer Institute) who edited the text. The Immune Monitoring Core Lab (IMCL; MD Anderson Cancer Center) who performed Luminex assays and the flow cytometry core lab (MD Anderson Cancer Center) assisted with FACS facility. Cancer Center Support Grant (CCCGS) supported STR DNA fingerprinting at MD Anderson Cancer center. D.C.D. is an American Legion Auxiliary Fellow in Cancer Research (ITC-55BS-Houston). Andrew Sowell-Wade Huggins Scholar in Cancer Research (Cancer Answers Foundation), and a Teal Pre-doctoral Scholar (Department of Defense Ovarian Cancer Research Program).

Grant Support

This work was supported by funding from Cancer Center Core Grant (CA16672); R01 (CA124782, CA120956, CA141303); R33 (CA111627); P01 (CA146600); S10RR026916; Alben J. Ward Foundation; Burroughs Wellcome Fund; Bone Marrow Transplantation and Research Institute of Texas; Global Research Foundation; Department of Defense; Estate of Noelan L. Bibler; Gillson Longenbaugh Foundation; Harry T. Mangurian, Jr. Fund for Leukemia Immunotherapy; Fund for Leukemia Immunotherapy; Institute of Personalized Cancer Therapy; Leukemia and Lymphoma Society; Lymphoma Research Foundation; Miller Foundation, Mr. Herb Simons, Mr. and Mrs. Joe H. Scales; Mr. Thomas Scott; MD Anderson Cancer Center Moon Shot, National Foundation for Cancer Research; Pediatric Cancer Research Foundation; Production Assistance for Cellular Therapies (PACT); Tumor Immunotherapy; Thomas Scott, William Lawrence and Blanche Hughs Children's Alliances.

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Received December 21, 2013; revised April 8, 2014; accepted April 27, 2014; published OnlineFirst May 15, 2014.

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