Neuroblastoma Killing Properties of Vδ2 and Vδ2-Negative γδT Cells Following Expansion by Artificial Antigen-Presenting Cells

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Abstract

**Purpose:** The majority of circulating human γδT lymphocytes are of the Vγ9Vδ2 lineage, and have T-cell receptor (TCR) specificity for nonpeptide phosphoantigens. Previous attempts to stimulate and expand these cells have therefore focused on stimulation using ligands of the Vγ9Vδ2 receptor, whereas relatively little is known about variant blood γδT subsets and their potential role in cancer immunotherapy.

**Experimental Design:** To expand the full repertoire of γδT without bias toward specific TCRs, we made use of artificial antigen-presenting cells loaded with an anti γδTCR antibody that promoted unbiased expansion of the γδT repertoire. Expanded cells from adult blood donors were sorted into 3 populations expressing respectively Vδ2 TCR chains (Vδ2+), Vδ1 chains (Vδ1+), and TCR of other δ chain subtypes (Vδ1negVδ2neg).

**Results:** Both freshly isolated and expanded cells showed heterogeneity of differentiation markers, with a less differentiated phenotype in the Vδ1 and Vδ1negVδ2neg populations. Expanded cells were largely of an effector memory phenotype, although there were higher numbers of less differentiated cells in the Vδ1+ and Vδ1negVδ2neg populations. Using neuroblastoma tumor cells and the anti-GD2 therapeutic mAb ch14.18 as a model system, all three populations showed clinically relevant cytotoxicity. Although killing by expanded Vδ2 cells was predominantly antibody dependent and proportionate to upregulated CD16, Vδ1 cells killed by antibody-independent mechanisms.

**Conclusions:** In conclusion, we have demonstrated that polyclonal-expanded populations of γδT cells are capable of both antibody-dependent and -independent effector functions in neuroblastoma. *Clin Cancer Res; 20(22); 5720–32. ©2014 AACR.*
Translational Relevance

γδT cells comprise less than 5% of peripheral blood T lymphocytes in most populations. Any strategy to exploit them in cancer therapy requires adoptive transfer, which must therefore involve large-scale ex vivo expansions. Previous studies have used aminobisphosphonates or functional equivalents to expand the Vγ9Vδ2 lineage, but strategies to expand γδT cells bearing other receptors have not been developed as clinical applications. Here, we show that artificial antigen-presenting cells (aAPCs) can result in the unbiased expansion of a wide range of repertoires. The Vδ1 lineages and the Vδ1<sup>low</sup>Vδ2<sup>high</sup> lineages in the expanded populations are less differentiated and show potent antibody-independent cytotoxicity against neuroblastoma cells. This demonstration of robust expansion and cytotoxicity of polyclonal γδT cells supports translation of the technology into adoptive transfer clinical trials.

levels of pathogens such as malaria, HIV, and mycobacteria. Cytomegalovirus (CMV) infection is also known to be a stimulus of Vδ<sup>1−</sup>γδT-cell expansion (10). The ligands of the Vδ1 TCR are less well understood than those of the Vδ2 TCR, but are thought to include MHC-associated proteins MICA (11) and MICB, CMV-associated UL16 binding proteins (ULPB1–4), and lipid antigens presented on CD1 (12, 13). Vδ1<sup>−</sup>γδT cells predominate in intestinal tissues, and studies of tumor-infiltrating Vδ1<sup>+</sup>γδT cells suggest a protective role against epithelial malignancies (14), whereas CMV-activated Vδ1<sup>+</sup>γδT cells are protective against HT29 colonic carcinoma growth in a xenograft model (15). Very little is known about the potential role of Vδ1<sup>low</sup>Vδ2<sup>high</sup> γδT cells in cancer protection.

Neuroblastoma is a cancer arising from the nervous system that primarily affects children. Fifty percent of cases fall into a high-risk category with 5-year survival of around 40% despite intensive and highly toxic therapy. Current immunotherapy strategies target GD2, a ganglioside expressed abundantly on all neuroblastomas. Immunotherapy using antibody on the surface of plastic tissue culture wells overnight before adding PBMC in medium (RPMI, 10% FCS, 1% PS) was supplemented with 100 U/mL IL2 (PeproTech 100–02) and 60 ng/mL IL21. For expansion of γδT cells, with anti-human VδTCR antibodies (Leaf-purified B1, BioLegend 331204; purified anti-Vδ TCR Vγ9 clone B3, BioLegend 333204; anti-TCR pan-γδ clone ImmunoS10, Beckman Coulter COIM1349; anti-Pan TCRγδ clone 5A6. E91, Pierce antibodies 106101) or control antibody (purified mouse IgG1 isotype control antibody, clone MG1–45; BioLegend 401402) was achieved by immobilizing the antibody on the surface of plastic tissue culture wells overnight before adding PBMC in medium (RPMI, 10% FCS, 1% penicillin/streptomycin [PSI]) containing 100 U/mL IL2. aAPC-only expansions used 2:1 aAPC:γδT cells, with aAPC added every 7 days. The aAPCs were irradiated before use (80 Gy). The medium (RPMI1640, 10% FCS, 1% PS) was supplemented with 100 U/mL IL2 (PeproTech 200–02) and 60 ng/mL IL21. For expansion of γδT cells with aAPC coated in B1, the same ratios and technique were used, but the aAPCs were coated in B1 anti-γδTCR antibody before adding them to the γδT cells. Cells were incubated at 37°C with 5% CO<sub>2</sub>. Fold changes of γδT cells were derived by calculating the percentage of live cells in each γδT cell line, but Vδ2<sup>+</sup> cytotoxicity depends on antibody opsonization of target cells, whereas Vδ2<sup>low</sup> cytotoxicity is predominantly antibody independent.

Materials and Methods

Cell lines

K562 artificial antigen-presenting cells (aAPC) engineered to express CD86, CD137L, and IL15 (clone 4) were provided by Laurence Cooper as described in the companion article. Human neuroblastoma cell lines Kelly, SKNAS, SKNDZ, IMR32, and LAN1 were originally obtained from the ATCC.

Isolation of γδT cells from peripheral blood mononuclear cell

Cells were obtained from patients with neuroblastoma at the point of diagnosis or from healthy donors (via NHS National Blood Service). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll density gradient separation. γδT cells were isolated using 2 rounds of positive selection for the γδTCR with the Anti-TCRγδ MicroBead Kit (130-050-701; Miltenyi) according to the manufacturer’s protocol. If depletion of CD14<sup>+</sup> and CD11c<sup>+</sup> cells was performed, PBMCs were first stained with mouse anti-human CD14-PE (BioLegend 301806; clone M5E2), mouse anti-human CD14-PE (BioLegend 301806; clone M5E2) before a phycoerythrin (PE) depletion step using Anti-PE MicroBeads (130-048-801), in accordance with the manufacturer’s protocol. The CD14<sup>−</sup>/CD11c<sup>−</sup> fraction was then subjected to 2 rounds of positive selection for the γδTCR as described above.

Expansion of γδT cells from PBMC

γδT cells were expanded from freshly isolated PBMCs or from isolated pure populations of γδT cells. Cells were obtained from healthy donors (via NHS National Blood Service) or from patients with neuroblastoma before exposure to cytotoxic chemotherapy. Phosphoantigen-based expansions were carried out as previously described (1). Expansion using anti-human VδTCR antibodies (Leaf-purified B1, BioLegend 331204; purified anti-Vδ TCR Vγ9 clone B3, BioLegend 333204; anti-TCR pan-γδ clone ImmunoS10, Beckman Coulter COIM1349; anti-Pan TCRγδ clone 5A6. E91, Pierce antibodies 106101) or control antibody (purified mouse IgG1 isotype control antibody, clone MG1–45; BioLegend 401402) was achieved by immobilizing the antibody on the surface of plastic tissue culture wells overnight before adding PBMC in medium (RPMI, 10% FCS, 1% penicillin/streptomycin [PSI]) containing 100 U/mL IL2. aAPC-only expansions used 2:1 aAPC:γδT cells, with aAPC added every 7 days. The aAPCs were irradiated before use (80 Gy). The medium (RPMI1640, 10% FCS, 1% PS) was supplemented with 100 U/mL IL2 (PeproTech 200–02) and 60 ng/mL IL21. For expansion of γδT cells with aAPC coated in B1, the same ratios and technique were used, but the aAPCs were coated in B1 anti-γδTCR antibody before adding them to the γδT cells. Cells were incubated at 37°C with 5% CO<sub>2</sub>. Fold changes of γδT cells were derived by calculating the percentage of live cells in each γδT cell line.
cell subset at a given time using flow cytometry and relating this to the number of Trypan blue-negative cells in the culture system.

Expansion of αβ T cells using CD3/CD28 Dynabeads

PBMCs from healthy donors were cocultured with Dynabeads Human T-cell activator CD3/28 (Life Technologies, 111.31D) in accordance with the manufacturer’s protocol. The beads were added at day 0 and every 7 days thereafter. The cells were maintained in medium (RPMI-1640, 10% FCS, 1% PS) supplemented with 100 U/mL II2.

Flow cytometry

Flow cytometry analysis was carried out on BD LSRII or BD FACSAria flow cytometers, and results were analyzed using BD FACSDiva Software (Version 6.1.3, build 2009 05 13 13 29). The following antibodies were used in this investigation: Mouse anti-human CD11c-PE (Biolegend 301606; clone 3.9), mouse anti-human CD14-PE (BioLegend 301806; clone M5E2), mouse anti-human CD14-APC (BioLegend 301806; clone M5E2), mouse anti-human CD3-PE/Cy7 (Biolegend 300316; clone HIT3a), mouse anti-human CD45RA-PECy7 (BioLegend 304126; clone H1100), mouse anti-human CD27-Violet 421 (BioLegend 302824; clone O323), mouse anti-human TCR Vβ1-FTC (Thermo Scientific TCR2750; clone TS8.2), mouse anti-human TCR Vβ2-PE (BioLegend 313408; clone B6), mouse anti-human CD45RA-APC (BioLegend 304114 clone H1100), mouse anti-human CD62L-APC (BioLegend 304814; clone DREG-56), mouse anti-Granzyme B-FTC (BioLegend 515403; clone GB11), mouse anti-human IFNy-APCCy7 (BioLegend 502530; clone 45.B3) mouse anti-human NKp30-APC (BioLegend 325210; clone P30-15), mouse anti-human FAS-L-PE (BioLegend 306407; clone NOK-1), and mouse anti-human NKG2D-APC (BioLegend 320808; clone 1D11). Compensation was carried out using single-color controls and ebioScience OneComp eBeads (ebioScience 01–1111). Where intracellular cytokine staining was performed, cells were prepared using BioLegend fixation and permeabilization buffers in accordance with the manufacturer’s protocol.

Fluorescence microscopy

Cells that were examined by fluorescence microscopy had already been stained with anti-CD11c-PE, anti-CD14-PE, and anti–Hapten-FTC (anti-γδTCR-Hapten) and fixed using Fixation Buffer. Cell suspension (20 μL) at a concentration of 2 × 10^6 cells per ml was applied to each slide and the cover slip applied directly before sealing with glue. The slides were stored in the dark at 4°C overnight to allow the glue to dry before viewing.

Slides were viewed on a Zeiss Axioplan 2 microscope using a Plan-Apochromat 63×/1.40 oil objective. Images were captured at room temperature (20°C) using a Quantix digital camera (Photometrics) and SmartCapture VP software. For immunofluorescence experiments, images were saved as TIFF files and viewed using Adobe Photoshop CS5. Brightfield images were collected using a Zeiss axiocam 200 M microscope with a Plan-Apochromat 63×/1.40 oil objective. The microscope stage was maintained at 37°C with 5% CO₂. Images were captured using a Zeiss axiocam and Axiovision 4.0 software.

Cytotoxicity assays

Expanded γδT cells were used as effector cells. Target cells were coated either with opsonizing antibody or a nontargeting isotype control and labeled with 100 μCi Na251CrO4 in cell culture and tested in a standard chromium release assay as previously described. A range of Effector: Target (E:T) ratios was used in each case (10:1, 5:1, 2.5:1, 1:2.5:1), and cells were incubated for 4 hours for each assay. Human IgG1 anti-GD2 antibody ch14.18 (clinical grade) was used as a GD2 opsonizing antibody, with human IgG1 rituximab anti-CD20 antibody (clinical grade) as a control.

Cytokine production assays

The BD cytokine bead array was used in accordance with the manufacturer’s protocol to analyze production of IFNγ, Granzyme B, and TNFα by γδT cells cocultured with neuroblastoma cells. Briefly, 0.25 × 10^6 γδT cells were cocultured with 0.25 × 10^6 target cells opsonized with ch14.18 anti-GD2 or control antibody (rituximab). The cells were cocultured for 24 hours and the supernatant analyzed for cytokine content.

High-throughput sequencing of γδT cells using targeted capture

RNA was isolated from PBMC, Vd1+, Vd2+, and double-negative cell populations using TRIzol (Invitrogen). RNA (1 μg) from each sample was used to prepare and multiplex libraries for 500 cycle paired-end sequencing on the MiSeq using the Ultra Library Prep Kit for Illumina and Multiplex Oligos for Illumina (NENext E7530 and E7335, respectively). A custom bait library of Agilent’s SureSelect targeted capture system, based on complementary V and J gene segment sequences, as downloaded from the IMGT database, was used to enrich for γδTCR chain sequences (18). Postcapture libraries were amplified from the Illumina P5 and P7 adapter sequences and sequenced on a MiSeq genetic analyzer (Illumina). Quality and quantity of libraries were assessed throughout using Agilent’s Bioanalyzer. FASTQ files were downloaded from BaseSpace following MiSeq runs. Fastx Toolkit was used to discard reads with a quality score of less than Q30, and paired reads were aligned using Fast Length Adjustment of Short Reads (19). Identification and analysis of γδTCR chain content was carried out using the Deconvolutor (20).

Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 6.0c. Error bars, where displayed, indicate the SE of the mean of data from replicate experiments. Significance of difference between samples within figures was confirmed using paired or unpaired t tests, depending on the experimental setting, with *P ≤ 0.05* indicating significance.
Results

Isolation of pure populations of γδT cells from thawed PBMC aliquots

Although isolation of γδT cells to a high degree of purity from fresh blood can be achieved with either one or two rounds of antibody-conjugated bead-positive selection, this is not possible from frozen aliquots of PBMCs due to nonspecific uptake of magnetic FITC-labeled beads. The major contaminating cells express CD14 and/or CD11c and fall within the monocyte forward and side scatter gate on flow cytometric profiles (Supplementary Fig. S1A). Flow cytometric microscopy of isolates from thawed samples demonstrates FITC-conjugated anti-γδTCR beads containing with CD14/11c (Supplementary Fig. S1B). These contaminants were eliminated by adding a CD14/11c depletion step before 2 rounds of positive selection for the γδTCR (Supplementary Fig. S1C and S1D). Subsequent experiments therefore used this initial depletion step before γδT-cell expansions from fresh PBMC, allowing for repeated experiments on aliquots from the same blood sample.

Expansion of polyclonal γδT cells from PBMC and from purified populations

The Vγ9Vδ2 subset of human γδT cells in peripheral blood can be preferentially expanded using phosphoantigens or aminobisphosphonates. There is a relative paucity of knowledge about other γδT-cell subsets, which use Vγ chains 1–8 and Vδ chains 1–9. Expansion of Vδ2negγδT cells using immobilized anti-γδTCR antibody has been previously demonstrated (21), but the authors used only one antibody clone. We compared the ability of 5 commercially available mAbs to expand Vδ1 and Vδ2 γδT cells from healthy donor PBMC with a view to identify antibodies that led to a balanced expansion of both subsets. The differences were not statistically significant in terms of mean fold change for each antibody. Anti-γδTCR Clone B1 (BioLegend) provided the greatest fold change overall, taking into account Vδ1 and Vδ2 expansion (Fig. 1A).

To effect more potent expansion of γδT cells in culture, we made use of aAPCs that had been generated through engineering of the K562 erythroblastic leukemia cell line to express costimulatory molecules (CD86, 41BB-L), membrane-bound IL15, and the high-affinity Fc receptor FcγRI (CD64; ref. 22). Coating aAPC in murine anti-human stimulatory antibodies such as OKT3 anti-CD3 via CD3ε as an effective means of expanding T cells (22). We demonstrated that B1-anti-γδTCR murine IgG1 mAbs bound the aAPC (Supplementary Fig. S2). We compared the ability to expand γδT cells from adult blood of (i) irradiated aAPC coated with B1 mAb in the presence of added IL2 and IL21, (ii) uncoated aAPC, or (iii) IPP. After 7 days stimulation, the combination of aAPC+B1 anti-γδTCR was significantly superior to IPP in terms of γδT cell fold change (Fig. 1B). The addition of B1 anti-γδTCR to the aAPC did not at initial analysis lead to a significant improvement in γδT-cell expansion (comparison of fold change by t test yielded \( P = 0.2 \)), but if nonresponders were eliminated (defined as fold change <3 within 7 days), the combination of aAPC+B1 was significantly better than aAPC alone (\( P = 0.03 \)). Our aim was to produce a balanced expansion of γδT-cell subsets without the pressure toward Vδ2+ expansion associated with using phosphoantigens or aminobisphosphonates. Although non–antibody-treated aAPC led to expansion of all γδT-cell subsets, greater percentages of the rarer Vδ1+ and Vδ1negVδ2neg γδT subsets were obtained by combining aAPC with B1 anti-γδTCR (Fig. 1C shows 2 representative donors), and there was a marked difference from the pattern seen following IPP expansion (Fig. 1D). Although the expansion potential of γδT cells overall varied between donors (Fig. 1E), the combination of aAPC+B1 anti-γδTCR generally maintained the relative proportions of each γδT-cell subset during expansions from each donor over 3 weeks, allowing study of the entire γδT-cell repertoire (Fig. 1F). As shown in Fig. 1F, there was a high degree of variation in γδT-cell repertoire between different donors; Supplementary Fig. S3 shows representative data from three individuals before any expansion stimulus was applied.

γδT-cell expansion from the blood of patients with cancer has been reported to be problematic. In one study, γδT cells from 88% (14/16) healthy donors were expanded in vitro in response to IL2 + pamidronate, whereas γδT cells from only 49% (20/41) patients with cancer were successfully expanded following the same stimuli (23). We investigated the expansion potential of γδT cells from 10-mL blood samples from newly diagnosed children with neuroblastoma. Over a 28-day expansion period using aAPC+B1, we achieved over 650-fold expansion of γδT-cell numbers (mean fold change 665; 95% confidence interval, 410–920; \( n = 4 \); Fig. 1G).

To obtain quantitative data on the repertoire of TCR gene usage in the expanded γδT-cell subsets, we flow-sorted the Vδ1+, Vδ2+, and Vδ1negVδ2neg populations from normal donors and performed next-generation sequencing of TCR sequences. We compared these with γδT cells expanded using IPP, and also with the γδT-cell repertoires found in unstimulated PBMCs from the same donors. The level of diversity in Vγ and Vδ chain usage of healthy donors was reduced following 7 days of stimulation with IPP, lymphoblastoid cell line (LCL), and IL2 (Fig. 2A). Using this technique, it is possible to determine the abundance of clones bearing distinct TCRγ or TCRδ chain rearrangements. We have shown the commonest hypervariable sequences of PBMC and expanded TCRδ chains in Supplementary Table S2. When γδT cells were expanded using aAPC+B1, and sorted into Vδ1+ and Vδ2+ populations, we discovered high levels of γ chain diversity within the Vδ1+ population, encompassing Vγ2+, Vγ3+, and Vγ9+ chain usage. There is even greater diversity within the Vδ1+ populations when the joining regions of the γ chain are considered. Interestingly, the diversity of the Vδ2+ subset expanded from the same donor in the same way is much less than that of the Vδ1+ subset—all almost of the Vδ2+ cells were Vγ9Vγ2, using γJP and δ1 (Fig. 2B). Although there seems to have been some loss of diversity in the expansion of γδT cells from PBMC donor 2, this may be explained as the missing Vγ and Vδ populations fell in the Vδ1negVδ2neg population, which is not shown. By characterizing the γδT-cell repertoire...
within the Vδ1negVδ2neg subset, we found that it contains γδT cells bearing the full range of Vγ chains (Vγ2–5 and Vγ8–9) and a range of Vδ chains, including Vδ3, Vδ5, and Vδ8. There was greater joining segment diversity in the Vδ chains than in the Vγ chains in this subset (Fig. 2C). Although it is impossible to exclude the presence of some bias in the expansion technique using aAPC+B1, it is clearly less biased than expansion with IPP + LCL.

**γδT-cell subsets have different differentiation phenotypes**

Although αβ memory phenotype has been studied in great detail, corresponding data on γδT cells are more limited and their memory phenotype is less well defined. Three memory phenotypes of γδT cells have been previously described, based on CD27 and CD45RA staining (CD45RA+/CD27+ naïve, CD45RA−/CD27+ central memory, CD45RA−/CD27− effector memory CD45RA+; ref. 24). L-selectin (CD62L) can also be used as a memory marker. Similar to αβ T cells, as Vδ2+ γδT cells become more differentiated from central memory (TCM) to effector memory (TEM), they downregulate expression of L-selectin (CD62L) and CD27. Vδ1 memory phenotypes have been reported to show a similar pattern following antigen exposure, as demonstrated by the comparison of CMV+ and CMV− individuals (25).

Figure 3A shows the distribution of differentiation phenotypes from a representative patient with neuroblastoma, using CD62L and CD45RA as markers. Comparison of the CD62L/CD45RA phenotype between Vδ1+ and Vδ2+ T cells staining of PBMC taken from healthy donors and patients with neuroblastoma (at point of diagnosis) yielded some consistent patterns, but the variation between patients with neuroblastoma was higher than that seen in healthy donors.
Fig. 3B and C; Vd1+ gd T cells are less differentiated than Vd2+ gd T cells, as reflected by lower numbers of CD45RA−/CD62L− (TEM) cells and higher numbers of CD62L+/CD45RA+ (TCM) and CD62L+/CD45RA+ (TN). This was confirmed using CD27/CD45RA staining of PBMC—an example of which is shown in Fig. 3D. Using this staining panel, the percentage of naïve (CD27+/CD45RA+) cells is significantly higher in the Vd1+ population than in Vd2+ cells, whereas the Vd2+ subset contains significantly more central memory (CD27−/CD45RA−) cells (Fig. 3E and F).

Interestingly, γδT cells isolated from fresh blood of healthy donors (mean age, 24; Fig. 3E) seemed to be less differentiated than those obtained from cryopreserved leucocyte cones provided by the National Blood Service (Fig. 3B, adult donor ages unspecified). This may be related to donor age, but could also indicate an effect of cryopreservation on memory marker expression. The general trend of Vd1+ gd T cells being significantly less differentiated remains in either sample type.

There are insufficient numbers of Vd1negVd2neg γδT cells to phenotype in peripheral blood, but we were able to determine their memory phenotype following expansion with aAPC+B1 stimulation. Interestingly, following stimulation, Vd1+ and Vd1negVd2neg γδT cells retain CD27, CD62L, and CD45RA to significantly higher levels than Vd2+ cells (Fig. 4A). The pattern of Vd1+ γδT cells being less differentiated and Vd2+ cells being more differentiated is preserved during and despite expansion. We hypothesized that this may be explained in terms of different degrees of antigen exposure and TCR stimulation. To explore this possibility, we evaluated expression of the death receptor PD1, which is regarded as a marker of T-cell exhaustion. Levels were comparable between unstimulated γδT cells and γδT cells in PBMC. Following 14 days of weekly

Figure 2. Joining region diversity and Vy/V6 chain usage in fresh PBMC and expanded γδT cells from the same donors. Heat maps demonstrating variable and joining gene segment usage, as revealed by next-generation RNA sequencing, in γδTCR chain in PBMC populations of healthy donors, before and after expansion using IPP or aAPC+B1. Relative frequency of V and J pairings is shown in blue (low abundance) through to red (high abundance). PBMC donor 1 (A) demonstrates a dominance of Vy9V62, which is reinforced following a 7-day expansion with IPP and IL2. PBMC donor 2 (B) demonstrates more diversity before expansion using aAPC and B1, and there is greater γ chain diversity in the Vδ1+ subset than in the Vδ2- subset. In the Vδ1−/Vδ2− population sorted from donor 3 (C), there is marked diversity in both δ and γ chain joining segment and V segment usage in both PBMC and expanded cells.
stimulation with aAPC+B1, expression of PD1 in Vd1+ and Vd1−Vd2− γδT cells was significantly lower than that in Vd2+ γδT cells or αβT cells from the same donors stimulated with weekly CD3/CD28 beads at the same time points. CD3/CD28 beads were used as a control because this repeated stimulus is a recognized means of inducing exhaustion in αβT cells (Fig. 4B).

**Differential cytotoxic function of γδT-cell subsets**

Vδ2+ γδT cells expanded using phosphoantigens or aminobisphosphonates will kill a variety of tumor cell lines in vitro, an effect which is augmented by the opsonization of the target cell (1, 2, 6, 7, 26, 27). Antibody-dependent and -independent cytotoxicity has been observed in several hematologic and solid tumor models, including CD20+ hematologic malignancies, CD52+ lymphoma, and HER2+ breast cancer lines. Nearly all neuroblastoma tumors express the ganglioside GD2, which has been successfully targeted with mAbs ch14.18 and 3F8 in numerous clinical trials (16, 28, 29). In the absence of ch14.18, the innate antibody-independent killing of GD2+ neuroblastoma cell lines (LAN1 or Kelly) by Vδ2+ cells expanded using IPP was minimal and significantly less than Vδ1+ γδT cells expanded using aAPC+B1 (Fig. 5A). Nontransformed allogeneic PBMCs were not killed (data not shown), indicating that this effect is not simply linked to an MHC mismatch. For clarity, in this and subsequent cytotoxicity assays, an E:T ratio of 10:1 is shown unless otherwise stated.

We then loaded the neuroblastoma cells with ch14.18 anti-GD2 antibody to determine if this would boost γδT-cell cytotoxicity. In these and subsequent experiments, ADCC was determined by subtracting the antibody-independent cytotoxicity (AIC) from total killing observed in the presence of antibody. The cytotoxicity of IPP-expanded Vδ2+ γδT cells was significantly increased by target opsonization. Moreover, increased killing following addition of ch14.18 antibody was observed only against three GD2-positive and not three GD2-negative neuroblastoma cell lines. Therefore, Vδ2+ cytotoxicity following IPP expansion is predominantly antibody dependent (Fig. 5B and C). However, the cytotoxicity of Vδ1+ γδT cells expanded with aAPC+B1 was not significantly increased by target opsonization (P = 0.07 at 10:1 E:T ratio), though this could be explained by the antibody-independent killing of Vδ2+ cells expanded using

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**Figure 3.** Memory phenotypes of unstimulated γδT cells from healthy donors or patients with neuroblastoma at the point of diagnosis. The representative FACS plots (A and D) show the total T-cell population from a patient with neuroblastoma. Memory phenotype of V61 and V62 γδT cells from healthy donors (B) and patients with neuroblastoma (C; n = 6) using CD45RA and CD62L staining. Memory phenotype of V61 and V62 γδT cells using CD27 and CD45RA from a different set of healthy donors (E) and some of the same patients with neuroblastoma (F; n = 4) is also shown. Error bars, SEM.
this condition being slightly higher than those expanded with IPP (Fig. 5D). In contrast, V61+ γδT cells expanded with aAPC+B1 had significantly less ADCC but significantly more AIC against the cell lines tested, and V61negV62neg γδT cells showed intermediate levels of both ADCC and AIC (Fig. 5E). A full range of E:T ratios from 10:1 to 1.25:1 against the neuroblastoma cell line Kelly is shown in Supplementary Fig. S4, and GD2 expression of neuroblastoma cell lines shown in Supplementary Fig. S5. In line with the dependence on opsonized target cells for cytotoxic function, production of Th1 cytokines TNFα and IFNγ by IPP-expanded V62 γδT cells was only seen when the γδT cells were cocultured with ch14.18-opsonized GD2+ neuroblastoma (Fig. 5F). NKG2D expression in expanded V61+ γδT cells was significantly higher than in V61+ or V61−/V62 γδT cells despite identical durations of expansion (21 days; Supplementary Fig. S6A). Although both V61+ and V62+ γδT cells seemed to increase their production of Granzyme B in the presence of opsonized targets, the increase was more significant in the case of V62+ cells (P = 0.005 and 0.01 vs. P = 0.02 and 0.04; Fig. 5G). In fact, expansion with aAPC+B1 seems to induce IFNγ and Granzyme B production in V61+ and V61negV62neg γδT cells, which is not seen when V62+ γδT cells are expanded with IPP + LCL, though all subtypes were capable of upregulating IFNγ and Granzyme B in response to stimulation with phorbol 12-myristate-13-acetate (PMA)/ionomycin (Supplementary Fig. S6B, representative of 3 donors). The difference is likely due to the increased level of stimulation provided by 41BB-L and CD86 on the aAPC. Staining for Nkp30 and FAS-L was negative in γδT cells cocultured with aAPC or tumor cells, and also in those stimulated with PMA/ionomycin (data not shown). Production of IL1, IL4, IL6, IL10, IL17a, and IL17a by V61+ or V62+ γδT cells in the presence of opsonized or nonopsonized Kelly or LAN1 was insignificant (mean detected levels <1 pg/μL, n = 3; data not shown).
Figure 5. Differential cytotoxic profile of Vd1, Vd2, and Vd1+/Vd2− γδT cells against neuroblastoma cell lines. A, antibody-independent killing of GD2− neuroblastoma cell lines by polyclonal populations of Vd1+ and Vd2+ γδT cells; n = 11 for Kelly data; n = 3 for LAN1 data. B and C, cytotoxicity of IPP-expanded Vd2+ cells is significantly enhanced by target opsonization with ch14.18; raw data shown in B (n = 3), and antibody-dependent and -independent components of killing shown in C (n = 16 for Kelly and n = 3 for LAN1 and SKNAS). (Continued on the following page.)
Temporal expression of Fcγ receptors with expansion in γδT subsets

IgG antibodies can recruit effector cells for cytotoxicity or phagocytosis of opsonized targets through engagement and cross linking of the low-affinity Fc receptors FcγRI (CD32) or FcγRII (CD16) or the high-affinity Fc receptor FcγRI (CD64). To investigate the mechanism of γδT-cell ADCC, we compared the Fcγ receptor expression of Vδ1+ and Vδ2+ γδT cells as they were expanded. Vδ1+ γδT cells demonstrated significantly greater surface expression of CD16 and CD32 than unexpanded Vδ2+ cells (Figures 6A and B), whereas CD64 expression was very low (Fig. 6C). Over a 3-week expansion, CD32 expression fell in both subsets (Fig. 6B). In contrast, in Vδ1+ cells, CD16 expression decreased significantly by day 21, whereas Vδ2+ CD16 expression increased to levels significantly greater than baseline, and also to significantly greater than that of Vδ1+ cells from the same donors at the same time points (Fig. 6A). Although CD16 expression on Vδ1+ cells falls, it persists at a low level even after 21 days of expansion and is also seen at low levels in the Vδ1+Vδ2min subset (Fig. 6D). CD16 expression in expanded γδT cells showed a highly significant positive correlation with their ability to exert ADCC against opsonized GD2+ neuroblastoma (R² = 0.67; P = 0.001; Fig. 6E). It has previously been reported that the more differentiated subsets of Vδ2+ γδT cells have greater expression of CD16 (30), a finding that is in keeping with our observations. CD62L loss is a recognized marker of T-cell differentiation, and there is an inverse relationship between the expression of CD62L and that of CD16 in expanding Vδ2+ cells (Fig. 6F).

Discussion

γδT cells have been recognized as multifaceted effector cells for immunotherapy of cancer. Their tumoricidal properties include targeting cells in an MHC-independent manner by differentiating between healthy and transformed cells (31) and expanding from peripheral blood in response to engagement of their TCR. Previous studies of cancer immunotherapy using γδT cells have either enriched Vγ9Vδ2+ numbers in patients through administration of aminobisphosphonates (32) or have expanded this population of cells ex vivo before adoptive transfer (33, 34). A limitation in the field has been the lack of protocols for expansion of subsets other than Vγ9Vδ2+, and lack of understanding of the tumoricidal properties of these cells. Hence, our demonstration of expansion and killing properties of the non-Vδ2 subset raises new prospects for translation into clinical studies.

We chose to use neuroblastoma as a model system for evaluation of γδT-cell immunotherapy. Neuroblastoma immune evasion mechanisms include production of soluble NKG2D ligands sMICA and sMICB (35) and low expression of MHC (36), which protect the tumor against MHC-dependent killing by CD8+ T cells and NKGD2-bearing NK cells. Moreover, neuroblastoma is amenable to immunotherapy as demonstrated by significant clinical benefit to patients treated with ch14.18 anti-GD2 mAb (16, 28, 29), and sensitivity of neuroblastoma cells to killing by Vγ9Vδ2+ γδT cells (31).

We have shown that γδT subsets undergo unbiased expansion to clinically useful numbers from blood donors or patients with neuroblastoma using anti-γδTCR antibody-coated aAPCs. This degree of expansion is compatible with that using bisphosphonates or their metabolic products (37, 38). Previous studies have shown activities of γδT cells against hematologic malignancies (2, 7), renal cell carcinoma (32, 39), non–small cell lung cancer (33), osteosarcoma (40), and prostate cancer (41), and tumoricidal properties of non-Vδ2 γδT cells (21, 37, 42, 43). Using aAPC expansion, we have generated Vδ1, Vδ2, and Vδ1+Vδ2min populations with distinctive properties.

Phosphoantigen-activated or -expanded Vδ2+ γδT cells have previously been shown to exert effective ADCC with lymphoma (2, 7) and breast cancer (6, 26) and variable innate killing activity against neuroblastoma cells (31). In our hands, IPP-expanded Vδ2+ Δ2 cells have little innate cytotoxicity against allogeneic neuroblastoma cell lines, but marked ADCC, associated with release of Th1 cytokines IFNγ and TNFα. In contrast, Vδ2+ cells expanded with aAPC+B1 had greater innate killing, but their capacity for ADCC was reduced. It might be that this differential polarization in the two different culture conditions is independent of TCR signaling and results from alternate signals. An alternate hypothesis is that IPP and the B1 antibody preferentially expand γδT cells with different Vδ2 TCR chains and associated with different killing properties. In favor of this, we note that some of the Vδ2 cells have non-Vγ9 pairings (Fig. 2). Similarly, Vδ1 cells consistently show greater antibody-independent killing, supporting the notion that different TCRs are associated with different killing properties. Further studies are required to determine the mechanisms of innate killing. Importantly, there is a strong correlation between CD16 expression and ADCC; whereas γδT bearing a non-Δ2 chain lose CD16 and ADCC properties as they expand, Δ2 TCR-positive cells retain relatively bright CD16 expression and remain ADCC competent. Whether this polarization of phenotype is due to qualitative or quantitative difference in signaling through the TCR remains an open question.

A possible explanation for lack of antibody-independent killing of neuroblastoma by Vδ2 cells relates to the production of soluble NKG2D ligands sMICA, sMICB, and

(Continued.) D, comparative antibody-dependent and -independent killing of Kelly cells by Vδ2+ γδT cells expanded with IPP (n = 9) or aAPC (n = 7). E, Vδ1-, Vδ2-, and Vδ1Vδ2min show different patterns of antibody-dependent and -independent cytotoxicity (n = 5 and 11 for Kelly and n = 3 or 4 for LAN1). F, IFNγ and TNFα secretion as determined by cytokine bead array following coculture of IPP-expanded Vδ2+ with opsonized and nonopsonized Kelly cells (n = 3). G, Granzyme B in supernatant of expanded Vδ1+ and Vδ2+ γδT cells cocultured with γδT-cell-sensitive GD2+ tumor cell lines Kelly and LAN1, expressed as fold change of negative control (γδT-cell-insensitive cell line with irrelevant antibody).
ULPB1–6 by neuroblastoma cells, which can block the NKG2D receptor (35, 44). There are significantly higher levels of NKG2D on expanded V<sup>d</sup>2<sup>+</sup>gd<sup>–</sup>T cells (Supplementary Fig. S5), which may make them particularly susceptible to suppression by soluble NKG2D ligands. This is the first demonstration of the cytotoxicity of V<sup>d</sup>1<sup>−</sup>V<sup>d</sup>2<sup>−</sup>gd<sup>+</sup>T cells against neuroblastoma, and it offers an alternative to NK cells for generation of large bulk populations of innate killer cells for adoptive transfer. The V<sup>d</sup>1<sup>−</sup>V<sup>d</sup>2<sup>−</sup>gd<sup>+</sup>T cells are a heterogeneous population and maybe not surprisingly their cytotoxicity shows both antibody-dependent and -independent elements, with a (non-significant) tendency toward innate killing. Future studies will require single-cell sequencing or expansion of clones of V<sup>d</sup>1<sup>−</sup>V<sup>d</sup>2<sup>−</sup>, as well as the more conventional V<sup>d</sup>1<sup>+</sup> and V<sup>d</sup>2<sup>+</sup>gd<sup>+</sup>T cells, to define the different V<sub>g</sub>/V<sub>d</sub> pairings, and to study the functional significance of each pairing. We propose that expanded V<sub>g</sub>9V<sub>d</sub>2<sup>+</sup> cells will have the most potential clinical benefit in combination with a tumor antigen-specific therapeutic antibody such as rituximab, herceptin, or anti-GD2. However, in bulk expansions, the more modest innate cytotoxicity of aAPC<sup>+</sup>B1-expanded V<sup>d</sup>2<sup>+</sup> could be combined with the much more potent cytotoxicity of V<sup>d</sup>1<sup>+</sup> and V<sup>d</sup>1<sup>−</sup>V<sup>d</sup>2<sup>−</sup>gd<sup>+</sup>T cells.

It is striking that following stimulation with the pan-g<sup>T</sup>TCR antibody and aAPC, V<sup>d</sup>2<sup>+</sup>gd<sup>+</sup>T cells develop a more differentiated phenotype than V<sup>d</sup>1<sup>+</sup> or V<sup>d</sup>1<sup>−</sup>V<sup>d</sup>2<sup>−</sup>gd<sup>+</sup>T cells. There are several possible explanations. First, the differences may reflect greater differentiation in the starting populations. This could be explained in terms of a relatively greater frequency of exposure to the ligands of the V<sub>g</sub>9V<sub>d</sub>2 TCR compared with the (largely unknown) ligands of V<sup>d</sup>1<sup>+</sup> or V<sup>d</sup>1<sup>−</sup>V<sup>d</sup>2<sup>−</sup>gd<sup>+</sup> TCRs. Second, there might be a quantitatively greater stimulus to the cells associated with engagement of the V<sub>g</sub>9V<sub>d</sub>2 TCR compared with alternate stimuli. Finally, engagement of the V<sub>g</sub>9V<sub>d</sub>2 TCR might result in qualitatively different signaling and a specific drive toward a more differentiated phenotype. Distinguishing these different explanations will require detailed studies of TCR ligand effects and intracellular signaling. The preservation of relatively undifferentiated non-V<sup>d</sup>2 subsets of gd<sup>+</sup>T cells, with a less exhausted phenotype, is potentially exciting, as these cells have characteristics associated with efficacious cellular therapy products.

The differentiation state of T cells has important implications for their efficacy in cellular therapy. It is recognized that adoptive cell therapy using less differentiated CD8<sup>+</sup> cells is more efficacious (45), a finding that may also be true in the case of gd<sup>+</sup>T cells. Phenotyping using CD27, CD45RA, and CD62L is commonly used to determine the memory phenotype of gd<sup>+</sup>T cells and has also been used for the same purpose in both V<sup>d</sup>2<sup>+</sup> and V<sup>d</sup>1<sup>+</sup>T cells (24, 25, 38). Using these, we have shown that V<sup>d</sup>1<sup>−</sup>gd<sup>+</sup>T cells from patients with neuroblastoma at the point of diagnosis are significantly
less differentiated than Vδ2+ γδT cells from the same blood samples, a finding which is also seen in healthy adult donors. Following 28 days of expansion, Vδ1+ and Vδ1negVδ2neg γδT cells express a less differentiated state. Vδ2+ γδT cells are uniformly CD27low/CD62Llow/CD45RAlow, falling into the effector memory subset. Interestingly, in contrast to the findings of Angelini and colleagues (30), expanded Vδ2+, which are predominantly CD27+/CD45RA+, also express CD16. Hence, in expanded bulk populations using antibody B1-coated aAPC, there are cells of central memory phenotype expected to provide longevity following adoptive transfer, and cells primed for ADCC, which will provide additional antitumor efficacy.

T-cell exhaustion refers to a state induced by chronic antigen exposure and characterized by a decline in T-cell function—specifically a loss of ability to lyse target cells and produce cytokines such as IFNγ and TNFα (46, 47). CTLA4 and PD-1 are inhibitory receptors associated with hyperstimulated T cells, which provide important immunologic checkpoints to immune activation (48). We chose to measure PD-1 as a representative marker of hyperstimulation/exhaustion. PD-1 expression by Vδ1+ and Vδ1negVδ2neg γδT cells was significantly lower than that seen in Vδ2+ γδT cells in the same samples. This finding suggests Vδ1+ and Vδ1negVδ2neg γδT cells might be more favorable in adoptive transfer, a hypothesis that requires testing in clinical studies. Importantly, interaction between PD-1 and PDL-1 is a recognized immune escape mechanism of many tumors (49).

In summary, we have identified a novel approach for unbiased expansion of γδT cells from peripheral blood of patients with cancer. aAPCs are readily available for translation into GMP manufacturing. Unlike previous expansion studies favoring Vδ2 cell expansion, our method will also expand Vδ1+ and Vδ1negVδ2neg γδT cells, which have a more favorable innate killing and memory phenotype. Adoptive transfer of a bulk population of expanded cells bearing a broad repertoire of γδTCR may allow both innate killing and ADCC function to be exploited. Combined with the antigen-presenting function of γδT cells, this may prove to be an optimal adoptive cell therapy approach for cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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