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Individual V γ 2-J γ 1.2+ T cells respond to both isopentenylpyrophosphate and Daudi cell stimulation: Generating tumor effectors with low molecular weight phosphoantigens

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Abstract

Human V γ 2V δ 2 T cells exhibit T cell receptor-dependent, MHC-unrestricted recognition of antigen and play important roles in tumor and pathogen immunity. To characterize antigen recognition by the V γ 2V δ 2 TCR, we used the combined approach of spectratyping and CDR3 sequence analysis that measures changes in the TCR repertoire before and after stimulation with a phosphoantigen (isopentenyl pyrophosphate) or an irradiated tumor cell line (Daudi B lymphoma). Here we describe common V γ 2 chains that are substantially involved in the response to both phosphoantigens and tumor cells. The recognition properties of common V γ 2 chains explains the observation that V γ 2V δ 2 T cells expanded by phosphoantigen stimulation specifically recognize and kill some but not all tumor cell lines. Our studies further justify efforts to stimulate tumor immunity by administering low molecular weight phosphoantigens and boosting the frequency and tumor effector functions of circulating V γ 2V δ 2 T cells.

Keywords

Human; Tumor Immunity; T cells, Cytotoxic; Repertoire Development; T Cell Receptors; Gammadelta

Introduction

A subset of CD3⁺ T cells marked by expression of the $\gamma\delta$ T cell receptor (TCR) is important for tumor surveillance [20] and for control of tolerance, inflammation and pathogen immunity [1]. Human $\gamma\delta$ T cells comprise 1-10% of circulating lymphocytes [5,7,13,34] and the major subset expresses the V γ 2V δ 2 TCR but not the lineage determinants CD4 or CD8 [2]. The V γ 2V δ 2 T cell population is largely effector memory [23] and is potently cytotoxic for the Daudi B cell lymphoma [30] and other tumor targets [10,21,26,39]. Because V γ 2V δ 2 T cells represent an abundant effector memory population that is cytotoxic for tumor cells, there is a growing interest in understanding these cells and exploiting their capabilities in the development of new immunotherapies against malignancies [27,41].

The lack of information about tumor antigens recognized by the V γ 2V δ 2 TCR impedes our understanding of $\gamma\delta$ T cell regulation. We know that V γ 2V δ 2 T cells respond to stimulation with low molecular weight, non-peptidic antigens including intermediates of sterol biosynthesis (such as isopentenyl pyrophosphate) [9,16,18,35], aminobisphosphonate drugs [11,27] and alkylamines [3]. V γ 2V δ 2 T cells proliferate after stimulation with these agents and respond similarly to some tumor cell lines including the Daudi B cell lymphoma [4,22,25,30]. Tumor recognition is independent of classical MHC molecules, there is no defined requirement for antigen processing [13], and various tumor ligands have been implicated including HSP60 [28] and an F1 ATPase-related structure [37]. Cell lines expanded after stimulation with phosphoantigens or tumors are enriched for the V γ 2-J γ 1.2 rearrangement [18] and are potently cytotoxic for some tumors [10,38]. Stimulation also induces expression of Type 1 cytokines (IFN- γ , TNF- α , RANTES, MIP-1 α , MIP-1 β and Lymphotactin among others) [8,14,29,40] and can lead to surface expression of CD107a, a marker for cytotoxic effector cells [14]. Experimental data argues against T cell receptor binding to phosphoantigens, aminobisphosphonates or alkylamines and we do not understand why some (but not all) tumor cell lines are stimulatory or why V γ 2V δ 2 T cell lines are potently cytotoxic for tumor cells. Part of the problem reflects unusual features of the $\gamma\delta$ TCR repertoire.

Beginning early in life, a strong bias emerges for the V γ 2V δ 2 TCR in blood $\gamma\delta$ T cell populations [7]. Within the V γ 2 repertoire there is selection for chains expressing the J γ 1.2 segment [17], one of 5 functional J γ segments. Repertoire bias is a product of chronic positive selection for V γ 2-J γ 1.2 that is driven by self or ubiquitous antigens. The same bias is common to most human beings irrespective of haplotype [12,17] and is nearly identical in human and nonhuman primates [6,18,36]. In contrast, the highly diverse $\alpha\beta$ TCR repertoire is shaped by MHC-dependent positive and negative selection mechanisms in the thymus and does not show an obvious bias in the absence of infection or immunization [33].

Studies on V γ 2V δ 2 T cells are hampered by having few of the tools that are used commonly to study $\alpha\beta$ TCR recognition. A lack of known presenting molecules coupled with the inability to define tumor antigens has prevented the use of tetramers, epitope peptides, or similar approaches to study the $\gamma\delta$ TCR. Accordingly, we looked directly at the V γ 2 repertoire and its changes after treatment *in vitro* with model tumor cell (Daudi B cell lymphoma) or phosphoantigen (isopentenyl pyrophosphate) stimulators to understand the mechanism for antigen recognition. We define similarities and differences in the response to tumor cells or phosphoantigens by looking for V γ 2 chain sequences that are expanded preferentially after exposure to either stimulus. What emerges is a map of the complex responses for an entire T cell population exposed to two different antigens. The balance of common and specific recognition describes the capacity of this unique T cell subset to be triggered by phosphoantigens and respond with tumor cytotoxicity

Materials and Methods

Collection and preparation of lymphocytes

Heparinized blood was collected from six healthy volunteers with approval from the Institutional Review Board at the University of Maryland Baltimore and informed consent of the donors. Peripheral blood mononuclear cells were isolated by centrifugation over Ficoll-Paque density gradients as described by the manufacturer (Pharmacia, Uppsala, Sweden). Approximately 5×10^5 cells were cultured in supplemented RPMI-1640 medium (R10) containing 10% fetal bovine serum (FBS), 2mM L-glutamine (Gibco, Grand Island, NY), 1 U/mL penicillin/streptomycin (GIBCO, Grand Island, NY) and 100 U/mL recombinant human interleukin-2 (rhIL-2) (Tecin, Biological Resources Branch, NIH, Bethesda, Maryland). Isopentenyl pyrophosphate (IPP) (Sigma, St. Louis, MO) was added to a final concentration of 15 μ M or irradiated (120 Gy) Daudi B cells were added at a ratio of 2:1 Daudi: PBMC.

Cultures were incubated for two weeks at 37°C with 5% CO₂ and were replenished every 3-4 days by adding IL2-supplemented medium without IPP or Daudi.

Tumor Cell Lines

Daudi B cells (CCL-213, ATCC) and MOLT -4 T cells (CRL-1582, ATCC) were cultured in R10 additionally supplemented with 4.5 g/L glucose, 1.5 g/L NaHCO₃, 10mM HEPES and 1mM sodium pyruvate. Several tumor cell lines were selected because of their derivation from HIV-associated malignancies, including: BC-1, BC-2, BL-5, BL-8, IBL-4 (provided kindly by William Harrington, University of Miami) and 2F7. These lines contain no HIV proviral DNA and express no detectable HIV antigens. BC-1 (CRL-2230, ATCC) and BC-2 (CRL-2231, ATCC) were cultured in RPMI-1640 supplemented with 2mM L-glutamine, 1 U/mL penicillin/streptomycin and 20% non-heat inactivated FBS. B cell tumor lines BL-5, BL-8 and IBL-4 were kindly provided by Dr. William Harrington (University of Miami, Miami, FL) and cultured in IMDM supplemented with 2mM L-glutamine, 1 U/mL penicillin/streptomycin and 10% non-heat inactivated FBS. T cell line 2F7 (CRL-10237, ATCC) is cultured in R10 supplemented with 0.05mM β -mercaptoethanol (GIBCO, Grand Island, NY).

Calcein-release Cytotoxicity Assay

To quantify the cytotoxic capacity of V γ 2V δ 2 T cells we used a non-radioactive, fluorometric cytotoxicity assay involving the dye calcein-acetoxymethyl (calcein-AM)[32]. Tumor targets were labeled for 15 minutes with 2 μ M calcein-AM (Molecular Probes, Eugene, Oregon) at 37°C in 5% CO₂ and then washed once with phosphate-buffered saline (PBS). Cells were combined at various effector:target (E:T) ratios in 96 well V-bottom microtiter plates (Corning Inc., Corning, New York) and incubated at 37°C in 5% CO₂ for four hours; assays were performed in triplicate. Following incubation, supernatants were transferred to a 96-well flat-bottomed microtiter plates and calcein content is measured using a Wallac Victor² 1420 multi-channel counter (λ 485/535nm). Percent specific lysis is calculated as: [(test release – spontaneous release)/(maximum release – spontaneous release)] $\times$ 100.

Flow Cytometry

Two weeks after Daudi+IL2, IPP+IL2 or control (IL2 alone) stimulation, cultures were harvested and viable counts were performed by the trypan blue exclusion assay. 3×10^5 cells were washed once in RPMI-1640 and stained at 4°C with fluorescein isothiocyanate (FITC)-conjugated CD3 (Clone UCHT1; BD Biosciences, San Diego, CA) and phycoerythrin (PE)-conjugated anti-V δ 2 (clone B6; BD Biosciences, San Diego, CA) or the appropriate isotype controls. After 20 minutes, cells were washed once with RPMI-1640 and resuspended in PBS containing 2% paraformaldehyde. At least 10^4 lymphocytes (gated on the basis of forward and side scatter profiles) were acquired for each sample on a FACSCalibur (BD Biosciences, San Diego, CA). Flow cytometry data were analyzed with FloJo software (Tree Star, San Carlos, CA). Stimulation index (SI) represents the fold increase for V δ 2 lymphocytes after IPP or Daudi stimulation compared to IL2 alone. SI is calculated as the ratio of the absolute number of V δ 2 lymphocytes on Day 14 to the absolute number of V δ 2 lymphocytes expanded with IL2 alone.

RNA Extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from at least 10^6 cells using the RNeasy Mini Kit as described by the manufacturer (Qiagen, Valencia, CA). One microgram of total RNA was then converted into cDNA using the Reverse Transcription System (Promega, Madison, WI) in a reaction containing: 500 ng of oligonucleotide A (T₁₅V), 1 mM deoxynucleotriphosphates, 5 mM MgCl₂, 10mM Tris-HCL pH 8.8, 50 mM KCL, 0.1% Triton X-100, 18 units of avian myeloblastosis virus reverse transcriptase and 10 units of RNasin ribonuclease inhibitor. Each

reaction was incubated at 42°C for 2 hours, then cDNA were diluted to 100 µL by adding to the reaction 80 µL deionized water. PCR reactions were performed using 5 µL cDNA as template and 500 nM each of forward and reverse primers, 0.2 mM dNTPs, 2 mM MgCl₂, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.1% Triton X-100 and 1 unit of AmpliTaq Gold (Applied Biosystems, Foster City, CA). The following primers were used: oligo-Vγ2 (5'-ATCAACGCTGGCAGTCC-3'), oligo-Cγ1 (5'-GTTGCTCTTCTTTTCTTGCC-3'), 5' β-actin (5'-GTGGGGCGCCCCAGGCACCA-3') and 3' β-actin (5'-CTCCTTAATGTCACGCACGATTTC-3'). PCR was run with the following profile: denaturation for 1 minute at 94°C; 5 minutes at 68°C; 45 cycles (45 seconds at 94°C, 1 minute at 60°C, 1 minute at 72°C); extension for 10 minutes at 72°C. PCR products were separated on a 2% agarose/Tris-acetate-ethylenediaminetetraacetic acid buffer (TAE) gels containing 0.5 µg/mL ethidium bromide.

Spectratype Analysis

Primer extension reactions were performed as described previously [18]. Each reaction contained 1 µL PCR product, 3 mM MgCl₂, 0.2 mM dNTP, 0.2 units *Taq* DNA polymerase (Promega, Madison, WI), 10 mM Tris-HCl pH 8.8, 50 mM KCl, and 0.1 µM – carboxyfluorescein (6-FAM)-labeled primer (Cγ6-FAM for Vγ2 chains: 5'-AATAGTGGGCTTGGGGGAAAC-3'; Cδ16-FAM for Vγ2 chains: 5'-ACGGATGGTTTGGTATGAGG-3'). Four microliters of run-off products were diluted in deionized formamide and 1 µL of N,N,N',N'-trimethyl-6-rhodamine-labelled molecular size standard was added to each sample. After a denaturation step (5 minutes at 95°C followed by immediate quenching on ice), products were loaded on an Applied Biosystem microcapillary genetic analyzer (Perkin-Elmer, Foster City, CA) and run for either 27 (Vγ2 chains) or 24 (Vγ2 chains) minutes on a performance optimized polymer (POP-4). Molecular size and relative frequency of extension products were determined using Genescan software (Perkin-Elmer, Foster City, CA). To standardize the data irrespective of the run-off primer position, CDR3 length variation was expressed in terms of the total Vγ2 or Vγ2 coding region lengths. Run-off product lengths were corrected by adding the length of the known Vγ2 or Vδ2 mRNA coding regions outside the run-off product. According to this calculation, the major peak for Vγ2 chains is 996 nucleotides and for Vδ2 chains is 1029 nucleotides with corresponding run-off product lengths of 447 and 193 nucleotides, respectively.

Cloning and Sequencing

PCR products were purified by gel extraction using a Qiaquick Gel Extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Purified products were denatured (1 minute at 94°C), incubated for 30 minutes at 72°C with 2 mM MgCl₂, 0.2 mM dATP and 2.5 units AmpliTaq Gold (AB, Foster City, CA), then ligated into the pCR2.1 TOPO TA cloning vector (Invitrogen, Carlsbad, CA). Ligated vector was transfected into TOP10F' competent cells using the TA Cloning kit (Invitrogen) and bacterial colonies were grown overnight on agar plates containing 50 µg/mL ampicillin, 500 µM isopropylthiogalactoside (IPTG) and 80 µg/mL bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Colonies containing recombinant plasmids were cultured overnight and plasmid DNA were purified using the Wizard Plus Minipreps DNA purification kit (Qiagen, Valencia, CA). Sequencing reactions were done with the Bigdye v3.1 fluorescent sequencing kit (AB, Foster City, CA) using both M13F and M13R oligonucleotide primers for each sample. Sequences were analyzed on an automated sequencer ABI3700 and aligned using Sequencher (GeneCodes Corp, Ann Arbor, MI) and MacClade software.

Vγ2, Jγ1.1, Jγ1.2, Jγ1.3/2.3, Jγ2.1, and Cγ1 were identified by comparison to GenBank accession numbers X07205, X08084, M12950, M12960/M12961, M16016, M14996, respectively and TCRγ locus reference sequences NG_001336 and AF159056.

Results

The Adult Human V γ 2V δ 2 Repertoire is Skewed Toward Longer V γ 2 Chain Lengths

Over several years we collected data on the V γ 2 repertoire in healthy, HIV-negative adults. At present, we have data for 44 healthy adult volunteers residing in North America or Africa; the distribution of V γ 2 and V δ 2 chain lengths were determined by spectratype analysis. Similar to previous results, we found that in healthy adult humans the V γ 2 repertoire is skewed toward longer V γ 2 chain lengths (Figure 1), without an overall bias in the distribution of V δ 2 chains (data not shown). Selection for longer V γ 2 chains also coincides with the specific accumulation of V γ 2-J γ 1.2 chains and is estimated by calculating the frequency of V γ 2 chains greater than 987 nucleotides from spectratype data. Taken together, approximately 68% of all V γ 2 chains co-express the J γ 1.2 segment in adults. Thus, selection for the J γ 1.2 segment is a common feature of the adult V γ 2 repertoire and occurs independently of ethnic or geographic background. Part of these data were reported previously [17,18].

Isopentenyl Pyrophosphate and Daudi Tumor Cells Expand V γ 2⁺ lymphocytes

We selected six representative donors and stimulated PBMC *in vitro* with interleukin-2 (IL2) alone, 15 μ M IPP and IL2, or irradiated (120 Gy) Daudi tumor cells plus IL2. Healthy donors varied in age, sex and ethnicity (Table 1). After two weeks in culture (and periodic supplementation with medium containing IL2), there were strong proliferative responses to IPP or Daudi stimulation (Table 1). Day 14 cultures were comprised of greater than 60% lymphocytes as determined by forward and side scatter flow cytometric profiles, with the remainder consisting of dead or dying cells. Staining for CD3⁺CD4⁺ and CD3⁺CD8⁺ lymphocytes showed no significant change in the absolute number of these subsets (data not shown) although we observed elevated NK lymphocytes (CD3⁺CD8⁺) only after Daudi stimulation. Additionally, we used irradiated cells from several tumor cell lines (BC-1, BC-2, 2F7, MOLT-4, BL-5, BL-8 and IBL-4) to stimulate V γ 2V δ 2 lymphocytes from PBMC. Using the conditions described here, only Daudi and IBL4 elicited proliferative responses.

Both IPP or Daudi Expand Potent V γ 2V δ 2 Tumor Effectors

We expanded V γ 2V δ 2 T cells from PBMC using IPP or Daudi stimulation and tested each line for the ability to lyse three tumor cell targets: Daudi B lymphoma cells, MOLT-4 T cells and IBL4 B lymphoma cells. Effector cells stimulated with either IPP or irradiated Daudi were cytotoxic for all three tumor cell lines tested though we noted differences in potency (Figure 2). MOLT-4 was unusual in that it did not stimulate V γ 2V δ 2 proliferation, but was lysed by V γ 2V δ 2 effector cells. The majority of lymphoma cell lines tested by us and others [38] neither stimulate V γ 2V δ 2 proliferation nor are killed by expanded effector cells. It is important to note that some expanded V γ 2V δ 2 lines contained other lymphocytes including NK cells that might contribute to tumor cytolysis. However, we have observed high levels of tumor killing with lines that were highly enriched (>95%) for V γ 2V δ 2 T cells and effectively lysed tumor cells with effector to target ratios below 1. Further, the potency of effectors (in terms of specific lysis at a given E:T ratio) was the same after IPP or Daudi stimulation despite slightly higher NK cell levels in the Daudi-expanded cultures.

IPP or Daudi Selectively Skew V γ 2 Chains Toward Longer Chain Lengths

To understand how IPP or Daudi stimulation remodeled the V γ 2V δ 2 repertoire, we performed spectratype analyses to assess the size distribution for V γ 2 and V δ 2 chains after alkylphosphate or tumor stimulation. In all donors, both IPP and Daudi stimulation and subsequent cell proliferation skewed the V γ 2 repertoire toward longer chain lengths but did not alter the length distribution for V δ 2 chains. This was evident when cumulative frequencies for both V γ 2 and V δ 2 chains from all six donors were averaged and plotted (Figure 3). Both IPP and Daudi

skewed the V γ 2 repertoire toward chain lengths greater than 987 nucleotides as evidenced by rightward shifts in the cumulative frequency plots. Comparisons of V γ 2 chain lengths before and after stimulation for all six donors did not reach statistical significance. However, the starting V γ 2 repertoires of donors A and E were almost exclusively V γ 2 chains longer than 987 nucleotides and it was difficult to detect a shift in these highly biased samples (data not shown). When these donors were excluded from the statistical analysis, we observed a significant shift for the remaining four donors after Daudi stimulation ($p < 0.05$). V γ 2 chain lengths were normally distributed in all samples and cumulative frequency plots for V δ 2 were similar before and after stimulation with IL2, IPP or Daudi (Figure 3), whether or not donors A and E were excluded. In terms of chain length, the effects of antigen stimulation were most apparent in the V γ 2 repertoire and we continued our study with emphasis on that chain.

IPP or Daudi Selectively Expand V γ 2-J γ 1.2⁺ Cells

We sequenced V γ 2 chains before or after IPP or Daudi stimulation for 4 donors, all of which had significant differences between the starting and expanded spectratypes. The occurrence of discrete V γ 2 CDR3 nucleotide sequences (nucleotypes), predicted amino acid sequences (clonotypes), J γ segment usage and predicted chain lengths were determined for each donor before and after stimulation. Confirming and extending our previous results [18], we observed a positive selection for V γ 2-J γ 1.2 chains following stimulation with either IPP or Daudi (Figure 4). This result was most dramatic in donors whose starting repertoire had a lower abundance of V γ 2-J γ 1.2 chains. In all cases, sequence analyses confirmed the results obtained by spectratyping (data not shown).

Unlike $\alpha\beta$ T cell repertoire studies where starting frequencies of particular clonotypes are below the threshold of detection by spectratyping or DNA sequencing, the V γ 2 model provides an unique opportunity to observe repertoire dynamics. We calculated the relative change for each clonotype during a proliferative expansion. The ratio of clonotype frequency before and after stimulation was multiplied by the stimulation index for that culture (Table 1). This produced a relative stimulation index for each clonotype that measured the strength of response for cells expressing individual V γ 2 chains (Figure 5A). Plotting the relative stimulation index for each clonotype after IPP or Daudi treatment (Figure 5A) demonstrated that the population contained clonotypes that varied greatly in their response to each stimulus. As expected, most responding clonotypes and all of those responding to both IPP and Daudi, used the V γ 2-J γ 1.2 rearrangement (data not shown). Additionally, V γ 2 clonotypes that responded to both stimuli (common recognition) were often public chains because they were present in multiple, unrelated donors (Figure 5B).

The V γ 2 chains that responded to both IPP and Daudi comprised a significant fraction of the starting V γ 2 repertoire for each of these four donors. There was a trend among all four donors that IPP or Daudi promoted the expansion of common recognition V γ 2 clonotypes, many of which were also public chains.

Sequences of V γ 2-J γ 1.2 Chains that Respond to IPP, Daudi or Both

We divided all V γ 2-J γ 1.2 clonotypes into 3 groups based on the calculated stimulation indices to IPP or Daudi. Groups comprised clonotypes involved in common (Figure 6A), Daudi-specific (Figure 6B) or IPP-specific (Figure 6C) recognition. Relative stimulation indices varied from 1.6 (Figure 6B, clonotype 3-02) to 1865 (Figure 6A, clonotype 4-01). Although the absolute values varied, the ratios of Daudi SI to IPP SI were generally consistent for individual clonotypes from different donors. For example, the ratio of stimulation indices to IPP versus Daudi were similar in donors A and F for the clonotype 4-01, LWETQELG (Figure 6A). A similar consistency was observed for clonotype 4-03 in donors A and F, even though this sequence behaved differently for donors B and C. Finally, the common clonotype 4-06,

LWEVRELG, reacted more to Daudi than to IPP for donors C and F. Common recognition clonotypes were encoded by 3 nucleotides on average, while the majority of IPP- (6/7) or Daudi-(4/6) specific clonotypes were encoded by a single nucleotide. The overall trend was for increased nucleotide redundancy among the common recognition V γ 2-J γ 1.2 clonotypes.

The three designated subsets of V γ 2 clonotypes were aligned separately to search for consensus sequences in their CDR3 regions. We could not define consensus V γ 2 CDR3 that predicted common or specific recognition (Figure 6).

The V γ 2 Repertoire is Relatively Stable for Years

In order to justify the development of therapies that target V γ 2V δ 2 T cells, it is important to know that the pool of potential tumor effector cells is stable over extended intervals. For these studies, we relied on DNA sequencing data to compare the V γ 2 repertoire among individual donors at more than one time point. Initially, we compared the V γ 2 repertoire in eight donors for samples collected two years apart (Fig. 7A). On average, we had approximately 40 sequences for each time point. We identified V γ 2 nucleotides that were present in both samples and then calculated the proportion of the V γ 2 repertoire occupied by these nucleotides at each time point. Two donors (C and E) had conserved sequences occupying between 25 and 50% of the total repertoire and represented the lowest stability. Other donors including A and D were most stable with more than 75% of the V γ 2 repertoire occupied by conserved sequences.

We performed a similar analysis for three donors where V γ 2 sequence data existed for samples collected 7 years earlier (Figure 7B). Data for the early timepoint were reported previously [18]; data for the recent timepoint were generated during this study. On average, we had approximately 45 sequences for each time point. Remarkably, these three donors also showed a high degree of repertoire stability over his extended interval, with greater than 50% of all detected V γ 2 sequences being conserved. Overall, the V γ 2 repertoire is highly stable in healthy adults. It will be important for future studies to show whether changes in this repertoire might presage an increased risk for malignancy.

Discussion

Circulating V γ 2V δ 2 T cells exhibit robust proliferative responses to IPP or Daudi stimulation and cells expanded in either condition are comparably cytotoxic for the same Daudi cells. Analysis of the V γ 2 chain repertoire showed a substantial, though incomplete overlap between clones expanded by either stimulus and this accounted for the reported ability to expand $\gamma\delta$ T cells from PBMC with phosphoantigens and obtain a line equipped for cytolytic recognition of Daudi [38]. Individual V γ 2 clonotypes that responded in both stimulatory conditions were designated “common recognition” chains; they constituted a substantial portion of the starting repertoire in multiple donors and were expanded preferentially in stimulated cultures.

The ability to distinguish common (responding to both stimuli) and specific (responding to one stimulus) recognition clonotypes derives from unique features of the V γ 2 repertoire. In adults, the major population of V γ 2 chains in circulating T cells is the product of chronic selection for cells expressing the V γ 2-J γ 1.2 rearrangement, likely in response to self or ubiquitous antigens [24]. Cells expressing other V γ 2-J γ segment rearrangements dominate the immature thymic or cord blood $\gamma\delta$ repertoire, but comprise only a minor portion of the mature V γ 2 repertoire (unpublished observations and [7]). Selective pressures favoring the V γ 2-J γ 1.2 rearrangement reduce diversity to such an extent that identical clonotypes are often present among unrelated donors. The V γ 2 repertoire is relatively stable over extended intervals but we also note that rare adult donors have few V γ 2-J γ 1.2 cells and consequently poor functional responses, despite being otherwise healthy.

The abundance of particular V γ 2 clonotypes in the starting $\gamma\delta$ TCR repertoire provides an unique opportunity to determine the magnitude of change during an expansion. Such measurements are not usually possible for $\alpha\beta$ T cells, because the starting frequency for any given clone is in the range of 10^{-5} to 10^{-6} , and it is impractical to find these rare events by a bulk cDNA sequencing effort. We were able to measure the extent of proliferation for individual V γ 2 T cell clones and determine whether they respond better to one or both of the model stimuli. Many V γ 2 clonotypes were expanded after IPP or Daudi stimulation. In most cases, the ratio of proliferation after IPP versus Daudi was similar among donors, even though the absolute stimulation indices varied. We interpret this to mean that the response was TCR-dependent but cell expansion required other factors in the culture, likely including monocytic and dendritic cell subsets that are known to promote V γ 2V δ 2 T cell proliferation [15,31]. It is important to note that few studies have ever compared the performance of identical, naturally occurring T cell receptor clonotypes among different individuals. The degree of consistent responses is a remarkable finding and suggests a fundamental role for V γ 2V δ 2 T cells.

The dominant selection for common recognition V γ 2 clonotypes explains why IPP or other phosphoantigen stimulators produce potent antitumor effectors. Quite apart from suggesting that the V γ 2 repertoire is an example of degenerate or non-specific recognition, these data show a high degree of specificity as evidenced by the reproducible behavior of public clonotypes in different donor PBMC.

This work highlights important differences between TCR repertoire for $\gamma\delta$ and $\alpha\beta$ T cell populations. Unlike the starting $\alpha\beta$ T cell repertoire, we found numerous examples of public V γ 2 clonotypes in the starting repertoire nearly all of which expressed the J γ 1.2 segment; selection produced an astonishingly similar repertoire among unrelated individuals. Similar to what has been observed for public $\alpha\beta$ T cell repertoire, public V γ 2 clonotypes have minimal CDR3 diversity and are predominantly composed of germline-encoded residues [19]. Historically, the disproportionate engagement of MHC molecules with CDR1/2 of the $\alpha\beta$ TCR was proposed to explain the phenomenon of public responses with minimal CDR3 diversity in these cells [19]. Our observation that both IPP and Daudi expand public V γ 2 clonotypes irrespective of haplotype argues against the MHC-imprinting hypothesis for $\gamma\delta$ T cells and may reflect the inherent capacity of germline-encoded TCR to recognize conserved, non-MHC restricted antigens.

The mature V γ 2V δ 2 T cell repertoire encodes an effector subset that is primed for rapid proliferative and cytolytic responses to phosphoantigens, tumors and infected cells. This finding supports the efforts to stimulate antitumor responses in cancer patients by treatment with phosphoantigens [27,41]. The V γ 2 repertoire evolves in response to stimulation by ubiquitous or self molecules, possibly including normal metabolites like IPP. The common selection in most persons and the absence of MHC restriction creates a repertoire dominated by public clonotypes that recognize diverse stimuli. V γ 2V δ 2 T cells provide a model for understanding chronic T cell responses that defy peripheral tolerance, and are poised for responses to pathogens and tumor cells.

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Figure 1. The Biased V γ 2 Chain Repertoire in Healthy Adults

Spectratype data for V γ 2 chain length distribution were compiled from 44 healthy adults in North America and Nigeria. The proportion of chains at each nucleotide length are represented in the histogram; error bars indicate the standard error of the mean.

Figure 2. IPP- or Daudi-generated V γ 2V δ 2 Effectors Potently Lyse Tumor Cell Line Targets
 V γ 2 effectors were generated by expanding PBMC with IPP or irradiated Daudi tumor cells. V δ 2 effectors were titrated over tumor cell targets Daudi (squares), MOLT-4 (circles) or IBL-4 (triangles) at various effector to target ratios. Targets were labeled intracellularly with 2 μ M calcein-AM and cytotoxicity was determined by assessing the presence of released fluorescent calcein in supernatants.

Figure 3. CDR3 Length Distribution in the Population of V γ 2 or V δ 2 Chains Before and after Stimulation with IL2 alone, 15 μ M IPP and IL2 or irradiated Daudi and IL2

Whole RNA was extracted from PBMC and used to amplify V γ 2 or V δ 2 TCR chains using appropriate primer sets. PCR-amplified TCR chains were spectratyped using a fluorophoreconjugated primer specific for the γ or δ chain. Peak area and distribution were compiled with GeneScan software and data plotted as the relative (left column) or cumulative frequency (right column) of each CDR3 length for V γ 2 (top row) and V δ 2 (bottom row) chains. Relative frequency values are the averaged relative frequency at each nucleotide chain length for six, normal healthy donors and graphs indicate chain length distribution before (empty) or after stimulation with IL2 alone (checkered), IPP and IL2 (gray) or Daudi (black). Cumulative frequency plots show the accumulation of the averaged relative frequencies for six, normal healthy donors and were calculated by sequentially adding averaged relative frequencies from the shortest to the longest CDR3 nucleotide length. The change in V γ 2 or V δ 2 chain length distribution before (empty circles, broken line) or after stimulation with IL2 alone (hash mark, solid line), IPP (solid diamonds) or Daudi (solid squares).

Figure 4. IPP or Daudi Selectively Expand V γ 2-J γ 1.2 chains

RNA was extracted, V γ 2 chains amplified and CDR3 regions sequenced from Donors A, C, F and B before (empty) and after stimulation with IPP (gray) or irradiated Daudi (black). For each condition there were approximately 42 individual sequences analyzed. J γ 1.3 and J γ 2.3 are distinct genes that are difficult to distinguish by nucleotide sequence due to allelic variation of J γ 1.3.

A)

B)

Figure 5. Examples of Common or Specific Recognition of IPP and Daudi by V γ 2-J γ 1.2 Chains

In each of the 4 donors, the fold increase for all V γ 2-J γ 1.2 clonotypes detected after stimulation with IPP or Daudi was calculated and plotted on a scatter diagram. Fold increase is calculated by multiplying the stimulation index for the culture by the ratio of the clonotype frequency after stimulation to the clonotype frequency before stimulation. Limits for common or specific clonotypes were established based on fold-increase thresholds: clonotypes were considered specific if the ratio of Daudi to IPP fold increase was greater than 25. Clonotype stimulation analysis is restricted to V γ 2 chains expressing the J γ 1.2 segment. (B) IPP or Daudi stimulation expands common recognition V γ 2 chains that are public. Wenn diagrams depict the overlap of nucleotypes that encode common recognition or public V γ 2 clonotypes following stimulation with IPP or irradiated Daudi. Analysis is limited to V γ 2 sequences detected after stimulation with IPP or Daudi.

Tab. 3.3.c

A)

Name	Clonotype			Nuctypes	A		B		C		F	
	VG2	N	CG		IPP	Daudi	IPP	Daudi	IPP	Daudi	IPP	Daudi
4-01	LWE	T	QELG	4	1865.1	944.7				<i>n.c.</i>	192.9	117.7
4-02	LWEV	QK	LG	1	266.4	1889.3						
4-03	LWEV		QELG	5	399.7	944.7	2.7	3.0	7.8	137.0	80.4	196.2
4-04	LWEV	T	ELG	2					<i>n.c.</i>		48.2	824.0
4-05	LWEV	G	ELG	3	<i>n.c.</i>						96.5	588.5
4-06	LWEV	R	ELG	4					19.6	411.0	48.2	176.6
4-07	LW	SR	ELG	1	48.9	462.7						
4-08	LWEV		ELG	3	133.2	236.2			<i>n.c.</i>	<i>n.c.</i>		<i>n.c.</i>
4-09	LWE	GM	G	1	44.4	157.4						
4-10	LWE	A	ELG	1	63.4	135.0		<i>n.c.</i>	<i>n.c.</i>			

B)

Name	Clonotype			Nuctypes	A		B		C		F	
	VG2	N	CG		IPP	Daudi	IPP	Daudi	IPP	Daudi	IPP	Daudi
3-01	LWEV	E	ELG	2	<i>n.c.</i>	944.7		<i>n.c.</i>				
3-02	LWE			1					1.6	438.4		
3-03	LWE	A	QELG	3		<i>n.c.</i>			13.0	365.3		<i>n.c.</i>
3-04	LWE	N	QELG	1					7.8	274.0		
3-05	LWE	AK	ELG	1					<i>n.c.</i>	182.7		
3-06	LWE	EK	ELG	1				3.0				

C)

Name	Clonotype			Nuctypes	A		B		C		F	
	VG2	N	CG		IPP	Daudi	IPP	Daudi	IPP	Daudi	IPP	Daudi
2-01	LW	AE	ELG	1	266.4	<i>n.c.</i>						
2-02	LWEV	RGLG	ELG	1	266.4	<i>n.c.</i>						
2-03	LWEV	RK	LG	1	266.4	<i>n.c.</i>						
2-04	LW	LS	QELG	1							96.5	<i>n.c.</i>
2-05	LWEV	L	ELG	3	76.1	<i>n.c.</i>					<i>n.c.</i>	
2-06	LW	VKE	ELG	1	66.6	<i>n.c.</i>						
2-07	LW	DSR	ELG	1					7.8	<i>n.c.</i>		

Figure 6. Multiple V γ 2 Nucleotides Converge on Clonotypes that Recognize both IPP and Daudi
 V γ 2-J γ 1.2 clonotypes were designated as common recognition (A), Daudi-specific (B) or IPP-specific (C). The fold increase after IPP or Daudi stimulation was listed for each V γ 2-J γ 1.2 clonotype from each of the four normal, healthy donors that were sequenced. The number of nucleotides that converge to encode the same V γ 2-J γ 1.2 clonotype is also indicated. The fold increase for clonotypes not detected in the starting repertoire but present following stimulation with IPP or Daudi could not be calculated and are indicated with the abbreviation *n.c.*

Figure 7. V γ 2 Repertoire is Stable For Many Years

A) We obtained PBMC from 7 healthy adult North American donors (A, B, C, D, E, F, G and H) over a 2 year interval and sequenced V γ 2 chains at both timepoints. For each donor, V γ 2 nucleotypes were compared between timepoints and those present in both were considered conserved. The proportion of V γ 2 repertoire encoding conserved nucleotypes was calculated for both timepoints and plotted for all donors. Each timepoint represents an average of 40 individual sequences (range: 32-114) but is a single determination and does not have a standard error. Truncated V γ 2 chains encoding in-frame stop codons were excluded from the analysis. B) In a retrospective analysis, newly determined V γ 2 sequences for 3 donors (A, H and I) were compared to V γ 2 sequences obtained 7 years earlier. The proportion of V γ 2 repertoire encoding nucleotypes conserved over the 7 year interval is plotted for both timepoints and for each of the donors. Approximately 45 sequences were analyzed per donor timepoint (range: 35-54) and truncated V γ 2 chains encoding in-frame stop codons were excluded from the analysis.

Table 1

Expansion Profile of Vδ2 T cells from Normal, Healthy Donors

Donor	Age	Sex	Ethnicity	Vδ2 Frequency ^a				Stimulation Index ^b		
				Ex vivo	IL2	IPP	Daudi	IPP	Daudi	
A	51	Male	Caucasian	4.7 (6.1)	1.7 (1.4)	50.4 (48.1)	62.2 (86.2)	23.6		90.9
B	53	Male	African-American	0.7 (0.2)	0.5 (0.1)	1.6 (1.5)	2.5 (17.4)	4.0		9.0
C	28	Female	Caucasian	2.2 (2.1)	4.7 (3.6)	8.7 (5.2)	45 (68.8)	3.7		142.1
D	26	Female	Caucasian	4.5 (5.0)	5.2 (3.1)	38.9 (33.9)	50.7 (69.5)	18.8		93.3
E	29	Male	Caucasian	21.3 (23.9)	17 (13.3)	86.9 (87.2)	84 (88.4)	13.3		19.2
F	23	Male	Afro-Caribbean	1.3 (2.2)	3.7 (2.7)	18.2 (14.6)	40.1 (71.5)	4.0		15.6
Average ^c				5.8±7.8 (6.6±8.7)	5.5±5.9 (4±4.7)	34.1±31.7 (31.8±32.4)	47.4±27 (67±25.8)	11.2±8.7		61.7±54.8

^aVδ2 frequency is expressed as the percentage of lymphocytes or percentage of CD3⁺ lymphocytes (as indicated within parentheses) in PBMC and after stimulation with IL2 alone, IPP or Daudi.

^bThe stimulation index (SI) represents the increase for Vδ2 lymphocytes after IPP or Daudi compared to IL2 alone. SI is calculated as the ratio of the absolute number of Vδ2 lymphocytes on Day 14 to the absolute number of Vδ2 lymphocytes on Day 14 expanded with IL2 alone.

^cThe average Vδ2 frequency or stimulation index is indicated ± standard error of the mean.