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Cytokine Requirements for the Differentiation and Expansion of IL-17A- and IL-22-producing Human V γ 2V δ 2 T Cells¹

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Abstract

Human $\gamma\delta$ T cells expressing the V γ 2V δ 2 TCR play important roles in immune responses to microbial pathogens by monitoring prenyl pyrophosphate isoprenoid metabolites. Most adult V γ 2V δ 2 cells are memory cytotoxic cells that produce IFN- γ . Recently, murine $\gamma\delta$ T cells were found to be major sources of IL-17A in anti-microbial and autoimmune responses. To determine if primate $\gamma\delta$ T cells play similar roles, we characterized IL-17A and IL-22 production by V γ 2V δ 2 cells. IL-17A-producing memory V γ 2V δ 2 cells exist at low but significant frequencies in adult humans (1:2,762 T cells) and at even higher frequencies in adult rhesus macaques. Higher levels of V γ 2V δ 2 cells produce IL-22 (1:1,864 T cells) although few produce both IL-17A and IL-22. Unlike adult humans where many IL-17A⁺ V γ 2V δ 2 cells also produce IFN- γ (T $\gamma\delta$ 1/17), the majority of adult macaques IL-17A⁺ V δ 2 cells (T $\gamma\delta$ 17) do not produce IFN- γ . To define the cytokine requirements for T $\gamma\delta$ 17 cells, we stimulated human neonatal V γ 2V δ 2 cells with the bacterial antigen, HMBPP, and various cytokines and mAbs *in vitro*. We find that IL-6, IL-1 β , and TGF- β are required to generate T $\gamma\delta$ 17 cells in neonates whereas T $\gamma\delta$ 1/17 cells additionally required IL-23. In adults, memory T $\gamma\delta$ 1/17 and T $\gamma\delta$ 17 cells required IL-23, IL-1 β , and TGF- β but not IL-6. IL-22-producing cells showed similar requirements. Both neonatal and adult IL-17A⁺ V γ 2V δ 2 cells expressed elevated levels of ROR γ t. Our data suggest that, like Th17 $\alpha\beta$ T cells, V γ 2V δ 2 T cells can be polarized into T $\gamma\delta$ 17 and T $\gamma\delta$ 1/17 populations with distinct cytokine requirements for their initial polarization and later maintenance.

Keywords

Interleukin-17A; gamma delta T cell; Vgamma2Vdelta2 T cells; Interleukin-22; human

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Introduction

Members of the IL-17 cytokine family (IL-17A through IL-17F) are pro-inflammatory cytokines that possess a diverse array of functions ranging from neutrophil recruitment to induction of wound repair and tissue remodeling. IL-17A induces a plethora of inflammatory cytokines (such as TNF- α , IL-1 β , IL-6, GM-CSF, and G-CSF), chemokines (including but not limited to CXCL1, CXCL8, and CXCL10), and matrix metalloproteinases and defensins (1–6). In addition to its role in mediating protection, IL-17A, when dysregulated, has severe pathogenic consequences. Elevated levels of IL-17A have been observed in many autoimmune diseases such as rheumatoid arthritis (7,8), systemic lupus erythematosus (9,10), psoriasis (11,12), and multiple sclerosis (13).

Th17 CD4 $\alpha\beta$ T cells have been well described in both humans and mice, and the cytokine requirements for their generation from naïve CD4 T cells have been determined. At present it is believed that IL-6 and/or IL-21 signaling through STAT-3 results in the induction and amplification of Retinoid-related orphan receptor γ^4 (ROR γ^4) (*rorc*) (14) and ROR α (*rora*) in naïve T cells (15). STAT-3, which binds both the *Il17A* and *Il17F* promoters (16), then mediates acquisition of IL-17A production capability. IL-6 also induces expression of IL-23R on these developing Th17 precursors (17), thus enabling further STAT-3 signaling through the IL-23R. IL-23/IL-23R signaling through STAT-3 is required by committed Th17 precursors for terminal differentiation of these cells into effector Th17 cells and further maintenance of their phenotype *in vivo* (18). TGF- β is also required for maximal differentiation of Th17 cells. However, rather than acting directly, TGF- β appears to mediate its effect indirectly by suppressing Th1 and Th2 differentiation by inhibiting STAT-4 and GATA-3, respectively (19). Human Th17 CD4 $\alpha\beta$ T cells also appear to require TGF- β for maximal differentiation of Th17 cells (20–22) probably through a similar mechanism (23).

Despite the extensive study of Th17 T cells, IL-17A production is not an exclusive characteristic of CD4 $\alpha\beta$ T cells. IL-17A can also be produced by unconventional T cells such as $\gamma\delta$ T (reviewed in 24) and $\alpha\beta$ NKT (25,26), as well as macrophages (27) and neutrophils (28). Among unconventional T cells, $\gamma\delta$ T cells represent a population of innate-like T cells that developed early in vertebrate phylogeny along with B cells and $\alpha\beta$ T cells (29). Much like conventional CD8 $\alpha\beta$ T cells, $\gamma\delta$ T cells exhibit antigen specificity, robustly proliferate in response to activation, produce pro-inflammatory cytokines (such as TNF- α and IFN- γ), and are highly cytolytic to their targets. However, certain murine $\gamma\delta$ T cell subsets are also potent IL-17A producers, and, in some disease settings, $\gamma\delta$ T cells constitute a greater fraction of the IL-17A producing cells and secrete IL-17A earlier in disease than conventional CD4 or CD8 $\alpha\beta$ T cells (30–35). Furthermore, murine $\gamma\delta$ T cells can produce IL-17A, IL-22, and IL-21 in response to IL-23 and IL-1 β (36).

Despite their conservation across species, mouse and human $\gamma\delta$ T cells demonstrate significant differences. One major difference is the existence of the V γ 2V δ 2 T cell subset (also termed V γ 9V δ 2) in humans and other primates (37), which comprises the majority (up to 90%) of circulating $\gamma\delta$ T cells. The orthologous V genes, which rearrange to generate the V γ 2V δ 2 TCR in primates, are absent from mice and other mammals. V γ 2V δ 2 T cells are distinct from conventional $\alpha\beta$ T cells in that they are almost exclusively memory cytotoxic T cells producing IFN- γ and TNF- α (38,39) which can expand to very high levels (commonly >50% of circulating T cells) during *in vivo* infections with bacteria and protozoa (reviewed in 40,41). We and others have identified HMBPP, an essential metabolite in isoprenoid

⁴Abbreviations used in this paper: CBMC, cord blood mononuclear cell; HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; iNKT, invariant NKT cells; IPP, isopentenyl pyrophosphate; MFI, mean fluorescence intensity; ROR, retinoid-related orphan receptor

synthesis in some bacteria and all Apicomplexan parasites (42–44), as an antigen for V γ 2V δ 2 T cells. By specifically recognizing a common essential microbial metabolite, V γ 2V δ 2 T cells can mount memory responses to many bacterial and parasitic protozoan infections that have never been encountered previously.

V γ 2V δ 2 T cells also recognize, isopentenyl pyrophosphate (IPP), an essential intermediate for isoprenoid synthesis that is common to both microbes and man (45). Under normal circumstances, IPP is sequestered inside host cells at low levels and therefore fails to activate host V γ 2V δ 2 T cells. Certain tumor cells or treatment of human cells with bisphosphonates (46) or alkylamines (47) causes increases in IPP resulting in activation of V γ 2V δ 2 T cells (reviewed in 41). The V γ 2V δ 2 T cell receptor can distinguish foreign HMBPP from self IPP since HMBPP is 30,000-fold more active, stimulating at picomolar concentrations. This recognition by V γ 2V δ 2 $\gamma\delta$ T cells allows for immediate memory T cell responses both to microbes and to self IPP when overproduced by malignant cells or after pharmacological treatments.

In contrast to mice, few studies have investigated IL-17A production by human $\gamma\delta$ T cells. Human $\gamma\delta$ T cells producing IL-17A have been shown to be present in peripheral blood and were slightly increased in patients with active TB infections (48). Similarly, HIV infected patients have an increased frequency of IL-17A-producing V δ 1 T cells (49). However, neither of these studies characterized IL-17A- and IL-22-producing V γ 2V δ 2 T cells in detail or examined the cytokine requirements for IL-17A production by human $\gamma\delta$ T cells. Thus, the potential role of $\gamma\delta$ T cells as sources of IL-17A and IL-22 in human immune responses is unclear.

In this study, we demonstrate that IL-17A and IL-22 producing V γ 2V δ 2 T cells exist at low but significant frequencies in human and non-human primates. Our data suggest that, like Th17 $\alpha\beta$ T cells, V γ 2V δ 2 $\gamma\delta$ T cells can be polarized into T $\gamma\delta$ 17, T $\gamma\delta$ 1/17, and T $\gamma\delta$ 22 populations with distinct cytokine requirements for their initial polarization and their later maintenance.

Materials and Methods

Antigen and cytokines

HMBPP was synthesized as described (50). Recombinant human IL-6, IL-1 β , IL-23, and TGF- β were all purchased from eBioscience. Recombinant human IL-2 (Proleukin) was purchased from Hoffman-La Roche. Neutralizing anti-human IFN- γ , anti-human IL-4, anti-human IL-6, anti-human IL-23 p19, and anti-human IL-1 β were purchased from R&D Systems.

Antibodies

FITC-conjugated anti-human V δ 2 TCR (clone B6), allophycocyanin-Cy7-conjugated anti-human CD3 (clone SK7), FITC-conjugated anti-human TCR $\gamma\delta$ (clone B1), and PE- or biotin-conjugated anti-human IFN- γ (both clone 4S.B3) were purchased from BD Biosciences. Alexa-Fluor⁶⁴⁷-conjugated anti-human T-Bet (clone eBio4B10) and Alexa-Fluor⁶⁴⁷ or PE-conjugated anti-human ROR γ t (both clone AFKJS-9) were purchased from eBioscience. PerCP-Cy5.5 -conjugated anti-human IL-17A (clone eBio64DEC17) and PE-conjugated anti-human IL-17A (clone eBio64CAP17) were purchased from eBioscience. PE-conjugated anti-human IL-22 (clone 142928) was purchased from R&D Systems. allophycocyanin-conjugated anti-human CD27 (clone O323), biotin-conjugated anti-human CD28 (clone CD28.2), biotin-conjugated anti-human CD4 (clone L3T4) and PE-Cy7 Streptavidin were purchased from eBioscience. For monkey studies, unconjugated anti-

human V δ 2 (clone 15D) was purchased from Endogen and FITC-conjugated goat anti-mouse (IgM+IgG Fab fragment) was purchased from Biosource.

Adult PBMC isolation and culture

Normal human or female rhesus macaque peripheral blood was collected by venipuncture and PBMC isolated using Ficoll-Paque Plus from Amersham Biosciences. PBMC in X-VIVO 15 serum free media (BioWhittaker) were cultured at 1×10^5 cells per well in 96 well round bottom tissue culture plates.

For differentiation and expansion experiments, PBMC were plated as above in X-VIVO 15 serum-free media (unless otherwise stated) and incubated in the presence or absence of 0.316 μ M HMBPP, 50 ng/ml rhIL-23, 50 ng/ml rhIL-1 β , 50–200 ng/ml rhIL-6, 1 ng/ml rhTGF- β , 10 μ g/ml anti-human IL-6, and 10 μ g/ml anti-human IL-23. On the third day, 1 nM IL-2 was added to cultures. Cells were cultured for 7–12 days. On the final day, cells were washed, and stimulated with PMA and ionomycin as for *ex vivo* analyses as described above. Note that the cytokines were titrated in pilot experiments to determine their optimal concentrations. Also, preliminary studies found that the addition of neutralizing anti-IFN- γ and anti-IL-4 were unnecessary for the polarization of IL-17A⁺ V γ 2V δ 2 T cells and therefore were not added in subsequent polarization experiments (except where otherwise noted). Since there was a high degree of variability between adult human donors, the number of IL-17A⁺ V γ 2V δ 2 for each donor and condition was normalized to the maximal number of IL-17A⁺ V γ 2V δ 2 T cells expanded for each particular donor. Such variability was not unexpected as the donors had highly variable frequencies of IL-17A⁺ V γ 2V δ 2 T cells *ex vivo*.

Umbilical cord blood mononuclear cell culture

Umbilical cord blood was obtained from normal term deliveries. Cord blood mononuclear cells (CBMC) were isolated from heparinized cord blood using Ficoll-Paque Plus density gradient centrifugation and frozen in liquid nitrogen until needed. For polarization experiments, CBMC were defrosted and plated in X-VIVO 15 serum free media and cultured with or without 200 μ M HMBPP and the same cytokine concentrations as were used for adult PBMC polarizations. IL-2 (1 nM) was added on day 3, and the cells re-stimulated with PMA and ionomycin on day 13 for intracellular cytokine staining. Note that neonatal V γ 2V δ 2 T cells from cord blood require higher concentrations of HMBPP and longer incubation periods than adult V γ 2V δ 2 T cells for expansion.

ELISA for IL-17A

To measure the quantity of IL-17A released from expanded T cell cultures, cells were re-stimulated with 50 ng/ml PMA (Sigma) and 2 μ g/ml Ionomycin (Sigma) for four-six hours, after which supernatants were collected. IL-17A was quantified in triplicate using the R & D Systems human IL-17 DuoSet ELISA kit.

Flow Cytometric Staining

To examine cytokine production *ex vivo*, PBMC were rested overnight and the next day stimulated with 50 ng/ml PMA (Sigma) and 2 μ g/ml Ionomycin (Sigma) for four-six hours in the presence of GolgiSTOP (monensin) (BD Biosciences) at the manufacturer's recommended concentration. PBMC were first stained with Live/Dead Blue (Invitrogen), to exclude dead cells, then stained with allophycocyanin-Cy7-conjugated anti-CD3, FITC conjugated anti-V δ 2 or FITC-conjugated anti-TCR $\gamma\delta$. The cells were then washed, fixed, and permeabilized using the BD Cytofix/Cytoperm Kit and then intracellularly stained with either PE-conjugated anti-IL-17A alone or PE-conjugated anti-IFN- γ or PE-conjugated anti-

IL-22 combined with PerCP-Cy5.5-conjugated anti-IL-17A. To determine the memory distribution of IL-17A⁺ cells, cells were stained as above with Live/Dead Blue, allophycocyanin-Cy7-conjugated anti-CD3 and FITC-conjugated anti-V δ 2, and then stained with biotin-conjugated anti-CD28 and allophycocyanin-conjugated anti-CD27. The cells were then fixed and permeabilized as described above, stained with PE-conjugated anti-IL-17A, and incubated with PE-Cy7 Streptavidin.

Similar ex vivo staining was performed for monkeys. Briefly, PBMC were stimulated as above, then stained with Live/Dead Blue and unconjugated anti-human V δ 2 (clone 15D), followed by detection with FITC-conjugated goat anti-mouse. Because the anti-human CD3 mAb clone SK7 does not cross-react with rhesus macaque CD3, it was not used. The cells were then blocked with normal mouse sera, fixed, and permeabilized with the BD Cytofix/Cytoperm Kit. The monkey cells were then intracellularly stained with PerCP-Cy5.5-conjugated anti-IL-17A, PE-conjugated anti-IFN- γ , and Alexa-Fluor⁶⁴⁷-conjugated anti-T-bet.

Post expansion, human PBMC and CBMC were re-stimulated with PMA (50 ng/ml) and ionomycin (2 μ g/ml) in the presence of GolgiSTOP for four-six hours. The cells were then stained with Live/Dead Blue, followed by surface staining with FITC-conjugated anti-V δ 2, APC-Cy7-conjugated anti-CD3, and biotin-conjugated anti-CD4. The cells were fixed and permeabilized as previously described and then intracellularly stained with either PE-conjugated anti-IFN- γ , Alexa-Fluor⁶⁴⁷-conjugated anti-IL-22, and PerCP-Cy5.5-conjugated anti-IL-17A or PE-conjugated anti-ROR γ t, and Alexa-Fluor⁶⁴⁷-conjugated anti-T-bet, and PerCP-Cy5.5-conjugated anti-IL-17A. Lastly the biotin label was detected with PE-Cy7 Streptavidin.

To assess V γ 2V δ 2 T cells, an anti-V δ 2 mAb was used for all analyses since in adults the vast majority (96.4–100%) of V δ 2 T cells express V γ 2V δ 2 TCRs (51,52). In fact, 17 out of 36 donors had greater than 99.6% of V δ 2 chains paired with V γ 2 chains (51). The anti-V δ 2 mAb was also used to determine V γ 2V δ 2 T cells after expansion by HMBPP since only $\gamma\delta$ T cells expressing V γ 2V δ 2 TCRs respond and expand to prenyl pyrophosphates (53,54). After expansion of $\gamma\delta$ T cells by prenyl pyrophosphates, 100% of V δ 2 T cells express V γ 2V δ 2 TCRs. In neonates prior to prenyl pyrophosphate expansion, about equal proportions of V δ :2 chains are paired with V γ 2 as paired with V γ 1 (51). However, we did not assess ex vivo production of IL-17A and IL-22 by neonates since the number of V γ 2V δ 2 T cells was too low. Therefore, we have used V γ 2V δ 2 to designate V δ 2 T cells in the text although a very small fraction of the cells may express V γ 1V δ 2 TCRs.

Statistical analyses

V γ 2V δ 2 T cell number was determined by multiplying the frequency of cells within the live cell gate by the total number of live cells in 20 round-bottom wells of a 96 well plate. Cell counts were determined by trypan blue exclusion prior to PMA and ionomycin stimulation. For statistical analyses, the nonparametric Kruskal-Wallis test, followed by Dunn's post-test was used with $p < 0.5$ considered statistically significant. Statistical analyses were done in Prism version 4.0c (GraphPad Software).

Results

Frequency of circulating IL-17A- and IL-22-producing V γ 2V δ 2 T cells in normal humans and rhesus macaques

Naïve $\gamma\delta$ T cells in mice are epigenetically programmed to be potent IFN- γ -producing Th1-like cells by virtue of constitutive expression of eomesodermin (Eomes) and poor methylation of the IFN- γ locus as compared with naïve CD4 T cells (55,56). Nonetheless,

although most human V γ 2V δ 2 T cells produce IFN- γ , minor populations have been identified that produce IL-4 and IL-10 (57). This suggests that V γ 2V δ 2 T cells, like $\alpha\beta$ T cells, can be polarized into different functional lineages. To investigate the existence of Th17-like V γ 2V δ 2 T cells in humans, we isolated peripheral blood mononuclear cells (PBMC) from 10 normal donors, stimulated the PBMC with the mitogen, ionomycin, in the presence of PMA, and performed intracellular cytokine staining. Ex vivo mitogen stimulation of T cells revealed that IL-17A-producing V γ 2V δ 2 T cells were present in most donors although the proportions varied widely ranging from 0.2 to 3% of V γ 2V δ 2 T cells with an average of $1.1 \pm 0.3\%$ (Fig. 1A, 1C, and Table 1). No IL-17A production from V γ 2V δ 2 T cells was observed in the absence of ionomycin and PMA stimulation (data not shown). An average of $0.9 \pm 0.2\%$ of peripheral blood $\gamma\delta$ T cells secreted IL-17A. These proportions were similar to $\alpha\beta$ T cells where an average of $1.1 \pm 0.1\%$ produced IL-17A (Fig. 1A and 1C). Thus, in one ml of blood an average of 389 ± 112 of V γ 2V δ 2 cells produced IL-17A (Fig. 1B and Table I) and the frequency of IL-17A-producing V γ 2V δ 2 T cells averages 1 out of every 2,762 T cells (Table I).

Because the V γ 2V δ 2 TCR is exclusively expressed in primates and not expressed by murine $\gamma\delta$ T cells, the rhesus macaque (*Macaca mulatta*) is a useful animal model to study V γ 2V δ 2 T cells *in vivo* (58). Therefore, we next asked whether V γ 2V δ 2 T cells in rhesus macaques produce IL-17A. PBMC were isolated from 8 macaques, stimulated with ionomycin in the presence of PMA, and cytokine production determined by intracellular staining. The frequency of peripheral blood IL-17A-producing V δ 2 T cells ex vivo was increased with a mean frequency of $5.6 \pm 1.3\%$ (ranging from 1.1 to 13.4%, see Fig. 1E) compared to $1.1 \pm 0.3\%$ in humans. We noted similar frequencies in splenic T cells from another 2 rhesus macaques (data not shown). Taken together, these results demonstrate that an IL-17A⁺ V δ 2 T cell population, parallel to the Th17 $\alpha\beta$ T cell subset, exists in humans and that this population is conserved in non-human primates albeit at higher levels.

IL-22 is believed to be produced by Th17-lineage T cells and is thought to help epithelial healing (59) and to mediate epithelial inflammation since it is elevated in the skin of patients with psoriasis (60,61) and in the colonic mucosa of patients with Crohn's disease (62). We found that IL-22-producing V γ 2V δ 2 T cells were a separate subset of cells distinct from IL-17A-producing V γ 2V δ 2 T cells since only 2.7% of IL-22-producing cells also produced IL-17A (Fig. 2A, 3B, 4B, Supplemental Fig. 1). The frequency of V γ 2V δ 2 T cells producing IL-22 averaged $1.2 \pm 0.2\%$ (ranging from 0.5 to 2.2%) of peripheral blood V γ 2V δ 2 T cells. The frequency of $\alpha\beta$ T cells producing IL-22 among these same donors averaged $2.3 \pm 0.5\%$. When the absolute cell numbers were calculated (Table I), there were 1.6-fold more IL-22-producing V γ 2V δ 2 T cells than IL-17A-producing V γ 2V δ 2 T cells (639 ± 328 cells/ml producing IL-22 versus 389 ± 112 cells/ml producing IL-17A) or 1 out of every 1,864 total T cells. Very few produced both IL-17A and IL-22 (17 ± 4 cells/ml). Thus, our results show that IL-17A- and IL-22-producing cells are separate populations of V γ 2V δ 2 T cells.

Phenotype of IL-17A⁺ V γ 2V δ 2 T cells

Classically defined murine Th17 have been reported to produce IL-17A, IL-17F, and IL-22 but not IFN- γ (63). Nonetheless, CD4 T cells producing both IL-17A and IFN- γ (64–66) and CD4 T cells producing IL-17A without IL-22 have been observed (67–70). Regardless of the other cytokines co-produced, both CD4 and CD8 $\alpha\beta$ T cells producing IL-17A have been exclusively detected within memory subsets (64,65,68,71,72). We therefore determined the spectrum of cytokines co-produced by IL-17A⁺ V γ 2V δ 2 T cells and the memory phenotype of IL-17A⁺ V γ 2V δ 2 T cells. After stimulation with PMA and ionomycin, most IL-17A⁺ V γ 2V δ 2 T cells co-produced IFN- γ , almost none co-produced IL-22 (Fig. 2A), IL-4, or IL-10 (data not shown). In contrast, fewer rhesus macaque IL-17A⁺ V γ 2V δ 2 T cells dual produced IFN- γ , with most being IL-17A single producers (representative staining in Fig.

2A). Consistent with published work (55,57,73,74), and their memory-like phenotype, we observed that the vast majority of human peripheral blood V γ 2V δ 2 T cells produced IFN- γ (> 90%) and a small fraction (< 5%) produced IL-4 (data not shown and 55, 57, 73, 74).

Unlike $\alpha\beta$ T cells, V γ 2V δ 2 T cells transition very early in life into phenotypically memory cells leaving few naïve V γ 2V δ 2 T cells in the adult circulation (< 2% naïve V γ 2V δ 2, C. Jin and C. T. Morita, unpublished observations and 75, 76). The process by which this occurs is not fully understood but probably is the result of stimulation by the ubiquitous foreign and self prenyl pyrophosphate antigens. Reminiscent of CD8 $\alpha\beta$ T cells, V γ 2V δ 2 T cells can be subdivided into memory subsets based on their expression of the CD27 and CD28 costimulatory receptors. Analogous to CD8 T cell development, V γ 2V δ 2 T cells can be divided into CD27⁺, CD28⁺ early memory cells (central memory), CD28⁻, CD27⁺ intermediate memory cells, and CD27⁻, CD28⁻ late memory cells (CD45RA⁺ effector memory) (77). Naïve V γ 2V δ 2 T cells represent < 2% of adult V γ 2V δ 2 T cells and constitute only a negligible proportion of CD27⁺ CD28⁺ V γ 2V δ 2 T cells that are distinguishable from central memory cells by their lack of CD45RO and their high level expression of CD45RA (C Jin and C. T. Morita, unpublished observations). To characterize the memory status of IL-17A⁺ V γ 2V δ 2 T cells, we performed staining for CD27 and CD28 on human PBMC after PMA and ionomycin re-stimulation. We observed similar proportions of T_{early} (+ T_{naïve}), T_{intermediate}, and T_{CD45RA late} cells in IL-17A⁺ V γ 2V δ 2 as found in total V γ 2V δ 2 T cells (Fig. 2B and 2C). Thus, in contrast to IL-17A⁺ Tc17 CD8 $\alpha\beta$ T cells that are almost exclusively restricted to the T_{early} and T_{intermediate} subsets (72), IL-17A⁺ V γ 2V δ 2 T cells were found within all three memory subsets without skewing.

Cytokine requirements for the differentiation and expansion of neonatal IL-17A⁺ V γ 2V δ 2 T cells

Unlike CD4 and CD8 $\alpha\beta$ T cells, little is known about the cytokines required to differentiate naïve $\gamma\delta$ T cells to produce IL-17A. Although umbilical cord blood represents the best source for naïve V γ 2V δ 2 T cells, even in cord blood only ~50% of the V γ 2V δ 2 T cells are phenotypically naïve (C Jin and C. T. Morita, unpublished observations). Given the low frequency of V γ 2V δ 2 T cells in cord blood (< 1% of T cells (51)), isolating pure naïve V γ 2V δ 2 T cells for *in vitro* polarization studies was not feasible. Therefore, we studied the polarization of total neonatal cord blood V γ 2V δ 2 T cells where ~50% have a naïve surface phenotype and none have been exposed to foreign antigens. Because a high proportion of V γ 2V δ 2 T cells react to the HMBPP antigen without prior selection (41), we were able to specifically expand V γ 2V δ 2 T cells directly from cord blood without purification. To determine the cytokine requirements for polarization of V γ 2V δ 2 T cells into IL-17A-producing cells, we cultured V γ 2V δ 2 T cells from 8 different donors in serum free media for 13 days with HMBPP and various combinations of the classical Th17 polarizing cytokines, IL-6, IL-1 β , IL-21, IL-23, and TGF- β and neutralizing cytokine antibodies. IL-2 was added on the third day. On the final day, cells were re-stimulated with PMA and ionomycin, surface stained for CD3 and V δ 2 (to identify V γ 2V δ 2 T cells, see Methods) and intracellularly stained for IL-17A, IFN- γ , and IL-22 as well as the transcription factors, ROR γ t and T-bet. We hypothesized that naïve V γ 2V δ 2 T cells, like naïve CD4 $\alpha\beta$ T cells, can be polarized under similar cytokine conditions (namely TGF- β , IL-6, IL-21, and IL-1 β) into IL-17A-producing T $\gamma\delta$ 17 V γ 2V δ 2 T cells.

Expansion of cord blood V γ 2V δ 2 T cells in response to antigen stimulation with HMBPP ranged from 8% to more than 20% of total CD3 T cells (Supplemental Fig. 2). Expanded V γ 2V δ 2 T cells were divided into IL-17A⁺ IFN- γ ⁻ (T $\gamma\delta$ 17), IL-17A⁺ IFN- γ ⁺ (T $\gamma\delta$ 1/17), IL-22⁺ IFN- γ ^{+/-} (T $\gamma\delta$ 22) subsets and their total numbers plotted (Fig. 3A). Representative staining for IL-17A, IFN- γ , and IL-22 is shown in Fig. 3B for condition 9. Because each donor differed in the magnitude of expansion, the number of IL-17A⁺ V γ 2V δ 2 for each

donor and condition was normalized to the maximal number of IL-17A⁺ V γ 2V δ 2 T cells expanded for each donor (Fig. 3A, bottom panel).

At baseline in the presence or absence of HMBPP (Fig. 3A, conditions 1 and 2), very few IL-17A⁺ (T γ δ 17 and T γ δ 1/17) and IL-22⁺ (T γ δ 22) V γ 2V δ 2 T cells were observed. IL-23 alone and IL-23 plus IL-1 β had minimal effects on the expansions of T γ δ 1/17 and T γ δ 17 V γ 2V δ 2 T cells (Fig. 3A, conditions 3 and 4). The combined effect of IL-23, IL-6, and IL-1 β also had little effect on the numbers of T γ δ 17 or T γ δ 1/17 (condition 5). However, when IL-23, IL-6, and IL-1 β were combined with TGF- β , a statistically significant increase in the number of T γ δ 1/17 cells (normalized to each donor's maximal response) was observed (Fig. 3A, bottom panel, compare condition 5 lacking TGF- β with condition 6 containing TGF- β). When endogenous IL-6 was neutralized in the presence of IL-23, IL-1 β , and TGF- β , the number of T γ δ 1/17 returned to moderate levels (Fig. 3A, compare conditions 6 and 7), suggesting an important role for IL-6 in the expansion of T γ δ 1/17 V γ 2V δ 2 T cells. However, exogenous IL-1 β , in combination with IL-6, IL-23, and TGF- β , was critical for expansion of both T γ δ 17 and T γ δ 1/17 (Fig. 3A, compare conditions 7 and 8). Taken together, these data suggest that neonatal T γ δ 17 and T γ δ 1/17 populations similarly require IL-1 β and TGF- β but that the T γ δ 1/17 population additionally requires IL-6.

Because IL-23 is considered a maintenance cytokine for memory Th17 T cells (78), we hypothesized that IL-23 would not be required for initial polarization of naïve cord blood V γ 2V δ 2 T cells into T γ δ 17 (or T γ δ 1/17) T cells. To test this hypothesis, we polarized V γ 2V δ 2 T cells in the presence of IL-1 β , IL-6, TGF- β , and neutralizing anti-IL-23. As predicted, we observed a statistically significant expansion in T γ δ 17 and T γ δ 1/17 V γ 2V δ 2 T cells even when IL-23 was neutralized (Fig. 3A, bottom, condition 9). These results support our hypothesis that IL-23 is not required for initial polarization of cord blood V γ 2V δ 2 into T γ δ 17, but that IL-1 β , IL-6, and TGF- β are required. Furthermore, our results suggest that exogenous IL-23 may actually inhibit T γ δ 17 development since more T γ δ 17 cells were found after its neutralization. Similar results were noted for a second small subset of cord blood V γ 2V δ 2 T cells expressing CD4. These cells showed very similar responses to cytokines with optimal expansion with IL-1 β , IL-6, TGF- β , and neutralization of IL-23 (Supplemental Fig. 1 and 2). In contrast, the effect of IL-23 on T γ δ 1/17 cells was different. This subset required IL-23 with IL-1 β , IL-6, and TGF- β for optimal expansion (compare condition 6 to condition 9). Thus, IL-23 favors the differentiation/and or expansion of neonatal V γ 2V δ 2 T cells as T γ δ 1/17 cells rather than T γ δ 17 cells.

IL-21 and TGF- β also polarize naïve CD4 $\alpha\beta$ T cells to a Th17 phenotype (22). Therefore, we tested whether IL-21 and TGF- β would similarly polarize neonatal V γ 2V δ 2 T cells into T γ δ 17 and/or T γ δ 1/17 cells. This combination (Fig. 3A, condition 10) failed to increase the number and/or percent of T γ δ 17 or T γ δ 1/17 V γ 2V δ 2 T cells, despite robust proliferation of V γ 2V δ 2 T cells (Supplemental Fig. 2, condition 10). Thus, unlike CD4 $\alpha\beta$ T cells, IL-21 and TGF- β were insufficient to support the development of IL-17A-producing V γ 2V δ 2 T cells.

Like IL-17A-producing V γ 2V δ 2 T cells, the numbers of T γ δ 22 V γ 2V δ 2 T cells tended to increase in the presence of IL-1 β , IL-6, and TGF- β (Fig. 3A, top, condition 9), although this increase did not reach statistical significance. Thus, our data suggest that although the T γ δ 22 cell population is distinct from T γ δ 1/17 and T γ δ 17 cell populations (Fig. 3B), T γ δ 22 V γ 2V δ 2 T cells require similar cytokines as those observed for T γ δ 17 cells.

Cytokine requirements for the expansion of adult IL-17A⁺ V γ 2V δ 2 T cells

We next asked whether IL-17A⁺ V γ 2V δ 2 T cells could be expanded from adult peripheral blood, and if similar cytokines were required to those required for neonatal IL-17A⁺ V γ 2V δ 2 T cells. Because the vast majority of adult V γ 2V δ 2 T cells are memory cells and

since IL-23R expression is restricted to memory CD4 T cells (79), we hypothesized that, like memory Th17 $\alpha\beta$ T cells, expansion of adult IL-17A⁺ V γ 2V δ 2 T cells would require IL-23. IL-1 β might also be required since it is important for the expansion of human CD4 Th17 T cells (80,81), and since the combination of IL-23 and IL-1 β induces IL-17A production by murine $\gamma\delta$ T cells (36). To determine the role of IL-23, we expanded adult V γ 2V δ 2 T cells in PBMC with HMBPP, IL-1 β , IL-6, neutralizing anti-IL-4, and neutralizing anti-IFN- γ in the presence or absence of IL-23 (Fig. 4). On the twelfth day, cells were restimulated with PMA and ionomycin and stained for IL-17A, IL-22, and IFN- γ intracellular cytokines. The addition of IL-23 to IL-6 and IL-1 β increased the frequency of IL-17A⁺ V γ 2V δ 2 T cells from 1.1% (roughly the same frequency of IL-17A⁺ V γ 2V δ 2 present *ex vivo*) to 9.4% (Fig. 4) due to an 8-fold increase in the number of IL-17A⁺ V γ 2V δ 2 T cells (Fig. 4B). Similarly, IL-17A levels increased 5.2-fold from 276 pg/ml in the absence to 1431 pg/ml in the presence of IL-23 (Fig. 4B). In these cultures, TGF- β was likely provided by serum included in the media. Thus, exogenous IL-23 can increase the numbers of IL-17A-producing V γ 2V δ 2 T cells.

To study this in more depth, V γ 2V δ 2 T cells from 10 adult donors were stimulated with HMBPP in the presence or absence of different cytokines to determine the cytokine requirements for the expansion of IL-17A-producing V γ 2V δ 2 T cells. To determine if TGF- β was required, serum free media was used. We found that the expanded adult V γ 2V δ 2 T cells, like neonatal V γ 2V δ 2 T cells, could be divided into IL-17A⁺ IFN- γ ⁺ (T γ δ 1/17), IL-17A⁺ IFN- γ ⁻ (T γ δ 17), and IL-22⁺ IFN- γ ^{+/-} (T γ δ 22) populations (representative staining for IL-17A and IFN- γ is shown in Fig. 4A and 5B). The vast majority of IL-17A⁺ V γ 2V δ 2 T cells expanded from adult blood were T γ δ 1/17 producing both IFN- γ and IL-17A (Fig. 5B). Fewer IL-17A⁺ IFN- γ ⁻ (T γ δ 17) V γ 2V δ 2 T cells were detected in adults with only 4 out of 10 adult donors exhibiting expansions of both T γ δ 17 and T γ δ 1/17 V γ 2V δ 2 T cells (compared with T γ δ 1/17 cells in Supplemental Fig. 3). These *in vitro* results were consistent with the *ex vivo* results since only these same four donors had detectable T γ δ 17 cells after stimulation.

IL-17A⁺ (T γ δ 1/17) or IL-22⁺ (T γ δ 22) V γ 2V δ 2 T cells were not preferentially expanded in the presence of HMBPP and IL-2 only (Fig. 5A, condition 2). Addition of IL-23 alone had minimal effect on the expansion of T γ δ 1/17 V γ 2V δ 2 T cells (Fig. 5A, condition 3) but did increase expansion of total V γ 2V δ 2 T cells from 12.7% to 25.1% of CD3 T cells (Supplemental Fig. 4A). In contrast to neonatal V γ 2V δ 2 T cells, there were moderate increases in T γ δ 1/17 cells with IL-23 and IL-1 β (condition 4). The addition of IL-6 to IL-23 and IL-1 β had little effect on T γ δ 1/17 V γ 2V δ 2 T cell numbers (Fig. 5A, top, condition 5) or the proportion of T γ δ 1/17 cells (Fig. 5A, bottom). However, the addition of TGF- β to IL-23 and IL-1 β further increased the expansion of T γ δ 1/17 V γ 2V δ 2 T cells (Fig. 5A, conditions 6 and 7, $p < 0.5$). Again the presence (condition 6) or absence (condition 7) of IL-6 had little effect on IL-17A⁺ cell numbers or in the expansion of total V γ 2V δ 2 T cells (Supplemental Fig. 4).

Like neonatal V γ 2V δ 2 T cells, IL-1 β appeared to also be critical for the expansion of adult T γ δ 1/17 V γ 2V δ 2 T cells, because in its absence (Fig. 5A, condition 8) the number of T γ δ 1/17 V γ 2V δ 2 T cells fell to low levels. However, unlike neonatal V γ 2V δ 2 T cells, neutralization of IL-23 in the presence of IL-1 β , IL-6, and TGF- β caused T γ δ 1/17 V γ 2V δ 2 T cell numbers to also drop to low levels (compare Fig. 5A, condition 9 to Fig. 3A, condition 9). These results support part of our hypothesis – that the expansion of T γ δ 1/17 V γ 2V δ 2 T cells from adult PBMC required IL-23 and IL-1 β – however we were surprised to see that TGF- β was also important for expansion of these cells.

A minority of adult donors (4 out of 11) had detectable expansions in T γ δ 17 V γ 2V δ 2 T cells. These individuals were analyzed separately, and the cytokine requirements for T γ δ 17 were compared with those for T γ δ 1/17 (Supplemental Fig. 3). As with the expansions of T γ δ 1/17 from the other adult donors, statistically significant increases in T γ δ 17 cells in these four donors were observed for the combination IL-23, IL-1 β , and TGF- β in the presence or absence of IL-6. This same combination (IL-23, IL-1 β , and TGF- β , in the absence of IL-6) also optimally expanded T γ δ 1/17 cells in these donors. Together, these results suggest that adult peripheral blood IL-17A⁺ V γ 2V δ 2 T cells, be they T γ δ 17 or T γ δ 1/17, have similar requirements for IL-23, IL-1 β , and TGF- β .

Although we were unable to expand significant numbers of T γ δ 22 V γ 2V δ 2 T cells from cord blood, significant numbers of T γ δ 22 V γ 2V δ 2 T cells were easily expanded from adult peripheral blood. IL-23, IL-1 β , and IL-6 were the minimal cytokines required for statistically significant expansion of T γ δ 22 V γ 2V δ 2 T cells (Fig. 5A, bottom, condition 5 and Fig. 5B). Addition of TGF- β to these cytokines enhanced expansion of T γ δ 22 V γ 2V δ 2 T cells (Fig. 5A, bottom, condition 6). As with neonatal V γ 2V δ 2 T cells after *in vitro* expansion and adult V γ 2V δ 2 T cells *ex vivo*, V γ 2V δ 2 T cells producing both IL-22 and IL-17A were extremely rare among expanded V γ 2V δ 2 T cells (Fig. 5B, bottom panel). These results provide further evidence that T γ δ 22 are a separate population distinct from the T γ δ 1/17 and T γ δ 17 populations.

Regulation of V γ 2V δ 2 T cells by ROR γ t and T-bet transcription factors

Differentiation of Th17 cells involves the coordinated upregulation of the key transcription factors, ROR γ t (RORC2) and ROR α , as well as the corresponding epigenetic changes to reinforce these genes and suppress others (82–84). Murine IL-17A-producing γ δ T cells are virtually absent from ROR γ t deficient mice, suggesting that IL-17A production by murine γ δ T cell requires this transcription factor. We therefore determined whether human IL-17A⁺ V γ 2V δ 2 T cells similarly express ROR γ t. We hypothesized that IL-17A⁺ V γ 2V δ 2 T cells have increased expression of ROR γ t and decreased expression of T-bet. To test this hypothesis, we performed intracellular staining for ROR γ t and T-bet on total IL-17A⁺ V γ 2V δ 2 T cells polarized from human neonates and adults. Because the *ex vivo* population of human IL-17A⁺ V γ 2V δ 2 T cells is relatively infrequent, we were not able to perform ROR γ t or T-bet staining on non-expanded V γ 2V δ 2 T cells from humans. However, because macaque blood contains a higher frequency of IL-17A⁺ V δ 2 T cells, we were able to analyze T-bet expression within monkey IL-17A⁺ V δ 2 T cells. Due to lack of confirmed antibody cross-reactivity for monkey ROR γ t we did not assess the expression of ROR γ t in monkey IL-17A⁺ V δ 2 T cells. Shown in Fig. 6A, is the staining from one representative macaque out of three studied demonstrating that IL-17A⁺, IFN- γ ⁻ V δ 2 T cells have somewhat decreased intracellular T-bet levels relative to IL-17A⁻, IFN- γ ⁺ V δ 2 T cells.

Next we wished to compare the expression ROR γ t and T-bet within expanded human IL-17A⁺ V γ 2V δ 2 T cells from cord blood. We chose to examine ROR γ t and T-bet expression on cord blood V γ 2V δ 2 T cells cultured under condition 9 (anti-IL-23, IL-1 β , IL-6, and TGF- β) because this combination yielded significant expansions of both T γ δ 17 and T γ δ 1/17 T cells (Fig. 3B, bottom). Total neonatal IL-17A⁺ cells (T γ δ 17 and T γ δ 1/17), expressed significantly more ROR γ t than IL-17A⁻ V γ 2V δ 2 T cells under the same culture conditions (Fig. 6B, left panels). We failed to detect statistically significant differences in the expression of T-bet by total IL-17A⁺ V γ 2V δ 2 T cells from cord blood. Similarly, total adult IL-17A⁺ V γ 2V δ 2 T cells cultured under condition 7 (IL-23, IL-1 β , anti-IL-6, TGF- β) expressed significantly more ROR γ t than IL-17A⁻ V γ 2V δ 2 T cells expanded from the same donors under the same culture conditions (Fig. 6B, right panels). And, as before, the expression of T-bet did not significantly differ between adult IL-17A⁺ and IL-17A⁻ V γ 2V δ 2 T cells. These data suggest that like murine IL-17A⁺ γ δ T cells, human IL-17A⁺ V γ 2V δ 2 T

cells up-regulate the ROR γ t transcription factor consistent with it playing a role in IL-17A production by human $\gamma\delta$ T cells.

Discussion

Although $\gamma\delta$ T cells are a major source of IL-17A in mice, the role of $\gamma\delta$ T cells in IL-17A production in humans has been unclear. In this study, we show that significant numbers of adult human blood V γ 2V δ 2 T cells produce IL-17A or IL-22 ex vivo although few produce both. IL-17A-producing adult V γ 2V δ 2 T cells are primarily memory cells distributed among early (central), intermediate, and late (effector) subsets similar to T γ δ 1 V γ 2V δ 2 T cells. Differentiation of IL-17A-producing cells from neonatal naive V γ 2V δ 2 T cells required the inflammatory cytokines, IL-1 β and IL-6, coupled with TGF- β but not IL-23. The addition of IL-23 favored differentiation to IL-17A-producing cells that also produced IFN- γ . Adult memory V γ 2V δ 2 T cells required IL-23 for maximal expansion of IL-17A-producing cells but did not require IL-6. For both neonatal and adult V γ 2V δ 2 T cells, cells producing IL-17A had higher levels of ROR γ t compared with cells that did not produce IL-17A, establishing a role for ROR γ t in IL-17A production. Although the frequency of IL-17A- and IL-22-producing V γ 2V δ 2 T cells are low, they could be significant sources of IL-17A and IL-22.

Consistent with this hypothesis, in mice, $\gamma\delta$ T cells and other unconventional T cells are important sources of IL-17A and IL-22. Murine $\gamma\delta$ T cells are rapidly mobilized and secrete IL-17A in response to a range of different pathogens including *Mycobacterium tuberculosis* (32), *M. bovis BCG* (85), *Listeria monocytogenes* (34), *Escherichia coli* (33), and *Salmonella enterica* serovar Enteritidis (86). The production of IL-17A by murine $\gamma\delta$ T cells precedes that of Th17 CD4 T cells (33) and IL-17A-producing $\gamma\delta$ T cells are protective in most infections (34,85). Besides their protective roles, murine IL-17A-producing $\gamma\delta$ T cells play pathologic roles in collagen-induced arthritis (87), experimental autoimmune uveitis (88), and experimental autoimmune encephalitis (36).

In mice, IL-17A-producing $\gamma\delta$ TCR subsets include invariant V γ 6V δ 1 T cells in the peritoneum of mice with bacterial infections (33), oligoclonal V γ 4V δ 4 T cells in collagen-induced arthritis (87), and V γ 4⁺ T cells in experimental autoimmune encephalitis (36). None of the antigens are known for these $\gamma\delta$ subsets. Additionally, some T10/T22-specific $\gamma\delta$ T cells, which express TCRs containing a CDR3 δ motif (89), produce IL-17A. Murine $\gamma\delta$ T cells acquire IL-17A potential in the neonatal thymus (90). For T10/T22-specific $\gamma\delta$ T cells, this was dependent on not encountering T10/22 in the thymus (89). In the periphery, $\gamma\delta$ T cells can be rapidly induced by IL-23 and IL-1 β (but not IL-6 and/or TGF- β in the presence or absence of TLR/Dectin-1 ligands to produce IL-17A, IL-21, and/or IL-22 (32,33,36,91,92). Although this is without apparent $\gamma\delta$ TCR triggering, this ability to release IL-17A could be due to the constant low-level activation of these cells by an endogenous TCR ligand since their antigen specificity is unknown. Nonetheless, IL-17A-production by murine $\gamma\delta$ T cells appears independent of exogenous antigen.

A second type of unconventional T cell is the invariant NKT cell (iNKT). Murine iNKT producing IL-17A develop in the adult thymus, independently of IL-6 and constitute <1% of iNKT cells in the spleen and liver but are greatly enriched in lymph nodes (25,26,93). IL-17A release by iNKT17 cells is stimulated by exposure to exogenous lipid antigens. However, similar to murine $\gamma\delta$ T cells, IL-23 alone (but possibly with self lipid antigens) also stimulates IL-17A. The combination has a synergistic effect (25). Human blood iNKT cells also produce IL-17A in response to IL-23 and agonistic anti-CD3 (25).

Unlike $\alpha\beta$ T cells which can recognize only a single pathogen's peptide/MHC complex, $V\gamma 2V\delta 2$ T cells are specific for many pathogens by virtue of their recognition of essential prenyl pyrophosphates. Almost all adult $V\gamma 2V\delta 2$ T cells recognize prenyl pyrophosphate antigens (for example, 91 out of 94 (97%) adult $V\gamma 2V\delta 2$ T cell clones responded (53,94,95)) due to the extensive use of germline encoded regions of the $V\gamma 2V\delta 2$ TCR for antigen recognition (96) and the selection for $J\gamma 1.2$ and a hydrophobic $V\delta 2$ CDR3 residue that occurs during infancy (51,94,97–99). The frequency of CD4 or CD8 $\alpha\beta$ T cells specific for a particular peptide/MHC complex among naïve cells is usually very low – 1:158,000–1:1,875,000 for CD4 (100) and 1:33,000–1:164,000 (4/6 were >1:142,000) for CD8 (101). In contrast, because all $V\gamma 2V\delta 2$ T cells respond to prenyl pyrophosphates, the frequency of reactive cells is high at 1 in 19 T cells (Table I). On average, 1.1% of $V\gamma 2V\delta 2$ T cells (1 in 2,762 T cells) produce IL-17A. The frequency of IL-17A- and IL-22-producing $V\gamma 2V\delta 2$ T cells maybe much higher in peripheral lymph nodes, in mucosa, or in the peritoneum as has been found in mice (35). Thus, for primary infections, $V\gamma 2V\delta 2$ T cells and other unconventional T cells may be important sources of early IL-17A and IL-22 until naïve CD4 and CD8 $\alpha\beta$ T cells can be expanded and differentiated into memory Th17/Tc17 and Th22/Tc22 cells. This suggests that $\gamma\delta$ T cells, like NK cells, may help bridge the gap between early innate and later adaptive immune responses.

IL-22 is an important cytokine produced by conventional and unconventional T cells in the Th17 lineage. We now describe $V\gamma 2V\delta 2$ T cells that produce IL-22 but not IL-17A. The existence of IL-22 single positive (Th22 cells) and IL-17A single positive cells has been described for CD4 $\alpha\beta$ T cells (59,69,70,102–104). The differentiation of T cells that exclusively produce IL-22 likely reflects the priming conditions during initial antigen exposure. For instance, naïve CD4 T cells primed by skin Langerhans cells or dermal dendritic cells preferentially differentiate into cells that exclusively produce IL-22 and not IL-17A or IFN- γ (102). Plasmacytoid dendritic cells also preferentially differentiate naïve CD4 T cells to Th22 cells (70). Although both $\alpha\beta$ and $V\gamma 2V\delta 2$ T cells have subsets that produce exclusively IL-17A or IL-22, only 2.7% of IL-22-producing $V\gamma 2V\delta 2$ T cells co-produce IL-17A whereas 10.4–18.2% of IL-22-producing CD4 $\alpha\beta$ T cells co-produce IL-17A (70) suggesting increased specialization for blood $V\gamma 2V\delta 2$ T cells.

Since essentially all adult $V\gamma 2V\delta 2$ T cells are memory cells, the relatively high frequency of $T\gamma\delta 22$ $V\gamma 2V\delta 2$ T cells (1 out of every 1,864 T cells) suggests that they make significant contributions to early IL-22 production during infections. In this role, they may function like innate $NKp44^+$ IL-22 $^+$ NK cells that are enriched at mucosal surfaces (105,106). Production of IL-22 mediates mucosal host defense against bacteria (107,108). Binding of IL-22 to IL-22 receptors expressed by epithelial cells of the digestive tract, skin, and lungs induces antimicrobial peptides, acute phase reactants, and matrix-metalloproteinases (109,110). Th22 T cells also produce fibroblast growth factors, CCL7 and CCL15 chemokines, and express the skin homing receptor, CCR10 (59). Similarly, $V\gamma 2V\delta 2$ T cells have been shown to produce FGF-7 (111) and connective tissue growth factor (112). Murine $\gamma\delta$ dendritic epidermal cells are primary sources of keratinocyte growth factor and IGF-1 and the presence of murine $\gamma\delta$ T cells speed wound healing in the skin and gut (113–116). These findings demonstrate parallels between murine and human $\gamma\delta$ T cells and suggest that subsets of both function as specialized $T\gamma\delta 22$ cells.

In contrast to humans, adult rhesus macaques exhibited ~5-fold higher frequencies of IL-17A-producing $V\gamma 2V\delta 2$ cells than humans. Moreover, the majority of rhesus macaque $V\gamma 2V\delta 2$ T cells exclusively produced IL-17A without IFN- γ . One possible explanation for this finding is the difference in $\gamma\delta$ TCR repertoires between humans and rhesus macaques. Unlike adult humans where $V\gamma 2V\delta 2$ T cells predominate (54% $V\gamma 2V\delta 2$ versus 15% $V\delta 1$ T cells), adult rhesus macaques exhibit a predominance of $V\delta 1$ T cells (24% $V\gamma 2V\delta 2$ versus

33% V δ 1 T cells) (58). This is similar to neonatal human $\gamma\delta$ T cells where human V γ 1 T cells constitute 40% of total $\gamma\delta$ T cells while V γ 2V δ 2 T cells only constitute 9% (51). Between 1–10 years of age, an expansion of human V γ 2V δ 2 T cells occurs due to environment factors causing a predominance of the V γ 2V δ 2 subset (75). Since the maturity of the rhesus monkeys and humans studied were similar, this lack of predominance of V γ 2V δ 2 T cells in rhesus macaques may reflect their housing in specific pathogen free environments where they are sheltered from significant infectious agents. As a consequence, their V γ 2V δ 2 T cells may be less antigen experienced allowing the persistence of naïve and early memory V γ 2V δ 2 T cells that can be differentiated or maintained as T $\gamma\delta$ 17 cells rather than converted to T $\gamma\delta$ 1/17 or T $\gamma\delta$ 1 cells.

Unexpectedly, the addition of exogenous IL-23 to IL-1 β , IL-6, and TGF- β appeared to inhibit T $\gamma\delta$ 17 development in neonates. The likely explanation for this observation is that IL-23 promotes the conversion of T $\gamma\delta$ 17 cells into T $\gamma\delta$ 1/17 cells. A similar effect has been seen for Th17 CD4 clones where IL-23 converted a subset of Th17 cells into Th1/17 cells (117). The same study found an even higher degree of conversion to Th1/17 with IL-12. Further studies are needed to confirm that T $\gamma\delta$ 17 V γ 2V δ 2 T cells give rise to T $\gamma\delta$ 1/17 in the presence of IL-23/IL-12. If this is so, then decreasing exposure to IL-12 or IL-23 may suppress conversion to T $\gamma\delta$ 1/17 and help maintain the T $\gamma\delta$ 17 phenotype.

Adult V γ 2V δ 2 T cells required IL-23 in addition to IL-1 β and TGF- β but not IL-6 for maximal expansion of T $\gamma\delta$ 1/17 (and in some donors, T $\gamma\delta$ 17) cells. This is in contrast to neonatal V γ 2V δ 2 T cells which required IL-6 with IL-1 β and TGF- β but not IL-23. The difference in cytokine requirements is consistent with the hypothesis that naïve V γ 2V δ 2 T cells (present in neonates), like naïve CD4 T cells, require IL-6 for initial up-regulation of IL-23R, ROR γ t, and ROR α (14,15,118), whereas memory IL-23R⁺ V γ 2V δ 2 T cells (present in adults) require only IL-23, IL-1 β , and TGF- β for re-expression of IL-17A. Similarly, production of IL-17A by murine IL-23R⁺ memory $\gamma\delta$ T cells has been shown to require only IL-23 and IL-1 β in the presence of fetal bovine serum, which contains TGF- β (36). TGF- β is likely not directly required for T $\gamma\delta$ 17 or T $\gamma\delta$ 22 cell differentiation but instead functions to inhibit differentiation to T $\gamma\delta$ 1 and T $\gamma\delta$ 2 lineages (19). In both neonates and adults, V γ 2V δ 2 T cells producing IL-17A had higher levels of ROR γ t compared with cells not producing IL-17A suggesting a role for ROR γ t in IL-17A production by $\gamma\delta$ T cells whereas levels of the Th1 transcription factor, T-bet did not significantly differ between IL-17A⁺ and IL-17A⁻ cells.

The conditions favoring IL-17A-producing V γ 2V δ 2 T cells also favored IL-22-producing V γ 2V δ 2 T cells although few produced both IL-17A and IL-22 (Fig. 3A, 4A, and S1). Th22 CD4 $\alpha\beta$ T cells optimally differentiate from naïve precursors in the presence of IL-6 and TNF- α . The combination of IL-1 β , IL-6, and TNF- α favors development of IL-22⁺, IL-17A⁺ CD4 $\alpha\beta$ T cells (70) whereas adding TGF- β to these cytokine combinations inhibits Th22 differentiation (70,119). In contrast to Th17, Th22 cells were characterized as expressing FOXO4 and lower levels of ROR γ t, high levels of the skin homing receptors, CCR4, CCR6, and CCR10, and cytokines/chemokines such as FGFs, CCL7, and CCL15 (59,70). Unlike some Th17 cells, Th22 T cells retained their ability to produce IL-22 upon repeated cell division (59,70) suggesting that the Th22 subset is a distinct functional subset that may be more stable than the Th17 subset. Our data suggests that T $\gamma\delta$ 22 and T $\gamma\delta$ 17 T cells, like some Th17 and Th22 CD4 $\alpha\beta$ T cells (59,70,120), belong to separate subsets that do not produce both cytokines. However, both T $\gamma\delta$ 17 and T $\gamma\delta$ 22 T cells can acquire IFN- γ production. Future experiments are needed to characterize the plasticity of T $\gamma\delta$ 22 and T $\gamma\delta$ 17 V γ 2V δ 2 T cells.

Based on our findings, we propose the following model for the development of $T\gamma\delta 17$ and $T\gamma\delta 1/17$ $V\gamma 2V\delta 2$ T cells (Fig. 7). In neonates and infants, microbial infections polarize some $V\gamma 2V\delta 2$ T cells into memory $T\gamma\delta 17$ through the actions of innate cell derived IL-6, IL-1 β , and TGF- β . Several studies have demonstrated that neonatal innate cells, including professional antigen presenting cells, produce insufficient levels of IL-12 to program Th1 effector T cells (121–123), and instead make IL-23, IL-1 β , and IL-6 (124,125). We propose that these cytokines, in combination with bacteria or parasite derived HMBPP, differentiate naïve $V\gamma 2V\delta 2$ T cells into $T\gamma\delta 17$ T cells that produce IL-17A and express ROR γ t, IL-23R, and IL-12R. Our data suggests that the presence of IL-23 (or possibly IL-12) causes $T\gamma\delta 17$ $V\gamma 2V\delta 2$ T cells to acquire IFN- γ production through the upregulation of T-bet, thereby converting to $T\gamma\delta 1/17$ cells. Since IL-12 production increases with age, responses to subsequent childhood infections are dominated by IL-12, converting most of the responding $T\gamma\delta 17$ to memory $T\gamma\delta 1/17$ by early adulthood (Fig. 7).

$V\gamma 2V\delta 2$ T cells are of considerable interest because many infections lead to large expansions of these cells (reviewed in 41) and cancer immunotherapies specifically expanding $V\gamma 2V\delta 2$ T cells have shown effectiveness against various tumors (126–129). $V\gamma 2V\delta 2$ T cells are attractive agents for cancer immunotherapy because they are not MHC-restricted like conventional T cells so a single vaccine can be used in all individuals regardless of MHC haplotype. Moreover, $V\gamma 2V\delta 2$ T cells are specifically stimulated by prenyl pyrophosphates and bisphosphonates; both of which are well tolerated *in vivo*. Once activated, $V\gamma 2V\delta 2$ T cells are broadly reactive to cancer cells of many tissue origins and to bacterially- and protozoan-infected cells while sparing normal cells. $V\gamma 2V\delta 2$ T cells have traditionally been considered Th1-like cytotoxic T cells. However, we now show that $V\gamma 2V\delta 2$ T cells can differentiate into $T\gamma\delta 17$ and $T\gamma\delta 22$ lineage cells. Others have demonstrated $V\gamma 2V\delta 2$ T cells with characteristics of follicular homing CD4 $\alpha\beta$ T cells (57,130) and regulatory CD25⁺ CD4 $\alpha\beta$ T cells (131). Taken together, these findings indicate that $V\gamma 2V\delta 2$ T cells exhibit more functional plasticity than previously appreciated. Understanding their plasticity will enable researchers to optimize existing therapies for the treatment of cancers and infections and to develop new therapies utilizing these alternative functional subsets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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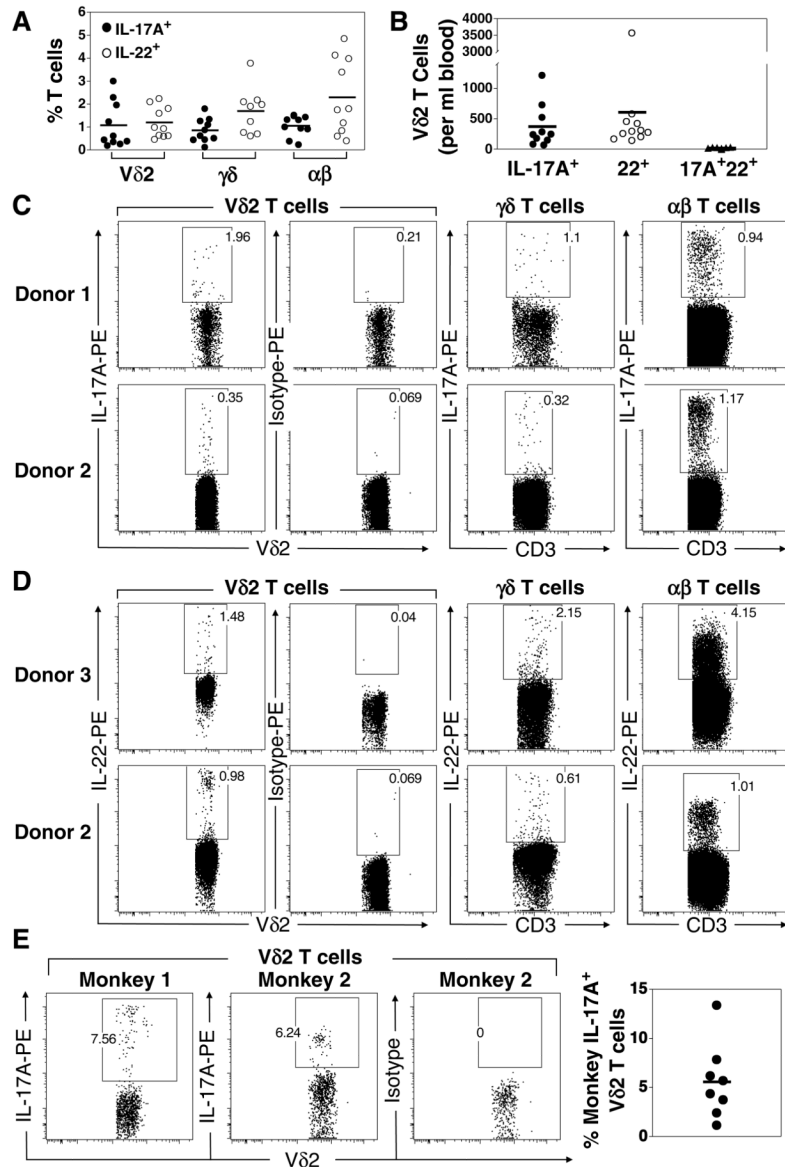


FIGURE 1. Frequency of $V\gamma 2V\delta 2$ T cells producing IL-17A and IL-22 in adult human and rhesus macaque donors

A. PBMC from 10 normal donors were stimulated with PMA and ionomycin and intracellular cytokine staining for IL-17A and IL-22 was performed. Viable T cells were gated using live/dead blue and anti-CD3 after which the different T cell subsets discriminated using anti- $V\delta 2$ (to identify $V\gamma 2V\delta 2$ T cells) and anti-pan $\gamma\delta$ (to identify total $\gamma\delta$ T cells). $\alpha\beta$ T cells were defined as $CD3^+, \gamma\delta^-$. B. Numbers of IL-17A⁺ $V\gamma 2V\delta 2$, IL-22⁺ $V\gamma 2V\delta 2$ and IL-17A⁺, IL-22⁺ $V\gamma 2V\delta 2$ T cells per milliliter of blood were calculated (Table 1). C. Representative IL-17A staining for $V\gamma 2V\delta 2$ T cells (abbreviated $V\delta 2$ T cells), total $\gamma\delta$ T cells, and total $\alpha\beta$ T cells. D. Representative IL-22 staining for $V\gamma 2V\delta 2$ T cells (abbreviated $V\delta 2$ T cells), total $\gamma\delta$ T cells, and total $\alpha\beta$ T cells. E. Representative IL-17A staining and average frequency of IL-17A⁺ $V\delta 2$ T cells among eight rhesus macaques. Each point refers to one donor and bars depict means.

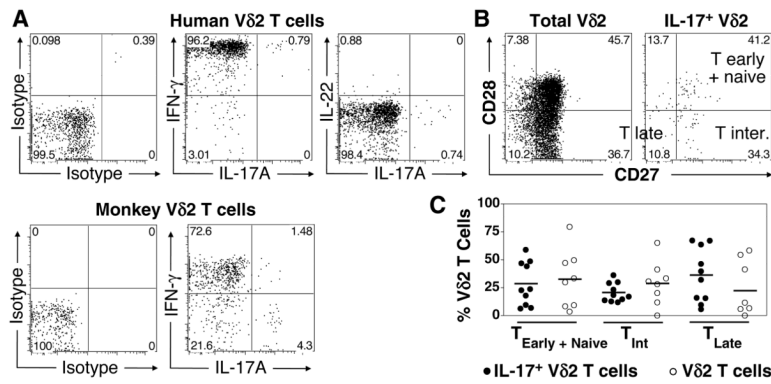


FIGURE 2. Cytokine profile and memory phenotype of IL-17A⁺ V γ 2V δ 2 T cells from adult human and monkey donors

A. IFN- γ production by IL-17A-producing V δ 2 T cells. PBMC were stimulated with PMA and ionomycin and stained intracellularly for IL-17A, IFN- γ , and IL-22. Shown is a representative human (top) and monkey (bottom) donor. **B.** Representative surface staining for memory markers, CD27 and CD28 on total V γ 2V δ 2 T cells or IL-17A⁺ gated V γ 2V δ 2 T cells. **C.** Frequency of total human V γ 2V δ 2 T cells or IL-17A⁺ gated V γ 2V δ 2 T cells belonging to T early + naïve (CD27⁺, CD28⁺), T intermediate (CD27⁺, CD28⁻) or T late (CD27⁻, CD28⁻) memory subsets. Each point represents one donor and bars depict means.

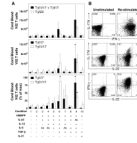


FIGURE 3. IL-1 β , TGF- β , and IL-6 induce maximal polarization of IL-17A⁺ neonatal CD4⁻ V γ 2V δ 2 T cells upon antigen stimulation with HMBPP

Umbilical cord blood mononuclear cells were expanded in the presence or absence of HMBPP, IL-23, IL-1 β , TGF- β , IL-6, neutralizing anti-IL-6, or neutralizing anti-IL-23 for 13 days ($n = 8$ individuals for IL-17A data and $n = 4$ for IL-22 data). IL-2 was added on day 3. On the final day, cells were restimulated with PMA and ionomycin and intracellular staining for IL-17A, IL-22, and IFN- γ performed. Expanded cord blood CD4⁻ V γ 2V δ 2 T cells (defined as V δ 2⁺, CD3⁺, CD4⁻) were divided into IFN- γ ⁻, IL-17A⁺ V γ 2V δ 2 T cells (termed T γ δ17), IFN- γ ⁺, IL-17A⁺ V γ 2V δ 2 T cells (termed T γ δ1/17), IFN- γ ⁺, IL-17A⁻ V γ 2V δ 2 T cells (termed T γ δ1), and IFN- γ ^{+/-}, IL-22⁺ V γ 2V δ 2 (termed T γ δ22). **A.** (Top) Median number of total IL-17A⁺ CD4⁻ V γ 2V δ 2 T cells (combined T γ δ1/17 and T γ δ17) or T γ δ22 V γ 2V δ 2 T cells among total CD4⁻ V γ 2V δ 2 T cells for each cytokine condition. (Middle) Median number of T γ δ17 or T γ δ1/17 CD4⁻ V γ 2V δ 2 T cells among total CD4⁻ V γ 2V δ 2 T cells for each condition. (Bottom) Median percent of maximum T γ δ17 or T γ δ1/17 CD4⁻ V γ 2V δ 2 T cells expanded for each condition. **B.** Representative cytokine staining on viable CD4⁻ V γ 2V δ 2 T cells expanded in the presence of HMBPP, IL-1 β , TGF- β , IL-6, and anti-IL-23, either unstimulated (left) or restimulated with PMA and ionomycin (right). Bars depict medians and error bars depict median absolute error. * $p < 0.05$, Kruskal-Wallis comparison with condition 2.

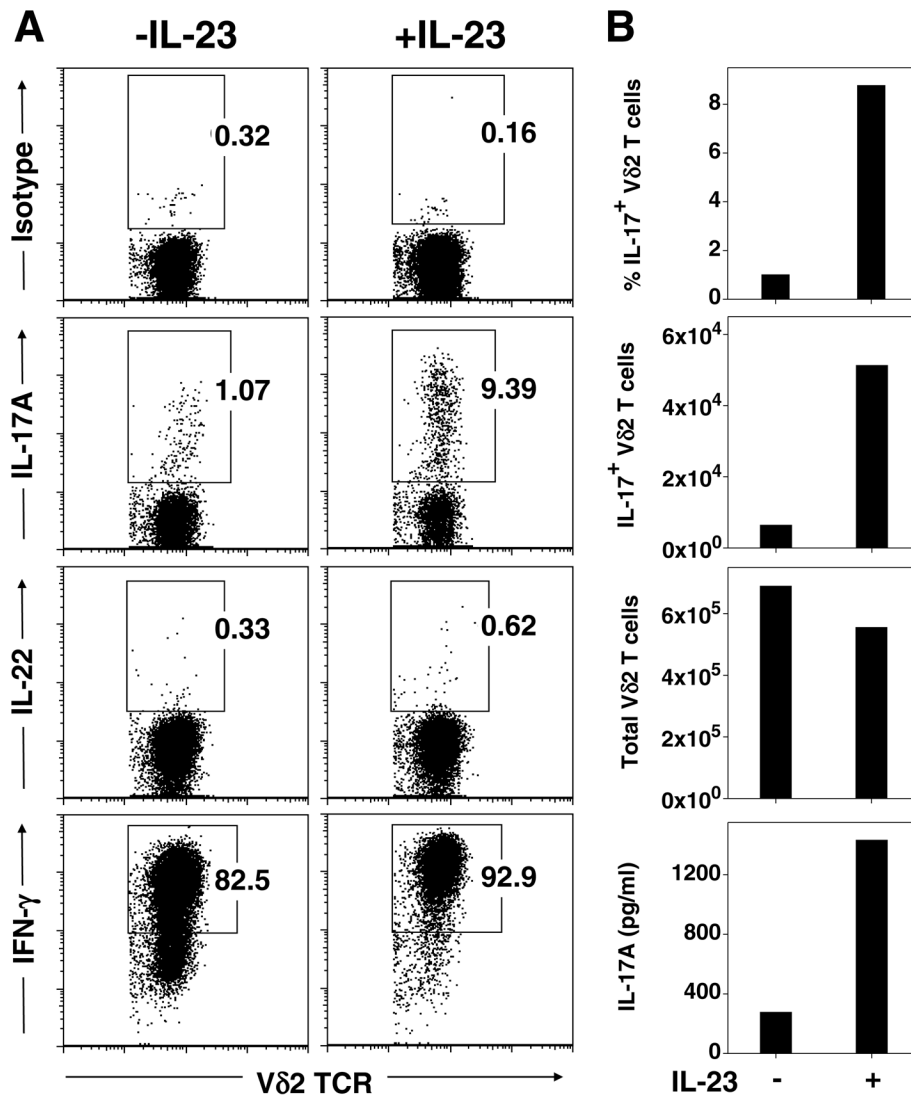


FIGURE 4. IL-23 is required for expansion of adult IL-17⁺ V γ 2V δ 2 T cells

IL-17A-producing V γ 2V δ 2 T cells, in serum-supplemented media, were measured in PBMC after expansion with HMBPP, IL-1 β , IL-6, neutralizing anti-IL-4, and neutralizing anti-IFN- γ in the presence or absence of IL-23. IL-2 was added on day 3. On day 12, cells were re-stimulated with PMA and ionomycin, after which the supernatants and cells were harvested for analysis. Expanded V γ 2V δ 2 T cells were defined as V δ 2⁺, CD3⁺. Representative of 2 donors. **A**. Cytokine profile of expanded V γ 2V δ 2 T cells. Intracellular staining for IL-17A, IL-22, and IFN- γ , (or isotype control) in the presence or absence of IL-23 is shown. **B**. Percent and total number of IL-17⁺ V γ 2V δ 2 T cells in the presence or absence of exogenous IL-23 (top two panels). Total expanded V γ 2V δ 2 T cells on day 12 (third panel). Total IL-17A protein released into culture as determined by ELISA (bottom panel).

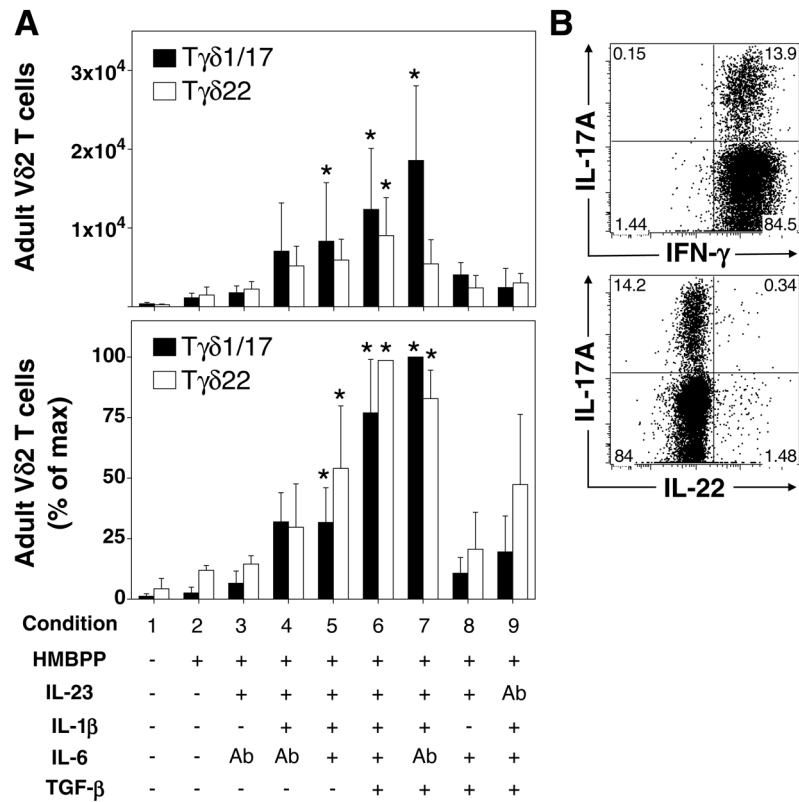


FIGURE 5. IL-23, IL-1 β , and TGF- β are sufficient for polarization of adult IL-17A⁺ V γ 2V δ 2 T cells after stimulation with HMBPP

Total PBMC, from 10 donors, were cultured in the presence or absence of HMBPP, IL-23, IL-1 β , TGF- β , IL-6, neutralizing anti-IL-6, or neutralizing anti-IL-23 for seven days. IL-2 was added on day 3. On the seventh day, cells were re-stimulated with PMA and ionomycin and intracellular staining for IL-17A, IL-22, and IFN- γ was performed. Expanded PBMC V γ 2V δ 2 T cells (defined as V δ 2⁺, CD3⁺, CD4⁻) could be divided into IFN- γ ⁺, IL-17A⁺ V γ 2V δ 2 T cells (termed T γ δ1/17), IFN- γ ⁺, IL-17A⁻ V γ 2V δ 2 T cells (termed T γ δ1), and IFN- γ ^{+/-}, IL-22⁺ V γ 2V δ 2 (termed T γ δ22). No IFN- γ ⁻, IL-17A⁺ V γ 2V δ 2 T cells (T γ δ17) were detected in these adult donors. **A.** (Top) Median number of T γ δ1/17 or T γ δ22 V γ 2V δ 2 T cells among total V γ 2V δ 2 T cells for each cytokine condition. (Bottom) Median percent of maximum T γ δ1/17 V γ 2V δ 2 T cells expanded for each cytokine condition. **B.**

Representative cytokine staining on V γ 2V δ 2 T cells expanded in the presence of HMBPP, IL-23, IL-1 β , TGF- β , and anti-IL-6, and restimulated with PMA and ionomycin. Bars depict medians and error bars depict median absolute error. * $p < 0.05$, Kruskal Wallis comparison with condition 2.

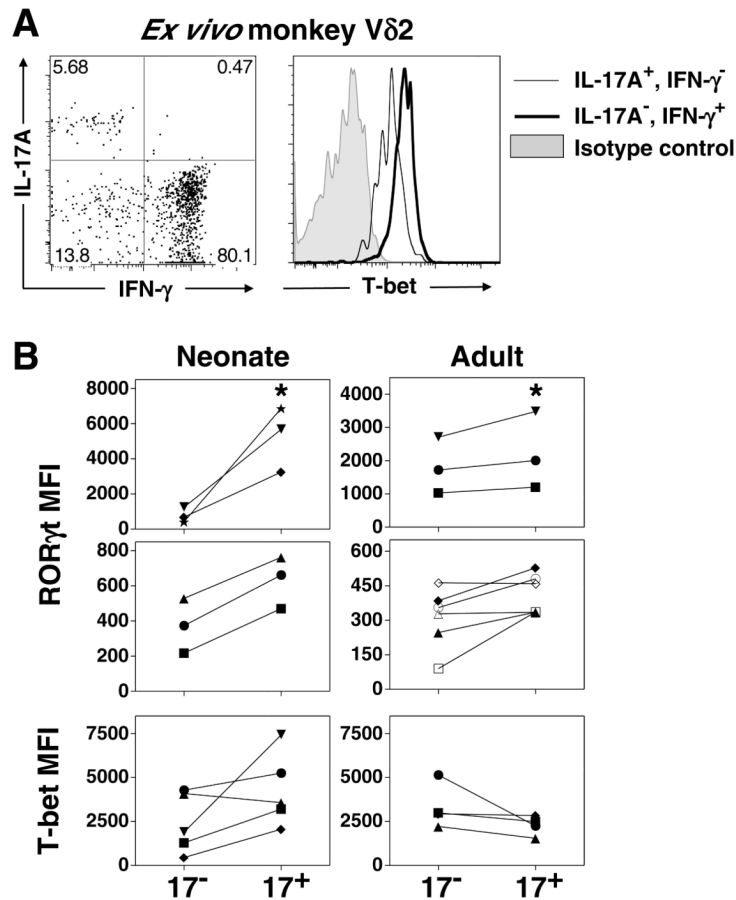
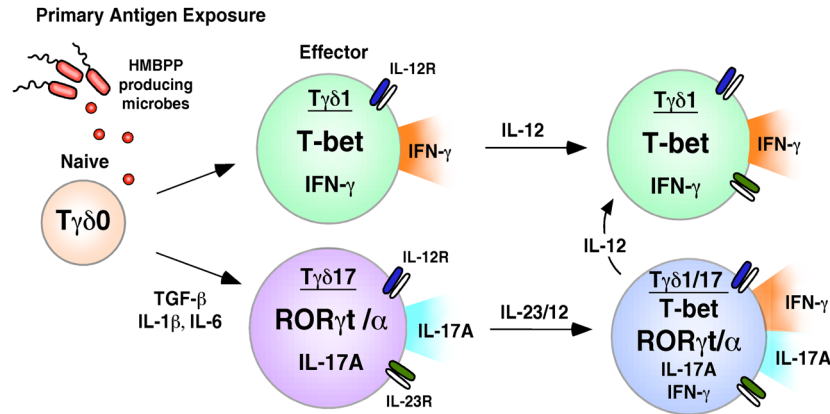


FIGURE 6. Expression of RORγt and T-bet by IL-17A⁺ Vγ2Vδ2 T cells

A. Representative staining for T-bet on monkey peripheral blood Vδ2 T cells, segregated into IL-17A⁺, IFN-γ⁻ Vδ2 T cells (Tγδ17) or IL-17A⁻, IFN-γ⁺ Vδ2 T cells (Tγδ1). Represents one of three monkeys examined. PBMC were isolated and stimulated with PMA and ionomycin and intracellular staining for IL-17A, IFN-γ, and T-bet performed. B. (Neonate, left) Cord blood mononuclear cells were polarized with HMBPP for 13 days in the presence of IL-1β, IL-6, TGF-β, and anti-IL-23, and (Adult, right) adult PBMC were polarized with HMBPP for 7 days in the presence of IL-1β, IL-23, TGF-β, and anti-IL-6. On the final day, cells were restimulated with PMA and ionomycin, surface stained for Vδ2 and CD3, and intracellularly stained for IL-17A, RORγt, and T-bet. Vγ2Vδ2 T cells were segregated into IL-17A⁺ and IL-17A⁻ and the MFI for each transcription factor minus the MFI of the respective isotype control is shown. Because donors had variable baseline RORγt staining, the donors are segregated into two graphs to accommodate the different magnitudes exhibited. Note that cells were not segregated based on IFN-γ production, therefore the neonatal IL-17A⁺ fraction refers to the sum of Tγδ1/17 and Tγδ17. * $p < 0.05$, Kruskal Wallis comparison with IL-17A⁻ group.

A. Neonates / Infants



B. Adults

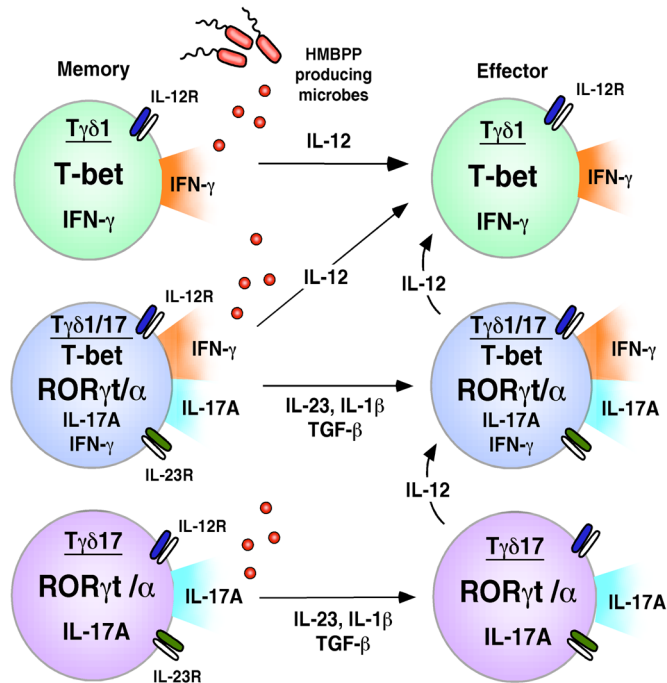


FIGURE 7. Steps in the differentiation and expansion of neonatal and adult $T\gamma\delta 17$ and $T\gamma\delta 1/17$ $V\gamma 2V\delta 2$ T cells

A. Neonates/Infants. Naïve $V\gamma 2V\delta 2$ T cells present in neonates are polarized to the $T\gamma\delta 17$ phenotype by antigen activation in the presence of IL-6, IL-1 β , and TGF- β . These early $T\gamma\delta 17$ cells are characterized by elevated expression of ROR γ t, IL-17A production, and minimal expression of IFN- γ and T-bet. The $T\gamma\delta 17$ cells up-regulate IL-23R (and likely IL-12R) enabling them to maintain their $T\gamma\delta 17$ phenotype in the presence of IL-23, IL-1 β , and TGF- β or to convert to a $T\gamma\delta 1/17$ phenotype in the presence of IL-23 or IL-12. **B. Adults.** Most adult $V\gamma 2V\delta 2$ T cells are memory cells and include small but significant populations of $T\gamma\delta 1/17$ and $T\gamma\delta 17$ cells. Expansion of adult memory $T\gamma\delta 1/17$ and $T\gamma\delta 17$ cells by HMBPP requires IL-23 in addition to IL-1 β and TGF- β but not IL-6. $T\gamma\delta 1/17$ and $T\gamma\delta 17$ likely have limited persistence and are either short-lived effector populations or are converted to $T\gamma\delta 1$ through the effects of IL-12.

Table 1

Frequency of adult IL-17A- and IL-22-producing human V γ 2V δ 2 T cells[†]

Donor	PBMC per ml blood	% V δ 2 among CD3 T cells	% IL-17A ⁺ among V δ 2	% IL-22 ⁺ among V δ 2	Frequency IL-17A ⁺ V δ 2 among CD3 T cells	IL-17A ⁺ V δ 2 per ml blood	Frequency IL-22 ⁺ V δ 2 among CD3 T cells	IL-22 ⁺ V δ 2 per ml blood
1	1.70 \times 10 ⁶	3.99	0.61	0.64	1:4,109	239	1:3,916	256
2	2.36 \times 10 ⁶	1.81	1.96	1.60	1:2,819	283	1:3,456	142
3	1.45 \times 10 ⁶	2.04	2.29	2.24	1:2,141	522	1:2,188	421
4	1.93 \times 10 ⁶	3.58	0.44	0.93	1:6,348	147	1:3,004	309
5	3.33 \times 10 ⁶	4.18	0.19	0.46	1:12,591	67	1:5,201	167
6	1.89 \times 10 ⁶	6.88	0.26	0.62	1:5,590	246	1:2,344	454
7	3.57 \times 10 ⁶	23.9	0.35	0.98	1:1,195	1213	1:427	3567
8	1.23 \times 10 ⁶	3.68	3.00	2.10	1:906	728	1:1,294	580
9	2.25 \times 10 ⁶	1.24	0.38	1.81	1:21,222	80	1:4,456	293
10	2.82 \times 10 ⁶	1.23	1.23	0.59	1:6,610	367	1:13,780	203
Mean \pm SEM	2.25 \pm 2.46 \times 10 ⁶	5.25 \pm 2.14	1.07 \pm 0.32	1.20 \pm 0.21	1:2,762 \pm 1:9,047	389 \pm 112	1:1,864 \pm 1:4,775	639 \pm 328

[†] PBMC were harvested from normal adult donors, counted, and stimulated with 50 ng/ml PMA and 2 μ g/ml Ionomycin for 4–6 h in the presence of GolgiSTOP (monensin). PBMC were then stained with Live/Dead Blue (Invitrogen) followed by APC-Cy7-conjugated anti-CD3, FITC conjugated anti-V δ 2 or FITC-conjugated anti-TCR $\gamma\delta$. The cells were then washed, fixed, and permeabilized using the BD Cytfix/Cytoperm Kit and then intracellularly stained with either PE-conjugated anti-IL-17A alone or PE-conjugated anti-IFN- γ or PE-conjugated anti-IL-22 combined with PerCP-Cy5.5-conjugated anti-IL-17A.