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Cytokine Requirements for the Differentiation and Expansion of IL-17A– and IL-22–Producing Human Vγ2Vδ2 T Cells

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Human γδ T cells expressing the Vγ2Vδ2 TCR play important roles in immune responses to microbial pathogens by monitoring prenyl pyrophosphate isoprenoid metabolites. Most adult Vγ2Vδ2 cells are memory cytotoxic cells that produce IFN-γ. Recently, murine γδ T cells were found to be major sources of IL-17A in antimicrobial and autoimmune responses. To determine if primate γδ T cells play similar roles, we characterized IL-17A and IL-22 production by Vγ2Vδ2 cells. IL-17A–producing memory Vγ2Vδ2 cells exist at low but significant frequencies in adult humans (1:2762 T cells) and at even higher frequencies in adult rhesus macaques. Higher levels of Vγ2Vδ2 cells produce IL-22 (1:1864 T cells), although few produce both IL-17A and IL-22. Unlike adult humans, in whom many IL-17A$^+$ Vγ2Vδ2 cells also produce IFN-γ (Tγδ1/17), the majority of adult macaques IL-17A$^+$ Vδ2 cells (Tγδ17) do not produce IFN-γ. To define the cytokine requirements for Tγδ17 cells, we stimulated human neonatal Vγ2Vδ2 cells with the bacterial Ag, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate, and various cytokines and mAbs in vitro. We find that IL-6, IL-1β, and TGF-β are required to generate Tγδ17 cells in neonates, whereas Tγδ1/17 cells additionally required IL-23. In adults, memory Tγδ1/17 and Tγδ17 cells required IL-23, IL-1β, and TGF-β, but not IL-6. IL-22–producing cells showed similar requirements. Both neonatal and adult IL-17A$^+$ Vγ2Vδ2 cells expressed elevated levels of retinoid-related orphan receptor γt. Our data suggest that, like Th17 αβ T cells, Vγ2Vδ2 T cells can be polarized into Tγδ17 and Tγδ1/17 populations with distinct cytokine requirements for their initial polarization and later maintenance. ***The Journal of Immunology*****, 2010, 184: 7268–7280.**

Members of the IL-17 cytokine family (IL-17A through IL-17F) are proinflammatory 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-1β, 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 αβ T cells have been well described in both humans and mice, and the cytokine requirements for their generation from naive 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 (ROR)γt (*rorc*) (14) and RORα (*rora*) in naive 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 αβ 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 αβ T cells. IL-17A can also be produced by unconventional T cells, such as γδ T (reviewed in Ref. 24) and αβ NKT (25, 26), as well as macrophages (27) and neutrophils (28). Among unconventional T cells, γδ T cells represent a population of innate-like T cells that developed early in vertebrate phylogeny along with B cells and αβ T cells (29). Much like conventional CD8 αβ T cells, γδ T cells exhibit Ag specificity, robustly proliferate in response to activation, produce proinflammatory cytokines (such as TNF-α and IFN-γ), and are highly cytolytic to their targets. However, certain murine γδ T cell subsets are also potent IL-17A producers, and in some disease settings, γδ T cells constitute a greater fraction of the IL-17A–producing cells and secrete IL-17A earlier in disease than do conventional CD4 or CD8 αβ T cells (30–35). Furthermore, murine γδ T cells can produce IL-17A, IL-22, and IL-21 in response to IL-23 and IL-1β (36).

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

Despite their conservation across species, mouse and human γδ T cells demonstrate significant differences. One major difference is the existence of the Vγ2Vδ2 T cell subset (also termed Vγ9Vδ2) in humans and other primates (37), which comprises the majority (up to 90%) of circulating γδ T cells. The orthologous V genes, which rearrange to generate the Vγ2Vδ2 TCR in primates, are absent from mice and other mammals. Vγ2Vδ2 T cells are distinct from conventional αβ T cells in that they are almost exclusively memory cytotoxic T cells that produce IFN-γ and TNF-α (38, 39) and that can expand to very high levels (commonly >50% of circulating T cells) during in vivo infections with bacteria and protozoa (reviewed in Refs. 40, 41). We and others have identified (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), an essential metabolite in isoprenoid synthesis in some bacteria and all Apicomplexan parasites (42–44), as an Ag for Vγ2Vδ2 T cells. By specifically recognizing a common essential microbial metabolite, Vγ2Vδ2 T cells can mount memory responses to many bacterial and parasitic protozoan infections that have never been encountered previously.

Vγ2Vδ2 T cells also recognize isopentenyl pyrophosphate (IPP), an essential intermediate for isoprenoid synthesis that is common to both microbes and humans (45). Under normal circumstances, IPP is sequestered inside host cells at low levels and therefore fails to activate host Vγ2Vδ2 T cells. Certain tumor cells or treatment of human cells with bisphosphonates (46) or alkylamines (47) causes increases in IPP resulting in activation of Vγ2Vδ2 T cells (reviewed in Ref. 41). The Vγ2Vδ2 TCR can distinguish foreign HMBPP from self IPP because HMBPP is 30,000-fold more active, stimulating at picomolar concentrations. This recognition by Vγ2Vδ2 γδ 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 γδ T cells. Human γδ 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 Vδ1 T cells (49). However, neither of these studies characterized IL-17A– and IL-22–producing Vγ2Vδ2 T cells in detail or examined the cytokine requirements for IL-17A production by human γδ T cells. Thus, the potential role of γδ 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 Vγ2Vδ2 T cells exist at low but significant frequencies in human and nonhuman primates. Our data suggest that, like Th17 αβ T cells, Vγ2Vδ2 γδ T cells can be polarized into Tγδ17, Tγδ1/17, and Tγδ22 populations with distinct cytokine requirements for their initial polarization and later maintenance.

Materials and Methods

Ag and cytokines

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

Abs

FITC-conjugated anti-human Vδ2 TCR (clone B6), allophycocyanin-Cy7–conjugated anti-human CD3 (clone SK7), FITC-conjugated anti-human TCR γδ (clone B1), and PE- or biotin-conjugated anti-human IFN-γ (both clone 4S.B3) were purchased from BD Biosciences (San Jose, CA). Alexa-Fluor^{647}–conjugated anti-human T-Bet (clone eBio4B10) and Alexa-Fluor^{647}– or PE-conjugated anti-human RORγt (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 Vδ2 (clone 15D) was purchased from Endogen (Rockford, IL), and FITC-conjugated goat anti-mouse (IgM+IgG Fab fragment) was purchased from BioSource (Carlsbad, CA).

Adult PBMC isolation and culture

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

For differentiation and expansion experiments, PBMCs were plated as above in X-VIVO 15 serum-free media (unless otherwise stated) and incubated in the presence or absence of 0.316 μM HMBPP, 50 ng/ml recombinant human IL-23, 50 ng/ml recombinant human IL-1β, 50–200 ng/ml recombinant human IL-6, 1 ng/ml recombinant human TGF-β, 10 μg/ml anti-human IL-6, and 10 μg/ml anti-human IL-23. On the third day, 1 nM IL-2 was added to cultures. Cells were cultured for 7–12 d. On the final day, cells were washed and then stimulated with PMA and ionomycin, as for ex vivo analyses described above. Note that the cytokines were titrated in pilot experiments to determine their optimal concentrations. Moreover, preliminary studies found that the addition of neutralizing anti–IFN-γ and anti–IL-4 was unnecessary for the polarization of IL-17A$^+$ Vγ2Vδ2 T cells; therefore, these were not added in subsequent polarization experiments (except where otherwise noted). Because of the high degree of variability between adult human donors, the number of IL-17A$^+$ Vγ2Vδ2 for each donor and condition was normalized to the maximal number of IL-17A$^+$ Vγ2Vδ2 T cells expanded for each particular donor. Such variability was not unexpected, as the donors had highly variable frequencies of IL-17A$^+$ Vγ2Vδ2 T cells ex vivo.

Umbilical cord blood mononuclear cell culture

Umbilical cord blood was obtained from normal term deliveries. Cord blood mononuclear cells (CBMCs) were isolated from heparinized cord blood, using Ficoll-Paque Plus density gradient centrifugation and frozen in liquid nitrogen until needed. For polarization experiments, CBMCs were defrosted and plated in X-VIVO 15 serum-free media and cultured with or without 200 μM 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 restimulated with PMA and ionomycin on day 13 for intracellular cytokine staining. Note that neonatal Vγ2Vδ2 T cells from cord blood require higher concentrations of HMBPP and longer incubation periods than do adult Vγ2Vδ2 T cells for expansion.

ELISA for IL-17A

To measure the quantity of IL-17A released from expanded T cell cultures, cells were restimulated with 50 ng/ml PMA (Sigma-Aldrich, St. Louis, MO) and 2 μg/ml ionomycin (Sigma-Aldrich) for 4–6 h, 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, PBMCs were rested overnight and the next day were stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 2 μg/ml ionomycin (Sigma-Aldrich) for 4–6 h in the presence of GolgiStop (monensin) (BD Biosciences) at the manufacturer's recommended concentration. PBMCs were first stained with LIVE/DEAD Blue (Invitrogen, Carlsbad, CA), to exclude dead cells, then stained with allophycocyanin-Cy7–conjugated anti-CD3, FITC-conjugated anti-Vδ2, or FITC-conjugated anti-TCR γδ. Next, the cells were 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-Vδ2, and then stained with biotin-conjugated anti-CD28 and allophycocyanin-conjugated anti-CD27. Next,

the cells were 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, PBMCs were stimulated as above, then stained with LIVE/DEAD Blue and unconjugated anti-human Vδ2 (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. Next, the cells were 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-Fluor^{647}–conjugated anti–T-bet.

Postexpansion, human PBMCs and CBMCs were restimulated with PMA (50 ng/ml) and ionomycin (2 μg/ml) in the presence of GolgiStop for 4–6 h. The cells were then stained with LIVE/DEAD Blue, followed by surface staining with FITC-conjugated anti-Vδ2, allophycocyanin-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-Fluor^{647}–conjugated anti–IL-22, and PerCP-Cy5.5–conjugated anti–IL-17A or PE-conjugated anti-RORγt, Alexa-Fluor^{647}–conjugated anti–T-bet, and PerCP-Cy5.5–conjugated anti–IL-17A. Lastly, the biotin label was detected with PE-Cy7 streptavidin.

To assess Vγ2Vδ2 T cells, an anti-Vδ2 mAb was used for all analyses because in adults the vast majority (96.4–100%) of Vδ2 T cells expressVγ2Vδ2 TCRs (51, 52). In fact, in 17 of 36 donors >99.6% of Vδ2 chains were paired with Vγ2 chains (51). The anti-Vδ2 mAb was also used to determine Vγ2Vδ2 T cells after expansion by HMBPP, as only γδ T cells expressing Vγ2Vδ2 TCRs respond and expand to prenyl pyrophosphates (53, 54). After expansion of γδ T cells by prenyl pyrophosphates, 100% of Vδ2 T cells express Vγ2Vδ2 TCRs. In neonates prior to prenyl pyrophosphate expansion, about equal proportions of Vδ2 chains are paired with Vγ2 as are paired with Vγ1 (51). However, we did not assess ex vivo production of IL-17A and IL-22 by neonates because the number of Vγ2Vδ2 T cells was too low. Therefore, we have used Vγ2Vδ2 to designate Vδ2 T cells in the text, although a very small fraction of the cells may express Vγ1Vδ2 TCRs.

Statistical analyses

Vγ2Vδ2 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 posttest, was used, with $p < 0.5$ considered statistically significant. Statistical analyses were done in Prism version 4.0c (GraphPad, San Diego, CA).

Results

Frequency of circulating IL-17A– and IL-22–producing Vγ2Vδ2 T cells in normal humans and rhesus macaques

Naive γδ 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 naive CD4 T cells (55, 56). Nonetheless, although most human Vγ2Vδ2 T cells produce IFN-γ, minor populations that produce IL-4 and IL-10 have been identified (57). This finding suggests that Vγ2Vδ2 T cells, like αβ T cells, can be polarized into different functional lineages. To investigate the existence of Th17-like Vγ2Vδ2 T cells in humans, we isolated PBMCs 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 Vγ2Vδ2 T cells were present in most donors, although the proportions varied widely, ranging from 0.2 to 3% of Vγ2Vδ2 T cells, with an average of 1.1 ± 0.3% (Fig. 1*A*, 1*C*, and Table I). No IL-17A production from Vγ2Vδ2 T cells was observed in the absence of ionomycin and PMA stimulation (data not shown). An average of 0.9 ± 0.2% of peripheral blood γδ T cells secreted IL-17A. These proportions were similar to αβ T cells, in which an average of 1.1 ± 0.1% produced IL-17A (Fig. 1*A*, 1*C*). Thus, in 1 ml of blood, an average of 389 ± 112 of Vγ2Vδ2 cells produced IL-17A (Fig. 1*B* and Table I), and the frequency of IL-17A–producing Vγ2Vδ2 T cells averages 1 of every 2762 T cells (Table I).

Because the Vγ2Vδ2 TCR is exclusively expressed in primates and not by murine γδ T cells, the rhesus macaque (*Macaca mulatta*) is a useful animal model to study Vγ2Vδ2 T cells in vivo (58). Therefore, we next asked whether Vγ2Vδ2 T cells in rhesus macaques produce IL-17A. PBMCs were isolated from eight macaques, stimulated with ionomycin in the presence of PMA, and cytokine production was determined by intracellular staining. The frequency of peripheral blood IL-17A–producing Vδ2 T cells ex vivo was increased, with a mean frequency of 5.6 ± 1.3% (ranging from 1.1 to 13.4%; see Fig. 1*E*) compared with 1.1 ± 0.3% in humans. We noted similar frequencies in splenic T cells from another two rhesus macaques (data not shown). Taken together, these results demonstrate that an IL-17A$^+$ Vδ2 T cell population, parallel to the Th17 αβ T cell subset, exists in humans and that this population is conserved in nonhuman 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 because 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 Vγ2Vδ2 T cells were a separate subset of cells distinct from IL-17A–producing Vγ2Vδ2 T cells because only 2.7% of IL-22–producing cells also produced IL-17A (Figs. 2*A*, 3*B*, 4*B*, Supplemental Fig. 1). The frequency of Vγ2Vδ2 T cells producing IL-22 averaged 1.2 ± 0.2% (ranging from 0.5 to 2.2%) of peripheral blood Vγ2Vδ2 T cells. The frequency of αβ T cells producing IL-22 among these same donors averaged 2.3 ± 0.5%. When the absolute cell numbers were calculated (Table I), there were 1.6-fold more IL-22–producing Vγ2Vδ2 T cells than IL-17A–producing Vγ2Vδ2 T cells (639 ± 328 cells/ml producing IL-22 versus 389 ± 112 cells/ml producing IL-17A) or 1 of every 1864 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 Vγ2Vδ2 T cells.

Phenotype of IL-17A$^+$ Vγ2Vδ2 T cells

Classically defined murine Th17 cells 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 coproduced, both CD4 and CD8 αβ T cells producing IL-17A have been exclusively detected within memory subsets (64, 65, 68, 71, 72). We therefore determined the spectrum of cytokines coproduced by IL-17A$^+$ Vγ2Vδ2 T cells and the memory phenotype of IL-17A$^+$ Vγ2Vδ2 T cells. After stimulation with PMA and ionomycin, most IL-17A$^+$ Vγ2Vδ2 T cells coproduced IFN-γ, and almost none coproduced IL-22 (Fig. 2*A*), IL-4, or IL-10 (data not shown). In contrast, fewer rhesus macaque IL-17A$^+$ Vγ2Vδ2 T cells dual produced IFN-γ, with most being IL-17A single producers (representative staining in Fig. 2*A*). Consistent with published work (55, 57, 73, 74), and their memory-like phenotype, we observed that the vast majority of human peripheral blood Vγ2Vδ2 T cells produced IFN-γ (>90%) and a small fraction (<5%) produced IL-4 (data not shown and Refs. 55, 57, 73, 74).

Unlike αβ T cells, Vγ2Vδ2 T cells transition very early in life into phenotypically memory cells, leaving few naive Vγ2Vδ2 T cells in the adult circulation (<2% naive Vγ2Vδ2; C. Jin and C.T. Morita, unpublished observations and Refs. 75, 76). The process by which this occurs is not fully understood but probably

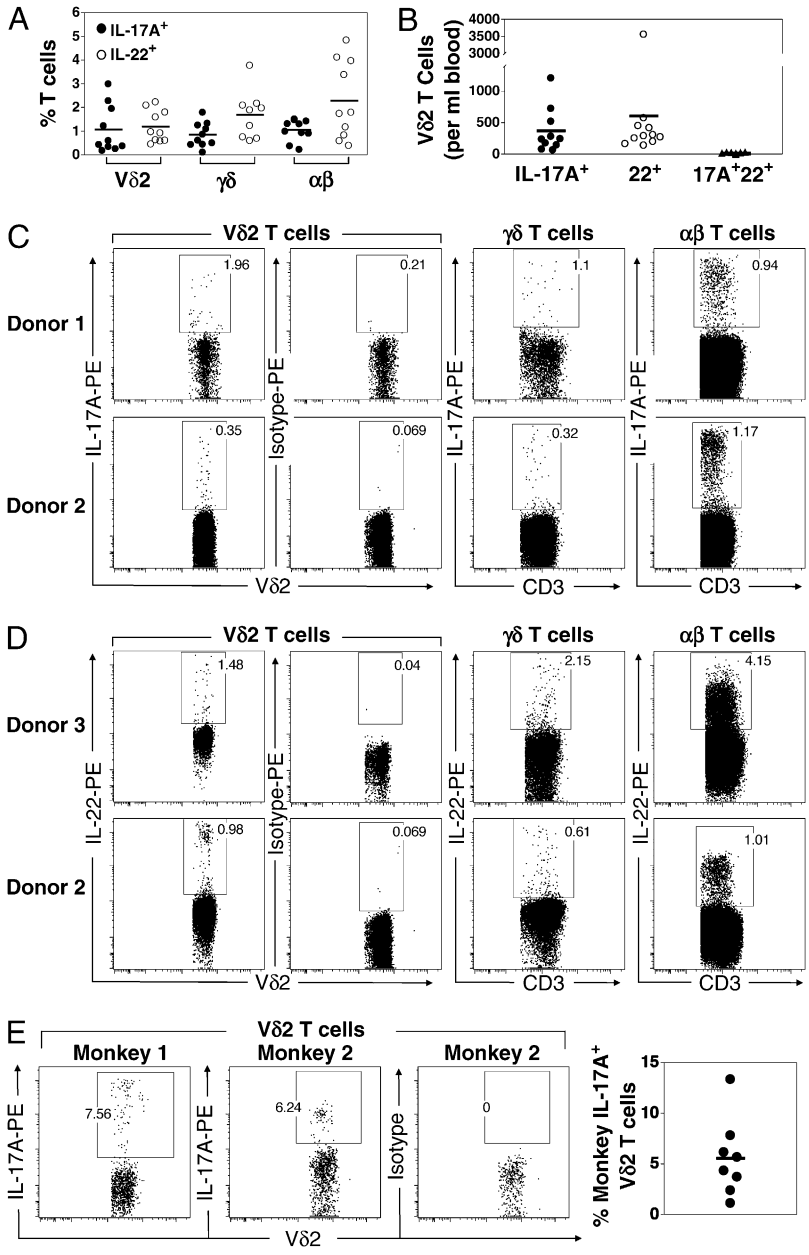

FIGURE 1. Frequency of Vγ2Vδ2 T cells producing IL-17A and IL-22 in adult human and rhesus macaque donors. *A*, PBMCs from 10 normal donors were stimulated with PMA and ionomycin, and intracellular cytokine staining for IL-17A and IL-22 was performed. Viable T cells were gated using LIVE/DEAD blue and anti-CD3, after which the different T cell subsets discriminated using anti-Vδ2 (to identify Vγ2Vδ2 T cells) and anti-pan γδ (to identify total γδ T cells). αβ T cells were defined as $CD3^+$, $\gamma\delta^-$. *B*, Numbers of $IL\text{-}17A^+$ Vγ2Vδ2, $IL\text{-}22^+$ Vγ2Vδ2, and $IL\text{-}17A^+$, $IL\text{-}22^+$ Vγ2Vδ2 T cells per milliliter of blood were calculated (Table I). *C*, Representative IL-17A staining for Vγ2Vδ2 T cells (abbreviated Vδ2 T cells), total γδ T cells, and total αβ T cells. *D*, Representative IL-22 staining for Vγ2Vδ2 T cells (abbreviated Vδ2 T cells), total γδ T cells, and total αβ T cells. *E*, Representative IL-17A staining and average frequency of $IL\text{-}17A^+$ Vδ2 T cells among eight rhesus macaques. Each point refers to one donor, and bars depict means.

is the result of stimulation by the ubiquitous foreign and self prenyl pyrophosphate Ags. Reminiscent of CD8 αβ T cells, Vγ2Vδ2 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, Vγ2Vδ2 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). Naive Vγ2Vδ2 T cells represent <2% of adult Vγ2Vδ2 T cells and constitute only a negligible proportion of $CD27^+$ $CD28^+$ Vγ2Vδ2 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 $IL\text{-}17A^+$ Vγ2Vδ2 T cells, we performed staining for CD27 and CD28 on human PBMCs after PMA and ionomycin restimulation. We observed similar proportions of T_{early} (+ T_{naive}), $T_{intermediate}$, and $T_{CD45RA\ late}$ cells in $IL\text{-}17A^+$ Vγ2Vδ2 as found in total Vγ2Vδ2 T cells (Fig. 2*B*, 2*C*). Thus, in contrast to $IL\text{-}17A^+$ Tc17 CD8 αβ T cells that are almost exclusively restricted to the T_{early} and $T_{intermediate}$ subsets (72), $IL\text{-}17A^+$ Vγ2Vδ2 T cells were found within all three memory subsets without skewing.

Cytokine requirements for the differentiation and expansion of neonatal $IL\text{-}17A^+$ Vγ2Vδ2 T cells

Unlike CD4 and CD8 αβ T cells, little is known about the cytokines required to differentiate naive γδ T cells to produce IL-17A. Although umbilical cord blood represents the best source for naive Vγ2Vδ2 T cells, even in cord blood only ~50% of the Vγ2Vδ2 T cells are phenotypically naive (C. Jin and C.T. Morita, unpublished observations). Given the low frequency of Vγ2Vδ2 T cells in cord blood [<1% of T cells (51)], isolating pure naive Vγ2Vδ2 T cells for in vitro polarization studies was not feasible. Therefore, we studied the polarization of total neonatal cord blood Vγ2Vδ2 T cells in which ~50% have a naive surface phenotype and none have been exposed to foreign Ags. Because a high proportion of Vγ2Vδ2 T cells react to the HMBPP Ag without prior selection (41), we were able to specifically expand Vγ2Vδ2 T cells directly from cord blood without purification. To determine the cytokine requirements for polarization of Vγ2Vδ2 T cells into IL-17A–

Table I. *Frequency of adult IL-17A– and IL-22–producing human Vγ2Vδ2 T cells*

Donor	PBMC per ml Blood	% Vδ2 among CD3 T Cells	% IL-17A$^+$ among Vδ2	% IL-22$^+$ among Vδ2	Frequency of IL-17A$^+$ Vδ2 among CD3 T Cells	IL-17A$^+$ Vδ2 per ml Blood	Frequency of IL-22$^+$ Vδ2 among CD3 T Cells	IL-22$^+$ Vδ2 per ml Blood
1	1.70×10^6	3.99	0.61	0.64	1:4,109	239	1:3,916	256
2	2.36×10^6	1.81	1.96	1.60	1:2,819	283	1:3,456	142
3	1.45×10^6	2.04	2.29	2.24	1:2,141	522	1:2,188	421
4	1.93×10^6	3.58	0.44	0.93	1:6,348	147	1:3,004	309
5	3.33×10^6	4.18	0.19	0.46	1:12,591	67	1:5,201	167
6	1.89×10^6	6.88	0.26	0.62	1:5,590	246	1:2,344	454
7	3.57×10^6	23.9	0.35	0.98	1:1,195	1213	1:427	3567
8	1.23×10^6	3.68	3.00	2.10	1:906	728	1:1,294	580
9	2.25×10^6	1.24	0.38	1.81	1:21,222	80	1:4,456	293
10	2.82×10^6	1.23	1.23	0.59	1:6,610	367	1:13,780	203
Mean ± SEM	$2.25 \pm 2.46 \times 10^6$	5.25 ± 2.14	1.07 ± 0.32	1.20 ± 0.21	1:2,762 ± 1:9,047	389 ± 112	1:1,864 ± 1:4,775	639 ± 328

PBMCs were harvested from normal adult donors, counted, and stimulated with 50 ng/ml PMA and 2 μg/ml ionomycin for 4–6 h in the presence of GolgiStop (monensin). PBMCs were then stained with LIVE/DEAD Blue (Invitrogen), followed by allophycocyanin-Cy7–conjugated anti-CD3, FITC-conjugated anti-Vδ2, or FITC-conjugated anti-TCR γδ. The cells were then washed, fixed, and permeabilized using the BD Cytofix/Cytoperm Kit; then, they were 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.

producing cells, we cultured Vγ2Vδ2 T cells from eight different donors in serum-free media for 13 d with HMBPP and various combinations of the classical Th17 polarizing cytokines, IL-6, IL-1β, IL-21, IL-23, and TGF-β, and neutralizing cytokine Abs. IL-2 was added on the third day. On the final day, cells were restimulated with PMA and ionomycin, surface stained for CD3 and Vδ2 (to identify Vγ2Vδ2 T cells, see *Materials and Methods*), and intracellularly stained for IL-17A, IFN-γ, and IL-22 as well as the transcription factors RORγt and T-bet. We hypothesized that naive Vγ2Vδ2 T cells, like naive CD4 αβ T cells, can be polarized under similar cytokine conditions (namely, TGF-β, IL-6, IL-21, and IL-1β) into IL-17A–producing Tγδ17 Vγ2Vδ2 T cells.

Expansion of cord blood Vγ2Vδ2 T cells in response to Ag stimulation with HMBPP ranged from 8% to >20% of total CD3 T cells (Supplemental Fig. 2). Expanded Vγ2Vδ2 T cells were divided into IL-17A$^+$ IFN-γ$^-$ (Tγδ17), IL-17A$^+$ IFN-γ$^+$ (Tγδ1/17), and IL-22$^+$ IFN-γ$^{+/-}$ (Tγδ22) subsets and their total numbers plotted (Fig. 3*A*). Representative staining for IL-17A, IFN-γ, and IL-22 is shown in Fig. 3*B* for condition 9. Because each donor differed in the magnitude of expansion, the number of IL-17A$^+$ Vγ2Vδ2 for each donor and condition was normalized to the maximal number of IL-17A$^+$ Vγ2Vδ2 T cells expanded for each donor (Fig. 3*A*, *bottom panel*).

At baseline in the presence or absence of HMBPP (Fig. 3*A*, conditions 1 and 2), very few IL-17A$^+$ (Tγδ17 and Tγδ1/17) and IL-22$^+$ (Tγδ22) Vγ2Vδ2 T cells were observed. IL-23 alone and IL-23 plus IL-1β had minimal effects on the expansions of Tγδ1/17 and Tγδ17 Vγ2Vδ2 T cells (Fig. 3*A*, conditions 3 and 4). The combined effect of IL-23, IL-6, and IL-1β also had little effect on the numbers of Tγδ17 or Tγδ1/17 (condition 5). However, when IL-23, IL-6, and IL-1β were combined with TGF-β, a statistically significant increase in the number of Tγδ1/17 cells (normalized to each donor's maximal response) was observed (Fig. 3*A*, *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-1β, and TGF-β, the number of Tγδ1/17 returned to moderate levels (Fig. 3*A*; compare conditions 6 and 7), suggesting an important role for IL-6 in the expansion of Tγδ1/17 Vγ2Vδ2 T cells. However, exogenous IL-1β, in combination with IL-6, IL-23, and TGF-β, was critical for expansion of both Tγδ17 and Tγδ1/17 (Fig. 3*A*; compare conditions 7 and 8). Taken together, these data suggest that neonatal Tγδ17 and Tγδ1/17 populations similarly require IL-1β and TGF-β but that the Tγδ1/17 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 naive cord blood Vγ2Vδ2 T cells into Tγδ17 (or Tγδ1/17) T cells. To test this hypothesis, we polarized Vγ2Vδ2 T cells in the presence of IL-1β, IL-6, TGF-β, and neutralizing anti–IL-23. As predicted, we observed a statistically significant expansion in Tγδ17 and Tγδ1/17 Vγ2Vδ2 T cells even when IL-23 was neutralized (Fig. 3*A*, *bottom panel,* condition 9). These results support our hypothesis that IL-23 is not required for initial polarization of cord blood Vγ2Vδ2 into Tγδ17, but that IL-1β, IL-6, and TGF-β are required. Furthermore, our results suggest that exogenous IL-23 may actually inhibit Tγδ17 development because more Tγδ17 cells were found after its neutralization. Similar results were noted for a second small subset of cord blood Vγ2Vδ2 T cells expressing CD4. These cells showed very similar responses to cytokines with optimal expansion with IL-1β, IL-6, TGF-β, and neutralization of IL-23 (Supplemental Figs. 1, 2). In contrast, the effect of IL-23 on Tγδ1/17 cells was different. This subset required IL-23 with IL-1β, IL-6, and TGF-β for optimal expansion

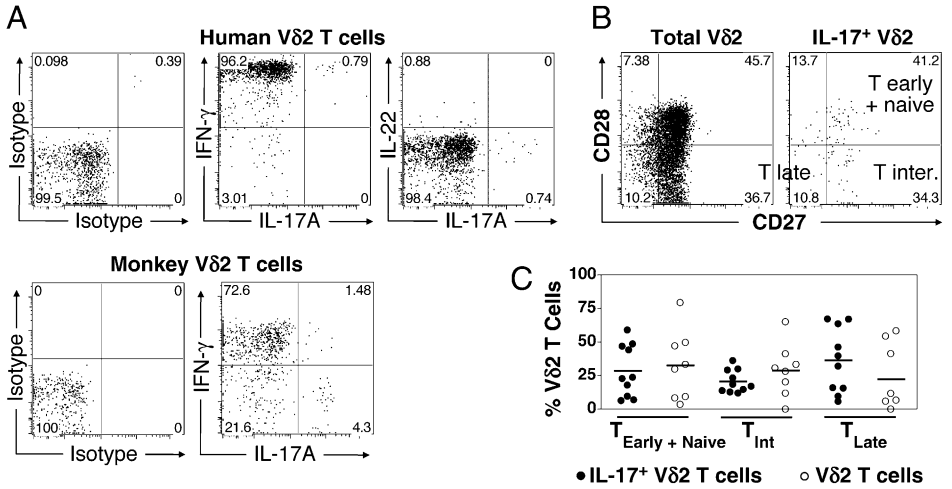

FIGURE 2. Cytokine profile and memory phenotype of IL-17A$^+$ Vγ2Vδ2 T cells from adult human and monkey donors. *A*, IFN-γ production by IL-17A–producing Vδ2 T cells. PBMCs were stimulated with PMA and ionomycin and stained intracellularly for IL-17A, IFN-γ, and IL-22. Shown is a representative human (*top panels*) and monkey (*bottom panels*) donor. *B*, Representative surface staining for memory markers CD27 and CD28 on total Vγ2Vδ2 T cells or IL-17A$^+$ gated Vγ2Vδ2 T cells. *C*. Frequency of total human Vγ2Vδ2 T cells or IL-17A$^+$ gated Vγ2Vδ2 T cells belonging to T early + naive (CD27$^+$, CD28$^+$), T intermediate (CD27$^+$, CD28$^-$) or T late (CD27$^-$, CD28$^-$) memory subsets. Each point represents one donor, and bars depict means.

(compare condition 6 with condition 9). Thus, IL-23 favors the differentiation/and or expansion of neonatal Vγ2Vδ2 T cells as Tγδ1/17 cells rather than Tγδ17 cells.

IL-21 and TGF-β also polarize naive CD4 αβ T cells to a Th17 phenotype (22). Therefore, we tested whether IL-21 and TGF-β would similarly polarize neonatal Vγ2Vδ2 T cells into Tγδ17 and/or Tγδ1/17 cells. This combination (Fig. 3*A*, condition 10) failed to increase the number and/or percent of Tγδ17 or Tγδ1/17 Vγ2Vδ2 T cells, despite robust proliferation of Vγ2Vδ2 T cells (Supplemental Fig. 2, condition 10). Thus, unlike CD4 αβ T cells, IL-21 and TGF-β were insufficient to support the development of IL-17A–producing Vγ2Vδ2 T cells.

Like IL-17A–producing Vγ2Vδ2 T cells, the numbers of Tγδ22 Vγ2Vδ2 T cells tended to increase in the presence of IL-1β, IL-6, and TGF-β (Fig. 3*A*, *top panel,* condition 9), although this increase did not reach statistical significance. Thus, our data suggest that although the Tγδ22 cell population is distinct from Tγδ1/17 and Tγδ17 cell populations (Fig. 3*B*), Tγδ22 Vγ2Vδ2 T cells require cytokines similar to those observed for Tγδ17 cells.

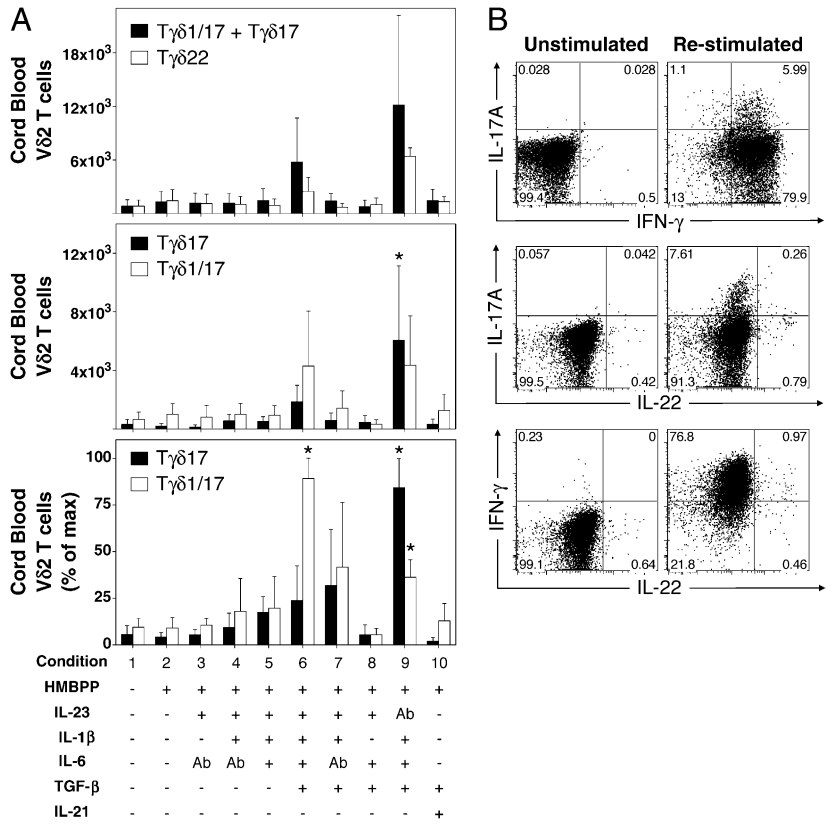

FIGURE 3. IL-1β, TGF-β, and IL-6 induce maximal polarization of IL-17A$^+$ neonatal CD4$^-$ Vγ2Vδ2 T cells upon Ag stimulation with HMBPP. Umbilical cord blood mononuclear cells were expanded in the presence or absence of HMBPP, IL-23, IL-1β, TGF-β, IL-6, neutralizing anti–IL-6, or neutralizing anti–IL-23 for 13 d ($n = 8$ 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 was performed. Expanded cord blood CD4$^-$ Vγ2Vδ2 T cells (defined as Vδ2$^+$, CD3$^+$, CD4$^-$) were divided into IFN-γ$^-$, IL-17A$^+$ Vγ2Vδ2 T cells (termed Tγδ17), IFN-γ$^+$, IL-17A$^+$ Vγ2Vδ2 T cells (termed Tγδ1/17), IFN-γ$^+$, IL-17A$^-$ Vγ2Vδ2 T cells (termed Tγδ1), and IFN-γ$^{+/-}$, IL-22$^+$ Vγ2Vδ2 (termed Tγδ22). *A*, Median number of total IL-17A$^+$ CD4$^-$ Vγ2Vδ2 T cells (combined Tγδ1/17 and Tγδ17) or Tγδ22 Vγ2Vδ2 T cells among total CD4$^-$ Vγ2Vδ2 T cells for each cytokine condition (*top panel*). Median number of Tγδ17 or Tγδ1/17 CD4$^-$ Vγ2Vδ2 T cells among total CD4$^-$ Vγ2Vδ2 T cells for each condition (*middle panel*). Median percent of maximum Tγδ17 or Tγδ1/17 CD4$^-$ Vγ2Vδ2 T cells expanded for each condition (*bottom panel*). *B*, Representative cytokine staining on viable CD4$^-$ Vγ2Vδ2 T cells expanded in the presence of HMBPP, IL-1β, TGF-β, IL-6, and anti–IL-23, either unstimulated (*left panels*) or restimulated with PMA and ionomycin (*right panels*). Bars depict medians, and error bars depict median absolute error. $*p < 0.05$, Kruskal-Wallis comparison with condition 2.

Cytokine requirements for the expansion of adult IL-17A$^+$ Vγ2Vδ2 T cells

We next asked whether IL-17A$^+$ Vγ2Vδ2 T cells could be expanded from adult peripheral blood, and if similar cytokines were required to those required for neonatal IL-17A$^+$ Vγ2Vδ2 T cells. Because the vast majority of adult Vγ2Vδ2 T cells are memory cells and IL-23R expression is restricted to memory CD4 T cells (79), we hypothesized that, like memory Th17 αβ T cells, expansion of adult IL-17A$^+$ Vγ2Vδ2 T cells would require IL-23. IL-1β might also be required, as it is important for the expansion of human CD4 Th17 T cells (80, 81) and as the combination of IL-23 and IL-1β induces IL-17A production by murine γδ T cells (36). To determine the role of IL-23, we expanded adult Vγ2Vδ2 T cells in PBMCs with HMBPP, IL-1β, IL-6, neutralizing anti–IL-4, and neutralizing anti–IFN-γ in the presence or absence of IL-23 (Fig. 4). On day 12, 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-1β increased the frequency of IL-17A$^+$ Vγ2Vδ2 T cells from 1.1% (roughly the same frequency of IL-17A$^+$ Vγ2Vδ2 present ex vivo) to 9.4% (Fig. 4) owing to an 8-fold increase in the number of IL-17A$^+$ Vγ2Vδ2 T cells (Fig. 4*B*). Similarly, IL-17A levels increased 5.2-fold from 276 pg/ml in the absence of IL-23 to 1431 pg/ml in its presence (Fig. 4*B*). 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 Vγ2Vδ2 T cells.

To study this observation in more depth, Vγ2Vδ2 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 Vγ2Vδ2 T cells. To determine if TGF-β was required, serum-free media were used. We found that the expanded adult Vγ2Vδ2 T cells, like neonatal Vγ2Vδ2 T cells, could be divided into IL-17A$^+$ IFN-γ$^+$ (Tγδ1/17), IL-17A$^+$ IFN-γ$^-$ (Tγδ17), and IL-22$^+$ IFN-γ$^{+/-}$ (Tγδ22) populations (representative staining for IL-17A and IFN-γ is shown in Figs. 4*A*, 5*B*). The vast majority of IL-17A$^+$ Vγ2Vδ2 T cells expanded from adult blood were Tγδ1/17 producing both IFN-γ and IL-17A (Fig. 5*B*). Fewer IL-17A$^+$ IFN-γ$^-$ (Tγδ17) Vγ2Vδ2 T cells were detected in adults, with only 4 of 10 adult donors exhibiting expansions of both Tγδ17 and Tγδ1/17 Vγ2Vδ2 T cells (compared with Tγδ1/17 cells in Supplemental Fig. 3). These in vitro results were consistent with the ex vivo results because only these same four donors had detectable Tγδ17 cells after stimulation.

IL-17A$^+$ (Tγδ1/17) or IL-22$^+$ (Tγδ22) Vγ2Vδ2 T cells were not preferentially expanded in the presence of HMBPP and IL-2 only (Fig. 5*A*, condition 2). Addition of IL-23 alone had minimal effect on the expansion of Tγδ1/17 Vγ2Vδ2 T cells (Fig. 5*A*, condition 3) but did increase expansion of total Vγ2Vδ2 T cells from 12.7 to 25.1% of CD3 T cells (Supplemental Fig. 4*A*). In contrast to neonatal Vγ2Vδ2 T cells, there were moderate increases in Tγδ1/17 cells with IL-23 and IL-1β (condition 4). The addition of

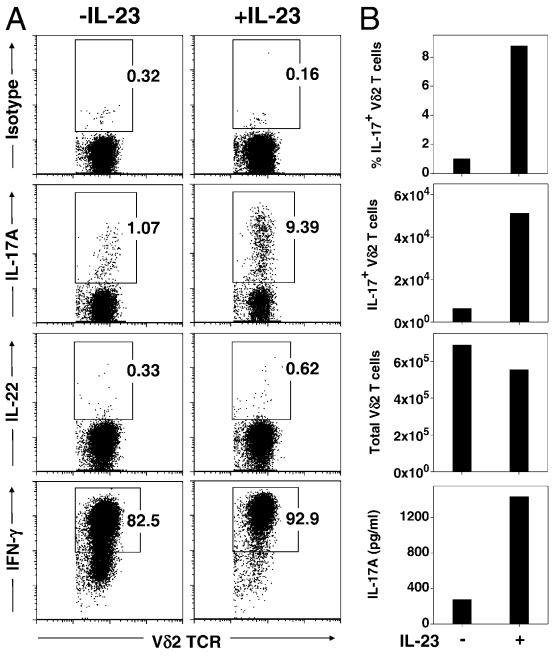

FIGURE 4. IL-23 is required for expansion of adult IL-17$^+$ Vγ2Vδ2 T cells. IL-17A–producing Vγ2Vδ2 T cells, in serum-supplemented media, were measured in PBMCs after expansion with HMBPP, IL-1β, IL-6, neutralizing anti–IL-4, and neutralizing anti–IFN-γ in the presence or absence of IL-23. IL-2 was added on day 3. On day 12, cells were restimulated with PMA and ionomycin, after which the supernatants and cells were harvested for analysis. Expanded Vγ2Vδ2 T cells were defined as Vδ2$^+$, CD3$^+$. Representative of two donors. *A*, Cytokine profile of expanded Vγ2Vδ2 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$^+$ Vγ2Vδ2 T cells in the presence or absence of exogenous IL-23 (*top two panels*). Total expanded Vγ2Vδ2 T cells on day 12 (*third panel*). Total IL-17A protein released into culture as determined by ELISA (*fourth panel*).

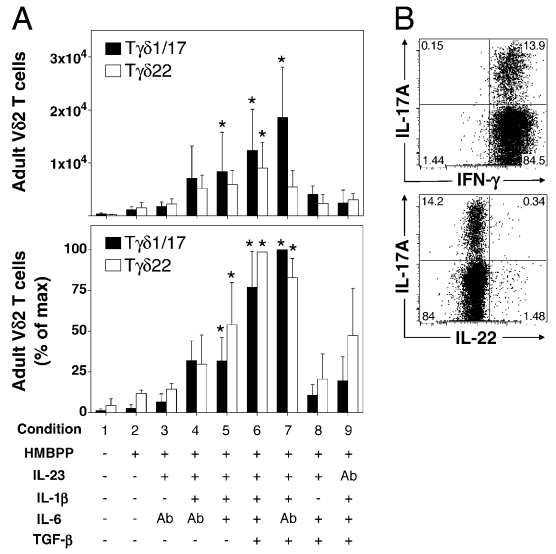

FIGURE 5. IL-23, IL-1β, and TGF-β are sufficient for polarization of adult IL-17A$^+$ Vγ2Vδ2 T cells after stimulation with HMBPP. Total PBMCs, from 10 donors, were cultured in the presence or absence of HMBPP, IL-23, IL-1β, TGF-β, IL-6, neutralizing anti–IL-6, or neutralizing anti–IL-23 for 7 d. IL-2 was added on day 3. On day 7, cells were restimulated with PMA and ionomycin, and intracellular staining for IL-17A, IL-22, and IFN-γ was performed. Expanded PBMC Vγ2Vδ2 T cells (defined as Vδ2$^+$, CD3$^+$, CD4$^-$) could be divided into IFN-γ$^+$, IL-17A$^+$ Vγ2Vδ2 T cells (termed Tγδ1/17), IFN-γ$^+$, IL-17A$^-$ Vγ2Vδ2 T cells (termed Tγδ1), and IFN-γ$^{+/-}$, IL-22$^+$ Vγ2Vδ2 (termed Tγδ22). No IFN-γ$^-$, IL-17A$^+$ Vγ2Vδ2 T cells (Tγδ17) were detected in these adult donors. *A*, Median number of Tγδ1/17 or Tγδ22 Vγ2Vδ2 T cells among total Vγ2Vδ2 T cells for each cytokine condition (*top panel*). Median percent of maximum Tγδ1/17 Vγ2Vδ2 T cells expanded for each cytokine condition (*bottom panel*). *B*, Representative cytokine staining on Vγ2Vδ2 T cells expanded in the presence of HMBPP, IL-23, IL-1β, 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.

IL-6 to IL-23 and IL-1β had little effect on Tγδ1/17 Vγ2Vδ2 T cell numbers (Fig. 5*A*, *top panel,* condition 5) or the proportion of Tγδ1/17 cells (Fig. 5*A*, *bottom panel*). However, the addition of TGF-β to IL-23 and IL-1β further increased the expansion of Tγδ1/17 Vγ2Vδ2 T cells (Fig. 5*A*, 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 on the expansion of total Vγ2Vδ2 T cells (Supplemental Fig. 4).

Like neonatal Vγ2Vδ2 T cells, IL-1β appeared also to be critical for the expansion of adult Tγδ1/17 Vγ2Vδ2 T cells, because in its absence (Fig. 5*A*, condition 8) the number of Tγδ1/17 Vγ2Vδ2 T cells fell to low levels. However, unlike neonatal Vγ2Vδ2 T cells, neutralization of IL-23 in the presence of IL-1β, IL-6, and TGF-β caused Tγδ1/17 Vγ2Vδ2 T cell numbers to also drop to low levels (compare Fig. 5*A*, condition 9, with Fig. 3*A*, condition 9). These results support part of our hypothesis—that the expansion of Tγδ1/17 Vγ2Vδ2 T cells from adult PBMCs required IL-23 and IL-1β; however, we were surprised to see that TGF-β was also important for expansion of these cells.

A minority of adult donors (4 of 11) had detectable expansions in Tγδ17 Vγ2Vδ2 T cells. These individuals were analyzed separately, and the cytokine requirements for Tγδ17 were compared with those for Tγδ1/17 (Supplemental Fig. 3). As with the expansions of Tγδ1/17 from the other adult donors, statistically significant increases in Tγδ17 cells in these four donors were observed for the combination IL-23, IL-1β, and TGF-β in the presence or absence of IL-6. This same combination (IL-23, IL-1β, and TGF-β, in the absence of IL-6) also optimally expanded Tγδ1/17 cells in these donors. Together, these results suggest that adult peripheral blood IL-17A$^+$ Vγ2Vδ2 T cells, be they Tγδ17 or Tγδ1/17, have similar requirements for IL-23, IL-1β, and TGF-β.

Although we were unable to expand significant numbers of Tγδ22 Vγ2Vδ2 T cells from cord blood, significant numbers of Tγδ22 Vγ2Vδ2 T cells were easily expanded from adult peripheral blood. IL-23, IL-1β, and IL-6 were the minimal cytokines required for statistically significant expansion of Tγδ22 Vγ2Vδ2 T cells (Fig. 5*A*, *bottom panel,* condition 5, and Fig. 5*B*). Addition of TGF-β to these cytokines enhanced expansion of Tγδ22 Vγ2Vδ2 T cells (Fig. 5*A*, *bottom panel,* condition 6). As with neonatal Vγ2Vδ2 T cells after in vitro expansion and adult Vγ2Vδ2 T cells ex vivo, Vγ2Vδ2 T cells producing both IL-22 and IL-17A were extremely rare among expanded Vγ2Vδ2 T cells (Fig. 5*B*, *bottom panel*). These results provide further evidence that Tγδ22 are a separate population distinct from the Tγδ1/17 and Tγδ17 populations.

Regulation of Vγ2Vδ2 T cells by RORγt and T-bet transcription factors

Differentiation of Th17 cells involves the coordinated upregulation of the key transcription factors RORγt (RORC2) and RORα, as well as the corresponding epigenetic changes to reinforce these genes and suppress others (82–84). Murine IL-17A–producing γδ T cells are virtually absent from RORγt-deficient mice, suggesting that IL-17A production by murine γδ T cell requires this transcription factor. We therefore determined whether human IL-17A$^+$ Vγ2Vδ2 T cells similarly express RORγt. We hypothesized that IL-17A$^+$ Vγ2Vδ2 T cells have increased expression of RORγt and decreased expression of T-bet. To test this hypothesis, we performed intracellular staining for RORγt and T-bet on total IL-17A$^+$ Vγ2Vδ2 T cells polarized from human neonates and adults. Because the ex vivo population of human IL-17A$^+$ Vγ2Vδ2 T cells is relatively infrequent, we were not able to perform RORγt or T-bet staining on nonexpanded Vγ2Vδ2 T cells from humans. However, because macaque blood contains a higher frequency of IL-17A$^+$ Vδ2 T cells, we were able to analyze T-bet expression within monkey IL-17A$^+$ Vδ2 T cells. Owing to lack of confirmed Ab cross-reactivity for monkey RORγt, we did not assess the expression of RORγt in monkey IL-17A$^+$ Vδ2 T cells. Shown in Fig. 6*A* is the staining from one representative macaque of three studied, demonstrating that IL-17A$^+$, IFN-γ$^-$ Vδ2 T cells have somewhat decreased intracellular T-bet levels relative to IL-17A$^-$, IFN-γ$^+$ Vδ2 T cells.

Next we wished to compare the expression of RORγt and T-bet within expanded human IL-17A$^+$ Vγ2Vδ2 T cells from cord blood. We chose to examine RORγt and T-bet expression on cord blood Vγ2Vδ2 T cells cultured under condition 9 (anti–IL-23, IL-1β, IL-6, and TGF-β) because this combination yielded significant expansions of both Tγδ17 and Tγδ1/17 T cells (Fig. 3*B*, *bottom panel*). Total neonatal IL-17A$^+$ cells (Tγδ17 and Tγδ1/17) expressed significantly more RORγt than did IL-17A$^-$ Vγ2Vδ2 T cells under the same culture conditions (Fig. 6*B*, *left panels*). We failed to detect statistically significant differences in the expression of T-bet by total IL-17A$^+$ Vγ2Vδ2 T cells from cord blood. Similarly, total adult IL-17A$^+$ Vγ2Vδ2 T cells cultured

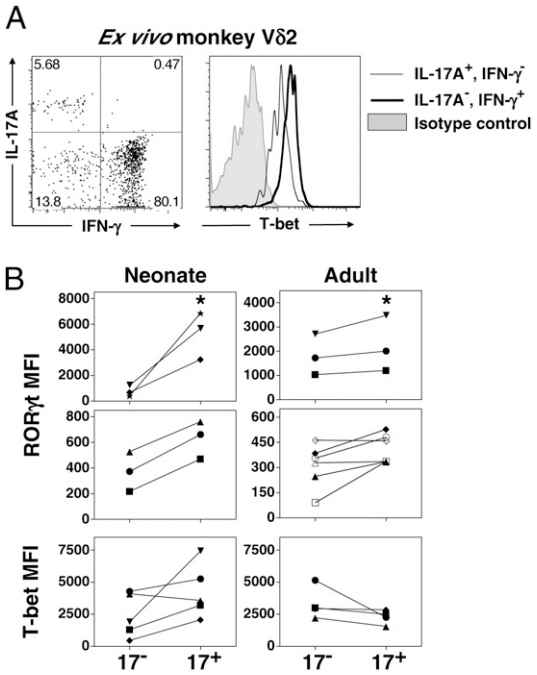

FIGURE 6. Expression of RORγt and T-bet by IL-17A$^+$ Vγ2Vδ2 T cells. *A*, Representative staining for T-bet on monkey peripheral blood Vδ2 T cells, segregated into IL-17A$^+$, IFN-γ$^-$ Vδ2 T cells (Tγδ17), or IL-17A$^-$, IFN-γ$^+$ Vδ2 T cells (Tγδ1). Represents one of three monkeys examined. PBMCs were isolated and stimulated with PMA and ionomycin, and intracellular staining for IL-17A, IFN-γ, and T-bet was performed. *B*. (Neonate, *left panels*) Cord blood mononuclear cells were polarized with HMBPP for 13 d in the presence of IL-1β, IL-6, TGF-β, and anti–IL-23, and (Adult, *right panels*) adult PBMCs were polarized with HMBPP for 7 d 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. MFI, mean fluorescence intensity.

under condition 7 (IL-23, IL-1β, anti–IL-6, TGF-β) expressed significantly more RORγt than did IL-17A$^-$ Vγ2Vδ2 T cells expanded from the same donors under the same culture conditions (Fig. 6*B*, *right panels*). And, as before, the expression of T-bet did not significantly differ between adult IL-17A$^+$ and IL-17A$^-$ Vγ2Vδ2 T cells. These data suggest that like murine IL-17A$^+$ γδ T cells, human IL-17A$^+$ Vγ2Vδ2 T cells upregulate the RORγt transcription factor, consistent with its role in IL-17A production by human γδ T cells.

Discussion

Although γδ T cells are a major source of IL-17A in mice, the role of γδ T cells in IL-17A production in humans has been unclear. In this study, we show that significant numbers of adult human blood Vγ2Vδ2 T cells produce IL-17A or IL-22 ex vivo, although few produce both. IL-17A–producing adult Vγ2Vδ2 T cells are primarily memory cells distributed among early (central), intermediate, and late (effector) subsets similar to Tγδ1 Vγ2Vδ2 T cells. Differentiation of IL-17A–producing cells from neonatal naive Vγ2Vδ2 T cells required the inflammatory cytokines IL-1β 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 Vγ2Vδ2 T cells required IL-23 for maximal expansion of IL-17A–producing cells but did not require IL-6. For both neonatal and adult Vγ2Vδ2 T cells, cells producing IL-17A had higher levels of RORγt compared with cells that did not produce IL-17A, establishing a role for RORγt in IL-17A production. Although the frequency of IL-17A– and IL-22–producing Vγ2Vδ2 T cells is low, these cells could be significant sources of IL-17A and IL-22.

Consistent with this hypothesis, in mice, γδ T cells and other unconventional T cells are important sources of IL-17A and IL-22. Murine γδ 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 γδ T cells precedes that of Th17 CD4 T cells (33), and IL-17A–producing γδ T cells are protective in most infections (34, 85). Besides their protective roles, murine IL-17A–producing γδ T cells play pathological roles in collagen-induced arthritis (87), experimental autoimmune uveitis (88), and experimental autoimmune encephalitis (36).

In mice, IL-17A–producing γδ TCR subsets include invariant Vγ6Vδ1 T cells in the peritoneum of mice with bacterial infections (33), oligoclonal Vγ4Vδ4 T cells in collagen-induced arthritis (87), and Vγ4$^+$ T cells in experimental autoimmune encephalitis (36). None of the Ags are known for these γδ subsets. In addition, some T10/T22-specific γδ T cells, which express TCRs containing a CDR3δ motif (89), produce IL-17A. Murine γδ T cells acquire IL-17A potential in the neonatal thymus (90). For T10/T22-specific γδ T cells, this was dependent on not encountering T10/22 in the thymus (89). In the periphery, γδ T cells can be rapidly induced by IL-23 and IL-1β (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 γδ TCR triggering, the ability to release IL-17A could be due to the constant low-level activation of these cells by an endogenous TCR ligand, because their Ag specificity is unknown. Nonetheless, IL-17A production by murine γδ T cells appears independent of exogenous Ag.

A second type of unconventional T cell is the invariant NKT cell (iNKT). Murine iNKT cells 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 Ags. However, similar to murine γδ T cells, IL-23 alone (but possibly with self lipid Ags) 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 αβ T cells, which can recognize only a single pathogen's peptide/MHC complex, Vγ2Vδ2 T cells are specific for many pathogens by virtue of their recognition of essential prenyl pyrophosphates. Almost all adult Vγ2Vδ2 T cells recognize prenyl pyrophosphate Ags [for example, 91 of 94 (97%) adult Vγ2Vδ2 T cell clones responded (53, 94, 95)] owing to the extensive use of germline-encoded regions of the Vγ2Vδ2 TCR for Ag recognition (96) and the selection for Jγ1.2 and a hydrophobic Vδ2 CDR3 residue that occurs during infancy (51, 94, 97–99). The frequency of CD4 or CD8 αβ T cells specific for a particular peptide/MHC-complex among naive cells is usually very low—1:158,000 to 1:1,875,000 for CD4 (100) and 1:33,000 to 1:164,000 (4/6 were $>$1:142,000) for CD8 (101). In contrast, because all Vγ2Vδ2 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 Vγ2Vδ2 T cells (1 in 2762 T cells) produce IL-17A. The frequency of IL-17A– and IL-22–producing Vγ2Vδ2 T cells may be much higher in peripheral lymph nodes, in mucosa, or in the peritoneum, as has been found in mice (35). Thus, for primary infections, Vγ2Vδ2 T cells and other unconventional T cells may be important sources of early IL-17A and IL-22 until naive CD4 and CD8 αβ T cells can be expanded and differentiated into memory Th17/Tc17 and Th22/Tc22 cells. This suggests that γδ 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 Vγ2Vδ2 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 αβ T cells (59, 69, 70, 102–104). The differentiation of T cells that exclusively produce IL-22 likely reflects the priming conditions during initial Ag exposure. For instance, naive 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 naive CD4 T cells to Th22 cells (70). Although both αβ and Vγ2Vδ2 T cells have subsets that produce exclusively IL-17A or IL-22, only 2.7% of IL-22–producing Vγ2Vδ2 T cells coproduce IL-17A, whereas 10.4–18.2% of IL-22–producing CD4 αβ T cells coproduce IL-17A (70), suggesting increased specialization for blood Vγ2Vδ2 T cells.

Because essentially all adult Vγ2Vδ2 T cells are memory cells, the relatively high frequency of Tγδ22 Vγ2Vδ2 T cells (1 of every 1864 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 Rs 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, Vγ2Vδ2 T cells have been shown to produce fibroblast growth factor 7 (111) and connective tissue growth factor (112). Murine γδ dendritic epidermal cells are primary sources of keratinocyte growth factor and IGF-1, and the presence of murine γδ T cells speeds wound healing in the skin and

gut (113–116). These findings demonstrate parallels between murine and human γδ T cells and suggest that subsets of both function as specialized Tγδ22 cells.

In contrast to humans, adult rhesus macaques exhibited ~5-fold higher frequencies of IL-17A–producing Vγ2Vδ2 cells than do humans. Moreover, the majority of rhesus macaque Vγ2Vδ2 T cells exclusively produced IL-17A without IFN-γ. One possible explanation for this finding is the difference in γδ TCR repertoires between humans and rhesus macaques. Unlike adult humans, in whom Vγ2Vδ2 T cells predominate (54% Vγ2Vδ2 versus 15% Vδ1 T cells), adult rhesus macaques exhibit a predominance of Vδ1 T cells (24% Vγ2Vδ2 versus 33% Vδ1 T cells) (58). This is similar to neonatal human γδ T cells, in which human Vδ1 T cells constitute 40% of total γδ T cells and Vγ2Vδ2 T cells constitute only 9% (51). Between 1 and 10 y of age, an expansion of human Vγ2Vδ2 T cells occurs owing to environmental factors causing a predominance of the Vγ2Vδ2 subset (75). Because the maturity of the rhesus monkeys and that of humans studied were similar, this lack of predominance of Vγ2Vδ2 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 Vγ2Vδ2 T cells may be less Ag experienced, allowing the persistence of naive and early memory Vγ2Vδ2 T cells that can be differentiated or maintained as Tγδ17 cells rather than converted to Tγδ1/17 or Tγδ1 cells.

Unexpectedly, the addition of exogenous IL-23 to IL-1β, IL-6, and TGF-β appeared to inhibit Tγδ17 development in neonates. The likely explanation for this observation is that IL-23 promotes the conversion of Tγδ17 cells into Tγδ1/17 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 Tγδ17 Vγ2Vδ2 T cells give rise to Tγδ1/17 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 Tγδ1/17 and help maintain the Tγδ17 phenotype.

Adult Vγ2Vδ2 T cells required IL-23 in addition to IL-1β and TGF-β, but not IL-6, for maximal expansion of Tγδ1/17 (and, in some donors, Tγδ17) cells. This is in contrast to neonatal Vγ2Vδ2 T cells, which required IL-6 with IL-1β and TGF-β, but not IL-23. The difference in cytokine requirements is consistent with the hypothesis that naive Vγ2Vδ2 T cells (present in neonates), like naive CD4 T cells, require IL-6 for initial upregulation of IL-23R, RORγt, and RORα (14, 15, 118), whereas memory IL-23R$^+$ Vγ2Vδ2 T cells (present in adults) require only IL-23, IL-1β, and TGF-β for re-expression of IL-17A. Similarly, production of IL-17A by murine IL-23R$^+$ memory γδ T cells has been shown to require only IL-23 and IL-1β in the presence of FBS, which contains TGF-β (36). TGF-β is likely not directly required for Tγδ17 or Tγδ22 cell differentiation but instead functions to inhibit differentiation to Tγδ1 and Tγδ2 lineages (19). In both neonates and adults, Vγ2Vδ2 T cells producing IL-17A had higher levels of RORγt compared with cells not producing IL-17A, suggesting a role for RORγt in IL-17A production by γδ 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 Vγ2Vδ2 T cells also favored IL-22–producing Vγ2Vδ2 T cells, although few produced both IL-17A and IL-22 (Figs. 3*A*, 4*A*, and Supplemental Fig. 1). Th22 CD4 αβ T cells optimally differentiate from naive precursors in the presence of IL-6 and TNF-α. The combination of IL-1β, IL-6, and TNF-α favors development of IL-22$^+$, IL-17A$^+$ CD4 αβ 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 RORγt; high levels of the skin homing receptors CCR4, CCR6, and CCR10; and cytokines/chemokines, such as fibroblast growth factors, 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 suggest that Tγδ22 and Tγδ17 T cells, like some Th17 and Th22 CD4 αβ T cells (59, 70, 120), belong to separate subsets that do not produce both cytokines. However, both Tγδ17 and Tγδ22 T cells can acquire IFN-γ production. Future experiments are needed to characterize the plasticity of Tγδ22 and Tγδ17 Vγ2Vδ2 T cells.

On the basis of our findings, we propose the following model for the development of Tγδ17 and Tγδ1/17 Vγ2Vδ2 T cells (Fig. 7). In neonates and infants, microbial infections polarize some Vγ2Vδ2 T cells into memory Tγδ17 through the actions of innate cell-derived IL-6, IL-1β, and TGF-β. Several studies have demonstrated that neonatal innate cells, including professional APCs, produce insufficient levels of IL-12 to program Th1 effector T cells (121–123), and instead make IL-23, IL-1β, and

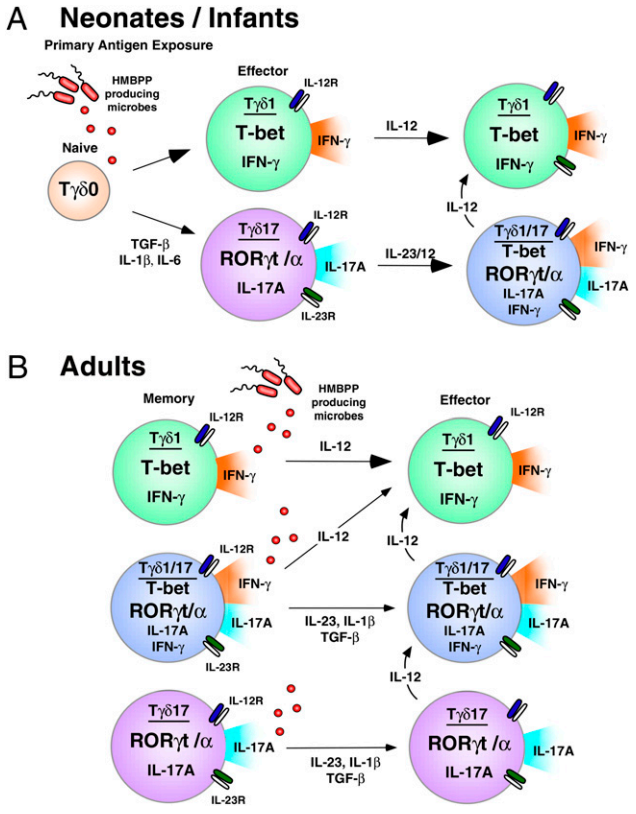

FIGURE 7. Steps in the differentiation and expansion of neonatal and adult Tγδ17 and Tγδ1/17 Vγ2Vδ2 T cells. *A*, Neonates/Infants. Naive Vγ2Vδ2 T cells present in neonates are polarized to the Tγδ17 phenotype by Ag activation in the presence of IL-6, IL-1β, and TGF-β. These early Tγδ17 cells are characterized by elevated expression of RORγt, IL-17A production, and minimal expression of IFN-γ and T-bet. The Tγδ17 cells upregulate IL-23R (and likely IL-12R), enabling them to maintain their Tγδ17 phenotype in the presence of IL-23, IL-1β, and TGF-β or to convert to a Tγδ1/17 phenotype in the presence of IL-23 or IL-12. *B*, Adults. Most adult Vγ2Vδ2 T cells are memory cells and include small but significant populations of Tγδ1/17 and Tγδ17 cells. Expansion of adult memory Tγδ1/17 and Tγδ17 cells by HMBPP requires IL-23 in addition to IL-1β and TGF-β, but not IL-6. Tγδ1/17 and Tγδ17 likely have limited persistence and are either short-lived effector populations or are converted to Tγδ1 through the effects of IL-12.

IL-6 (124, 125). We propose that these cytokines, in combination with bacteria- or parasite-derived HMBPP, differentiate naive Vγ2Vδ2 T cells into Tγδ17 T cells that produce IL-17A and express RORγt, IL-23R, and IL-12R. Our data suggest that the presence of IL-23 (or possibly IL-12) causes Tγδ17 Vγ2Vδ2 T cells to acquire IFN-γ production through the upregulation of T-bet, thereby converting to Tγδ1/17 cells. Because IL-12 production increases with age, responses to subsequent childhood infections are dominated by IL-12, converting most of the responding Tγδ17 to memory Tγδ1/17 by early adulthood (Fig. 7).

Vγ2Vδ2 T cells are of considerable interest because many infections lead to large expansions of these cells (reviewed in Ref. 41), and cancer immunotherapies specifically expanding Vγ2Vδ2 T cells have shown effectiveness against various tumors (126–129). Vγ2Vδ2 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, Vγ2Vδ2 T cells are specifically stimulated by prenyl pyrophosphates and bisphosphonates; both of which are well tolerated in vivo. Once activated, Vγ2Vδ2 T cells are broadly reactive to cancer cells of many tissue origins and to bacteria- and protozoan-infected cells while sparing normal cells. Vγ2Vδ2 T cells have traditionally been considered Th1-like cytotoxic T cells. However, we now show that Vγ2Vδ2 T cells can differentiate into Tγδ17 and Tγδ22 lineage cells. Others have demonstrated Vγ2Vδ2 T cells with characteristics of follicular homing CD4 αβ T cells (57, 130) and regulatory $CD25^+$ CD4 αβ T cells (131). Taken together, these findings indicate that Vγ2Vδ2 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.

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Disclosures

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