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Ontogeny of γδ T Cells in Humans

Stephen C. De Rosa,* James P. Andrus,† Stephen P. Perfetto,* John J. Mantovani,‡ Leonard A. Herzenberg,‡ Leonore A. Herzenberg,‡ and Mario Roederer[1]*

T cell receptors consist either of an α-chain combined with a β-chain or a γ-chain combined with a δ-chain. αβ T cells constitute the majority of T cells in human blood throughout life. Flow cytometric analyses presented in this study, which focus on the representation of the developmental (naive and memory) subsets of γδ T cells, show by function and phenotype that this lineage contains both naive and memory cells. In addition, we show that the representation of naive T cells is higher among αβ than γδ T cells in adults and that the low frequency of naive γδ T cells in adults reflects ontological differences between the two major γδ subsets, which are distinguished by expression of Vδ1 vs Vδ2 δ-chains. Vδ1 cells, which mirror αβ cells with respect to naive representation, predominate during fetal and early life, but represent the minority of γδ cells in healthy adults. In contrast, Vδ2 cells, which constitute the majority of adult γδ cells, show lower frequencies of naive cells than Vδ1 early in life and show vanishingly small naive frequencies in adults. In essence, nearly all naive Vδ2 cells disappear from blood by 1 year of life. Importantly, even in children less than 1 year old, most of the nonnaive Vδ2 cells stain for perforin and produce IFN-γ after short-term in vitro stimulation. This represents the earliest immunological maturation of any lymphocyte compartment in humans and most likely indicates the importance of these cells in controlling pathology due to common environmental challenges. *The Journal of Immunology,* **2004, 172: 1637–1645.**

T cell receptors either have an α-chain combined with a β-chain or a γ-chain combined with a δ-chain. T cells expressing αβ receptors represent the majority of T cells in human blood. The mechanisms of Ag processing and presentation for the CD4 and CD8 αβ T cell lineages are now well established. In contrast, T cells expressing γδ receptors represent a minority of blood T cells, and many questions remain unanswered with respect to the functions of these cells and the mechanisms through which they recognize Ags (reviewed in Refs. 1 and 2).

Compared with αβ TCRs, there are many fewer γ and δ genes available for recombination. Therefore, much of the γδ repertoire is achieved through TCR junctional diversity. In human T cells, only two δ genes, Vδ1 and Vδ2, are commonly used. mAbs specifically recognize TCRs using either of these δ genes. Thus, Vδ1- or Vδ2-expressing cells can be identified and studied using flow cytometry.

The representation of Vδ1 and Vδ2 γδ T cells varies with location and with disease status. Although Vδ2 cells typically constitute the majority among γδ T cells in blood, Vδ1 cells predominate at epithelial sites such as in the intestine and skin (3, 4). In certain diseases, the representation of Vδ1 or Vδ2 cells shifts dramatically. For example, in HIV disease Vδ2 cells are lost and Vδ1 cells expand (3, 5, 6). The significance of these changes is not known.

Functional differences have also been demonstrated between Vδ1 and Vδ2 cells. Vδ2 cells respond in vitro to small m.w. nonpeptidic phosphoantigens, originally described for Ags derived from mycobacteria (1, 7–9). Less is known about the Ag specificities of Vδ1 cells. However, Vδ1 cells in the intestinal epithelium can respond to stress-induced MHC class I-related molecules independent of Ag processing (10, 11).

Although some phenotypic analyses of γδ T cells beyond the subdivision into Vδ1 and Vδ2 subsets have been reported (12–14), few provide detailed phenotypic, functional, and developmental characterization of γδ T cells assessing multiple markers simultaneously (15). In this study, we apply immunophenotypic classification methods used to distinguish naive and memory αβ T cells (16, 17) to characterize the cells within the γδ T cell compartment early in life and in healthy adults. With these methods, we show that independent of T cell lineage (CD4, CD8), the γδ T cell compartment contains naive and memory subsets that are phenotypically and functionally comparable to those in the αβ compartment. In addition, we show a remarkably consistent assignment of functional attributes to phenotypically defined subsets among CD4, CD8, and γδ lineage T cells, demonstrating that functional and phenotypic maturation is highly parallel across lineage.

We also find distinct differences between the Vδ1 and Vδ2 subpopulations of γδ T cells. Although both Vδ1 and Vδ2 cells display signs of early activation in neonates, Vδ2 cells in particular are activated very early, with the majority of Vδ2 cells showing evidence of prior activation in individuals before 1 year. This represents the earliest immunological maturation of any lymphocyte compartment in humans and most likely reflects the importance of these cells in controlling pathology due to common environmental challenges.

Materials and Methods

Subjects

Subjects were recruited from the clinics and inpatient units of the Lucille Packard Children's Hospital at Stanford University. Blood was drawn at Stanford University and shipped to the National Institutes of Health for analysis. Cord blood was obtained from full-term newborn infants without outside factors for infection or immunosuppression. Other blood samples were drawn from infants and children undergoing elective surgery. All patients were essentially healthy and were presenting for elective surgical

procedures. All samples were drawn in the operating room or in the pre-anesthesia unit. No patients had undercurrent febrile illness or immune deficiency syndrome. Adult blood samples were drawn from healthy volunteers who worked in the hospital clinics or in the laboratory. A complete blood count was obtained for each sample by the clinical laboratory at Stanford University. The study was approved by the Institutional Review Boards both at Stanford and at the National Institutes of Health/National Institute of Allergy and Infectious Diseases.

In vitro stimulation

PBMC were isolated by Ficoll density centrifugation. Cells to be analyzed without stimulation were either prepared immediately for flow cytometric analysis (see below) or placed in culture overnight without stimulation for concurrent analysis with the stimulated cells. For IFN-γ and IL-2 analysis, cells at $1–2 \times 10^6$/ml were stimulated for 6 h with PMA (10 ng/ml; Sigma-Aldrich, St. Louis, MO) and ionomycin (2 μM; Sigma-Aldrich) in 24- or 48-well plates. For phosphoantigen stimulation, cells were stimulated overnight with isopentenyl pyrophosphate (IPP,[2] 30 μg/ml; Sigma-Aldrich) in 96-well V-bottom plates at a density of 1 million cells per 200 μl well. Monensin (2 μM; BD PharMingen, San Jose, CA) was also included in all stimulations. Depending on the number of cells available, between 3 and 6 million cells were cultured for each stimulation condition in separate wells. After stimulation, the cells from multiple wells for each condition were combined.

Flow cytometric analysis

For analysis of perforin expression, cells were surface stained with all the Ab reagents (18) except for those directed to cytokines, perforin, and CD3. In addition, during this staining, ethidium monoazide bromide (EMA, 1 μg/ml; Molecular Probes, Eugene, OR) was included to label dead cells, and CD163 and CD19, detected in the same channel as EMA, were included to label and exclude monocytes and B cells. An Ab to glycophorin A, CD235, was often also included in this dump channel to exclude red cells. Cells were stained for 10 min at room temperature in the dark, and then placed under an intense fluorescent light source for 10 min to photo-activate the EMA (leading to covalent cross-linking with DNA). After washing, fixation, and permeabilization (Cytofix/Cytoperm kit; BD Phar-Mingen), cells were stained intracellularly for cytokines or perforin, and CD3 (to detect both surface and internalized CD3). The multicolor FACS methods have been described elsewhere (19–22).

All mAb reagents, either purified or preconjugated, except for Texas Red-PE (TRPE) CD3 and CD45RO, were obtained from BD PharMingen. TRPE CD3 and CD45RO were obtained from Immunotech/Beckman Coulter (Miami, FL). Abs conjugated in our laboratory were done using standard protocols (http://drmr.com/abcon), and validated by comparison with commercial conjugates after appropriate titration. The fluorescent dyes used in each staining combination include the following: FITC, PE, TRPE, Cy5PE, Cy5.5PE, Cy7PE, Alexa 594, allophycocyanin, Cy5.5 allophycocyanin, Cy7 allophycocyanin, Cascade Blue, and Alexa 430. PE and allophycocyanin were obtained from ProZyme (San Leandro, CA). Cy5, Cy5.5, and Cy7 were obtained from Amersham Life Science (Pittsburgh, PA). The Alexa dyes and Cascade Blue were obtained from Molecular Probes.

A typical 12-color staining combination used for the cytokine analysis was: Cascade Blue CD45RO, Alexa 430 CD57, FITC Vδ1, PE Vγ9, TRPE CD3, EMA/CD163/CD19 (detected in Cy5PE channel), Cy5.5PE CD4, Cy7PE Vδ2, Alexa 594 IFN-γ, allophycocyanin IL-2, Cy5.5 allophycocyanin CD11a, and Cy7 allophycocyanin CD8. An alternate combination included CD27 and did not include Vγ9. For this panel, the following substitutions were made: Cascade Blue CD27, PE IL-2, Cy7PE CD11a, allophycocyanin Vδ2, Cy5.5 allophycocyanin CD8, and Cy7 allophycocyanin CD45RO. The combination for perforin analysis was similar to this latter panel, substituting: PE Vγ9 and Alexa 594 perforin. Because the perforin analysis used unstimulated PBMC processed by Ficoll, and therefore was not likely to include many dead cells, a panel not including a dump channel was sometimes used: Cascade Blue CD3, Alexa 430 CD57, FITC Vδ1, PE Vγ9, TRPE CD62L, Cy5PE CD27, Cy5.5PE CD4, Cy7PE CD11a, Alexa 594 perforin, allophycocyanin Vδ2, Cy5.5 allophycocyanin CD8, and Cy7 allophycocyanin CD45RO. An alternate panel, not including perforin, was also used for unstimulated cells: Cascade Blue CD62L, Alexa 430 CD57, FITC Vδ1, PE Vγ9, TRPE CD45RO, Cy5PE dump (as above), Cy5.5PE CD4, Cy7PE Vδ2, Alexa 594 CD45RA, allophycocyanin CD3, Cy5.5 allophycocyanin CD11a, and Cy7 allophycocyanin CD8. For Fig.

1*A*, the following panel was used: Cascade Blue CD57, FITC Vδ1, PE Vδ2, TRPE CD45RO, Cy5PE CD62L, Cy5.5PE CD45RA, Cy7PE CCR7, allophycocyanin CD27, Cy5.5 allophycocyanin CD11a, Cy7 allophycocyanin CD8, and biotin CD3. Biotin was revealed with streptavidin Quantum-Dot 605, obtained from QuantumDot (Hayward, CA).

Cells were analyzed on a FACS Digital Vantage, DiVa (BD Biosciences, San Jose, CA), equipped for detection of 12 fluorescences or the LSRII for Fig. 1*A* (BD Biosciences). Between 0.5 and 2.5 million events were collected for each sample. Data were analyzed using FlowJo versions 3.6 and 4.1 (Tree Star, San Carlos, CA).

Statistical analysis

All FACS plots are displayed with five-decade logarithmic axes (except forward scatter, linear) with a data transformation that allows for improved visibility of events that fall at the lower end of the log scale (D. Parks, W. Moore, and M. Roederer, manuscript in preparation). Except for Figs. 3 and 4, FACS plots are displayed as 5% contour graphs with outlying events beyond the last contour shown as dots. In Figs. 3 and 4, the FACS plots are shown as pseudocolor density plots. The γδ subsets in these figures are shown at lower resolution so that the lower frequency events appear as larger dots. For the overlay plots in these figures, one subset is shown either as white contours or white dot plots, depending on the number of events in the subset.

Data were analyzed using JMP software, version 5 (SAS Institute, Cary, NC). Comparisons between groups were determined by the Wilcoxon rank sums test. Data are shown as box plots in which the ends of the box are the 25th and 75th percentiles, and the line across the middle indicates the median. The lines above and below the box extend to the outermost data point that falls within 1.5× interquartile range.

Results

Naive and memory γδ T cell subsets can be identified by surface phenotype

For αβ T cells, there are several surface markers that are capable of differentiating naive cells from cells that are activated or that have previously encountered Ag (i.e., effector/memory cells). Two or more of these markers must be used in combination to achieve the most accurate discrimination of these subsets. Often CD45RA or CD45RO is used in combination with additional markers such as CD62L, CD11a, CD27, and CCR7.

In a small number of adult subjects, we examined the expression of six naive-defining markers simultaneously on αβ and γδ T cells (Fig. 1*A*). Because either expression of CD45RA, or lack of expression of CD45RO provides similar information regarding naive cell identification (data not shown), we typically use only one of these markers. Therefore, in Fig. 1, we gated on CD45RO⁻ cells and then examined two other markers, CD11a and CD27.

Naive cells express the brightest levels of CD27 and express CD11a at dull levels. Cells expressing low levels of CD27 are CD11a^bright, and are therefore not naive. Because distinguishing bright from dull levels of CD27 is difficult without an additional marker, we prefer to use CD11a over CD27 when limited to two naive-defining markers. After gating on three markers (CD45RO⁻, CD11a^dull, CD27^bright), use of an additional marker such as CD62L is generally not necessary, because nearly all of these cells are CD62L⁺ (Fig. 1*A*, *right panels*). CD62L provides clear distinction of naive and memory subsets, but its use is limited by the fact that this marker is usually lost when cells are stimulated or when cells are cryopreserved. In addition, we found that CD62L was lost from a large number of T cells after whole blood was shipped overnight to our laboratory at the National Institutes of Health. We suspect this is likely a consequence of temperature variations during transit. CCR7 is useful as a naive-defining marker for αβ T cells, but not for γδ T cells (Fig. 1*A*, *right panels*).

Therefore, we chose to use CD45RO in combination with CD11a as the primary naive-defining markers. We included CD27 as a third naive-defining marker in over one-third of the samples we analyzed. As previously demonstrated for CD4 and CD8 αβ T

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[2] Abbreviations used in this paper: IPP, isopentenyl pyrophosphate; EMA, ethidium monoazide bromide; TRPE, Texas Red-PE.
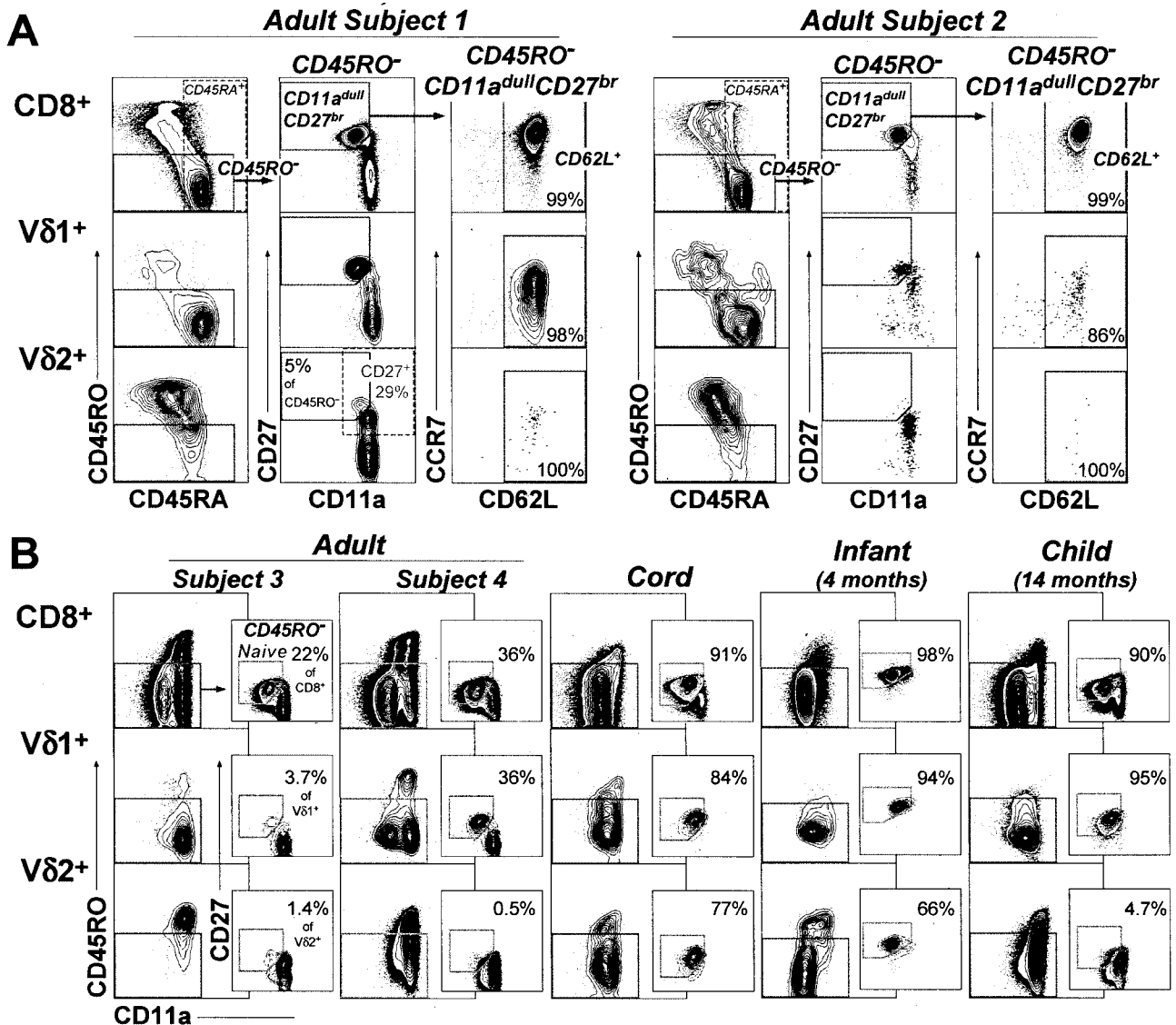
FIGURE 1. Naive and memory subsets of Vδ1⁺ and Vδ2⁺ γδ T cells can be identified by surface staining with CD45RO, CD11a, and CD27. *A*, For two adult subjects, PBMC were stained for six naive-defining markers. CD3⁺CD8⁺ (*upper panels*), Vδ1⁺ (*middle*), and Vδ2⁺ (*lower*) T cells are shown after scatter gating for lymphocytes. For each subject and for each subset, the plots on the *left* show the expression of CD45RA (abscissa) and CD45RO (ordinate). After gating on all CD45RO⁻ cells (which include the naive cells), the next column of panels shows the expression of CD11a (abscissa) and CD27 (ordinate) for the CD45RO⁻ cells. Finally, the *third column* of panels shows the expression of CD62L (abscissa) and CCR7 (ordinate) for the cells gated on three markers (CD45RO⁻ CD11a^dull CD27^bright). The percentages of the triple-gated cells that express CD62L are shown. For the Vδ2 cells for subject 1, the percentages of the CD45RO⁻ cells that are CD11a^dull/CD27^bright or that are CD27⁺ (including CD27^bright and CD27^low, without gating for CD11a) are listed for comparison. A few panels are shown as dot plots because of the small number of cells in the subset. *B*, CD3⁺CD8⁺ (*upper panels*), Vδ1⁺ (*middle*), and Vδ2⁺ (*lower*) T cells are shown after gating for EMA⁻ (live), scatter-gated lymphocytes. For each subject and for each subset, the plots on the *left* show the expression of CD11a (abscissa) and CD45RO (ordinate). After gating on all CD45RO⁻ cells (which include the naive cells), the *inset* shows the expression of CD11a (abscissa) and CD27 (ordinate) for the CD45RO⁻ cells. The percentages of cells within each CD8⁺, Vδ1⁺, or Vδ2⁺ subset that are naive (CD45RO⁻ CD11a^dull CD27^bright) are listed. Examples of two adult subjects demonstrating different staining profiles are shown on the *left*. A cord blood sample is shown in the *middle*, and two pediatric samples are shown on the *right*.

cells, naive cells do not express CD45RO, express low levels of CD11a, and express high levels of CD27 (16, 23, 24). All other cells are defined as nonnaive.

Fig. 1*B* shows examples of the expression of CD45RO, CD11a, and CD27 on T cell subsets in two adult blood samples, a cord blood sample, and two pediatric blood samples. Cells are first gated as CD45RO⁻, and then the CD45RO⁻ cells are further gated as CD11a^dull CD27^bright to identify naive cells. For the CD8 T cells, the naive cells are clearly identified as a separate population.

We used these same markers to determine whether comparable populations can be identified among γδ T cells. Because two major

subsets of γδ T cells, Vδ1 and Vδ2, account for the vast majority of γδ T cells in blood and can be identified by surface staining with mAbs, the naive-defining markers have been examined separately on the Vδ1 and Vδ2 subsets. Prior studies have noted a difference in the expression of CD45RO on Vδ1 and Vδ2 cells (12–14): in the adult, most Vδ2 cells express CD45RO and most Vδ1 cells do not.

As demonstrated by the expression of CD45RO on the γδ subsets in the two adult donors in Fig. 1, we also find that in adults, most Vδ1 cells do not express CD45RO, and most Vδ2 cells express high or intermediate levels of CD45RO. In addition, we find

that the lack of expression of CD45RO is not sufficient to pheno-typically identify cells as naive. Although there are Vδ1 and Vδ2 cells that are CD45RO⁻, many are CD11a^bright (and CD27⁻ or CD27^dull), and therefore do not fit the naive phenotype. Therefore, as for αβ T cells, two or more markers must be used or used to phenotypically identify γδ cells as naive.

For both αβ and γδ subsets in adult samples, two markers, CD45RO and CD11a, work very well to identify naive cells because there is a clear demarcation between CD11a^dull and CD11a^bright cells. CD11a works less well for cord blood and blood samples from infants. In part, this is due to the fact that there are few CD11a^bright cells to contrast with the CD11a^dull cells. In addition, naive cells stain slightly brighter for CD11a than do naive cells in adult and older pediatric samples. However, even with these differences, staining for CD11a is brighter on activated/memory cells. This is demonstrated for the Vδ2 cells from the 14-mo-old child in Fig. 1, in which the lower level of staining for CD27 confirms the transition from the naive phenotype. For ∼40% of our samples, we include CD27 as a third naive-defining marker (7 of 14 cord blood, 9 of 37 pediatric, 16 of 27 adult). For these samples, the naive frequency determined by all three markers correlates well with the naive frequency determined by CD45RO and CD11a alone (R^2 = 0.98, 0.94, 0.79, and 0.91 for the CD4, CD8, Vδ1, and Vδ2 subsets, respectively). Therefore, the naive frequency is accurately predicted by using only CD45RO and CD11a for the remainder of our samples that were not stained with CD27.

Vδ2 naive cells are lost early in life

Although lack of CD45RO is by itself not sufficient to identify cells as naive, CD45RO expression is sufficient to identify cells as nonnaive. Several previous studies demonstrated that the vast majority of Vδ2 cells in the adult are CD45RO⁺ and, thus, nonnaive (12–14). One study has shown that Vδ2 cells in cord blood do not express CD45RO and that CD45RO expression on Vδ2 cells increases with age in children (12). Because there is such a dramatic difference in CD45RO expression between Vδ1 and Vδ2 cells in adults, we examined in greater detail the changes in the naive and memory subpopulations of γδ T cells in early life.

Examples of the staining of a cord blood sample and two pediatric samples are shown in Fig. 1. The majority of CD8, Vδ1, and Vδ2 cells are naive in cord blood and in the infant sample. However, in the sample from a child 14 mo old, most of the Vδ2 cells are already nonnaive. This is in sharp contrast to the other T cell subsets, in which a majority of cells are still naive.

The *right panels* in Fig. 2 show the kinetics of the disappearance of naive T cells from blood. For both Vδ1 and Vδ2 cells, the major loss of naive cells as a percentage of the γδ subset occurs during the first year of life. This loss is most dramatic for Vδ2 cells in which a very small percentage of the Vδ2 cells remains naive after 1 year. In contrast, a relatively high proportion of naive Vδ1 cells persists through childhood. The proportion of naive CD4 and CD8 αβ T cells also decreases early in life; however, this does not occur as early as for γδ T cells and not to the extent observed for Vδ2 cells. Even before birth, as demonstrated in cord blood, in contrast to αβ T cells, many γδ cells are already nonnaive (Fig. 2, *right panels*).

The absolute counts compared between cord blood and the pediatric samples indicate a significant drop after birth in overall Vδ1 cells, but no change in Vδ2 cells (Fig. 2). This results in an inversion of the Vδ1-Vδ2 ratio, from a median of 3.0 for cord blood to 0.4 for the pediatric group. Naive Vδ1 counts drop between cord blood and the pediatric group, and again between the pediatric and adult groups. For Vδ2 cells, the naive cell drop between the cord blood and pediatric groups is not significant, most likely due to the large number of cells already nonnaive in cord blood (a median of 50%).

Naive and memory γδ T cells have functional similarities to their αβ counterparts

Naive and memory αβ T cells have distinct cytokine profiles in response to short-term polyclonal stimulation: naive cells produce IL-2 and few other cytokines, while memory/effector cells produce multiple cytokines, including the prototypical effector cytokine, IFN-γ. To determine whether the phenotypic definition of naive and memory γδ cells correlates with function, production of IFN-γ and IL-2 was examined following in vitro stimulation with PMA and ionomycin. As demonstrated in Fig. 3 for one adult, cells

FIGURE 2. The percentages and absolute counts of αβ and γδ T cells that are naive drop early in life. Data for CD4⁺ (*upper row*), CD8⁺ (*second row*), Vδ1⁺ (*third row*), and Vδ2⁺ (*lower row*) T cells are shown. Subsets have been gated as CD3⁺, EMA⁻ (live), scatter-gated lymphocytes. Naive cells have been additionally gated by two markers in some subjects (CD45RO⁻ CD11a^dull, squares), or by three markers in other subjects (CD45RO⁻ CD11a^dull CD27^bright, ○). The absolute counts for the overall subset (*left panels*) and the naive subsets (*middle*) are plotted for the cord, pediatric, and adult blood samples. Absolute counts have been calculated by multiplying the subset frequency relative to lymphocytes by the absolute lymphocyte count determined from the complete blood count. An asterisk indicates a significant difference ($p < 0.05$) between adjacent groups. The graphs on the *right* indicate the percentage of cells within the indicated subset that is naive plotted vs age. The age axis is displayed with three scales (0–2, 2–14, above 14 years) so that the infant and pediatric scales are expanded. The percentage of cord blood cells that are naive is grouped on the *left side* in these graphs.
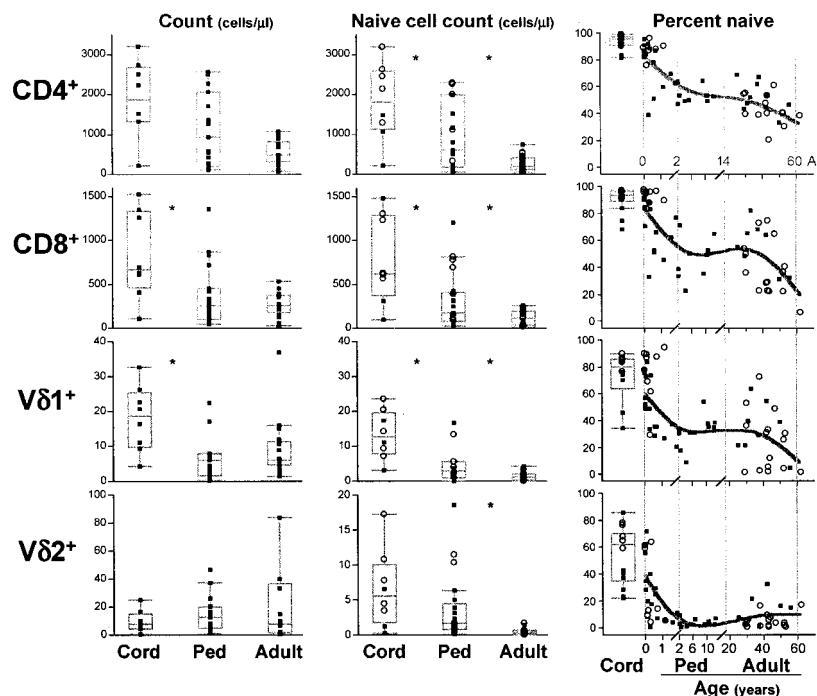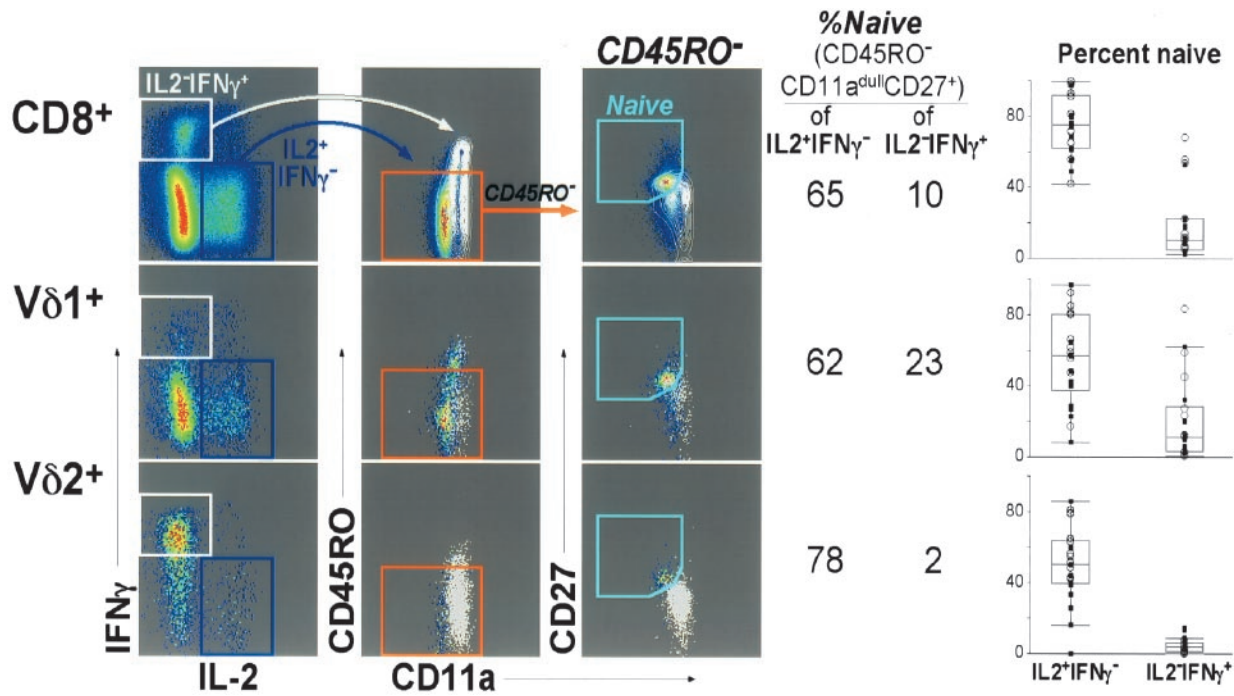
FIGURE 3. Naive and memory subsets of both $\alpha\beta$ and $\gamma\delta$ T cells have similar expression of IFN-γ and IL-2 in response to stimulation with PMA and ionomycin. CD3$^+$CD8$^+$ (*upper panels*), Vδ1$^+$ (*middle*), and Vδ2$^+$ (*lower*) T cells are shown after gating for EMA$^-$ (live), scatter-gated lymphocytes in one adult. *Left panels*, Show the expression of IL-2 (abscissa) and IFN-γ (ordinate) for the entire subset indicated to the *left*. Gates are drawn to show IL-2$^+$ IFN-γ^- cells (blue) and IL-2$^-$ IFN-γ^+ cells (white). The second set of panels shows the expression of CD11a (abscissa) and CD45RO (ordinate) for cells that express only IL-2 (pseudocolor) or only IFN-γ (white contours or dots). These cells are further gated as CD45RO$^-$ (which include naive cells), and the expression of CD11a (abscissa) and CD27 (ordinate) for the CD45RO$^-$ cells is shown in the third set of panels. In both the second and the third sets of panels, the IL-2$^-$ IFN-γ^+ cells overlay the IL-2$^+$ IFN-γ^- cells. The percentages of cells within each of these cytokine-defined subsets that have the naive phenotype (CD45RO$^-$ CD11a^{dull} CD27^{bright}) are listed. *Right panels*, Summarize these percentages for all adult and pediatric samples analyzed. For some samples, three naive-defining markers were used (CD45RO$^-$ CD11a^{dull} CD27^{bright}, $\bigcirc$, $n = 11$); for other samples, two naive-defining markers were used (CD45RO$^-$ CD11a^{dull}, squares, $n = 11$). Data for cord blood samples are not included because the frequency of IFN-γ-producing cells was very low. All three comparisons are significant at $p < 10^{-4}$.

producing only IL-2 are enriched for naive cells, and cells producing only IFN-γ are enriched for nonnaive cells. This is true for CD8 $\alpha\beta$ T cells as well as for the $\gamma\delta$ T cells. The tendency for IL-2$^+$ IFN-γ^- cells to be enriched for naive cells is significant when all the adult and pediatric samples examined for cytokine production are included in the analysis (Fig. 3, *right panels*).

Another difference observed between naive and memory $\alpha\beta$ T cells is the expression of perforin. Perforin is expressed in cytotoxic effector cells, but not in naive cells. Fig. 4 shows the expression of perforin as determined by intracellular staining in an adult and an infant. For both the CD8 $\alpha\beta$ cells and the $\gamma\delta$ cells, the subset of cells not expressing perforin is enriched for naive cells. This figure demonstrates that there are two levels of expression of perforin, high and low, and the data presented for the two subjects shown in this figure suggest that when perforin is expressed by $\gamma\delta$ T cells, the Vδ1 cells express high levels and the Vδ2 cells express low levels. When all the perforin data for adult samples are analyzed, there is a significant difference between the percentage of perforin-expressing Vδ1 and Vδ2 cells that express high perforin levels (Fig. 5). This pattern is evident for most subjects, but this figure also shows that several subjects do not fit this profile, e.g., there are three subjects for whom less than 50% of the perforin-expressing Vδ1 cells express high levels of perforin.

Vδ2 cells show evidence of activation early in life

Consistent with the early loss of most Vδ2 cells of the naive phenotype, by ~1 year of age, a large proportion of Vδ2 cells stains for perforin and produces IFN-γ in response to polyclonal stimu-

lation. Staining examples in two adults are shown in Figs. 3 and 4. In four pediatric samples analyzed, unlike the CD8$^+$ and Vδ1$^+$ cells, the majority of the Vδ2$^+$ cells stain for IFN-γ (Fig. 6). All four samples are from subjects less than 1.5 years of age, indicating that the Vδ2 cells become responsive to PMA/ionomycin stimulation (in terms of IFN-γ production) very early in life. This responsiveness persists into adulthood. Interestingly, even in cord blood, a substantial proportion of Vδ2 cells produces IFN-γ (Fig. 6).

For the four pediatric samples examined for perforin expression (different from the four samples analyzed for IFN-γ production), two of the four show a high proportion of Vδ2 cells staining for perforin (Fig. 6). These two pediatric samples are from subjects 2 and 4 mo old, indicating that it is in this early time that many Vδ2 cells acquire an activated phenotype.

Vδ2 is preferentially paired with Vγ9 in the adult, and these cells respond to phosphoantigen stimulation

The Ag specificities of $\gamma\delta$ T cells are not well understood; however, $\gamma\delta$ T cells expressing the Vδ2Vγ9 TCR are known to respond to in vitro stimulation with small m.w. phosphoantigens. After overnight stimulation with one such Ag, IPP, responding Vδ2 cells can be detected by intracellular staining with IFN-γ. Although a variable percentage of Vδ2 cells in adults and children responds to this stimulation, few cord blood cells respond (Fig. 7).

This would indicate that either Vδ2 cells with this specificity are not present in cord blood or that they have not been previously activated and therefore cannot respond within the short time frame

FIGURE 4. High and low levels of perforin expression can be identified on comparable subsets of CD8$^+$, Vδ1$^+$, and Vδ2$^+$ T cells: naive T cells are enriched among cells not expressing perforin. Data are shown for an adult (*upper set* of panels) and an infant (*lower set* of panels). CD3$^+$CD8$^+$ (*upper row*), Vδ1$^+$ (*middle*), and Vδ2$^+$ (*lower*) T cells are shown after gating for EMA$^-$ (live), scatter-gated lymphocytes. *Left panels*, Show the expression of perforin (abscissa) and CD45RO (ordinate) for the entire subset indicated to the *left*. Gates are drawn to show perforin$^-$ and perforin$^+$ cells. The second set of panels shows the expression of CD11a (abscissa) and CD45RO (ordinate) for cells that express perforin at high or intermediate levels (white contours or dots) or do not express perforin (pseudocolor). These cells are further gated as CD45RO$^-$ (which include naive cells), and the expression of CD11a (abscissa) and CD27 (ordinate) for the CD45RO$^-$ cells is shown in the third set of panels. In both the second and the third sets of panels, the perforin-expressing cells overlay the perforin$^-$ cells. The percentages of cells within the perforin$^-$ or the perforin$^+$ subsets that have the naive phenotype (CD45RO$^-$ CD11a^{dull} CD27^{bright}) are listed.
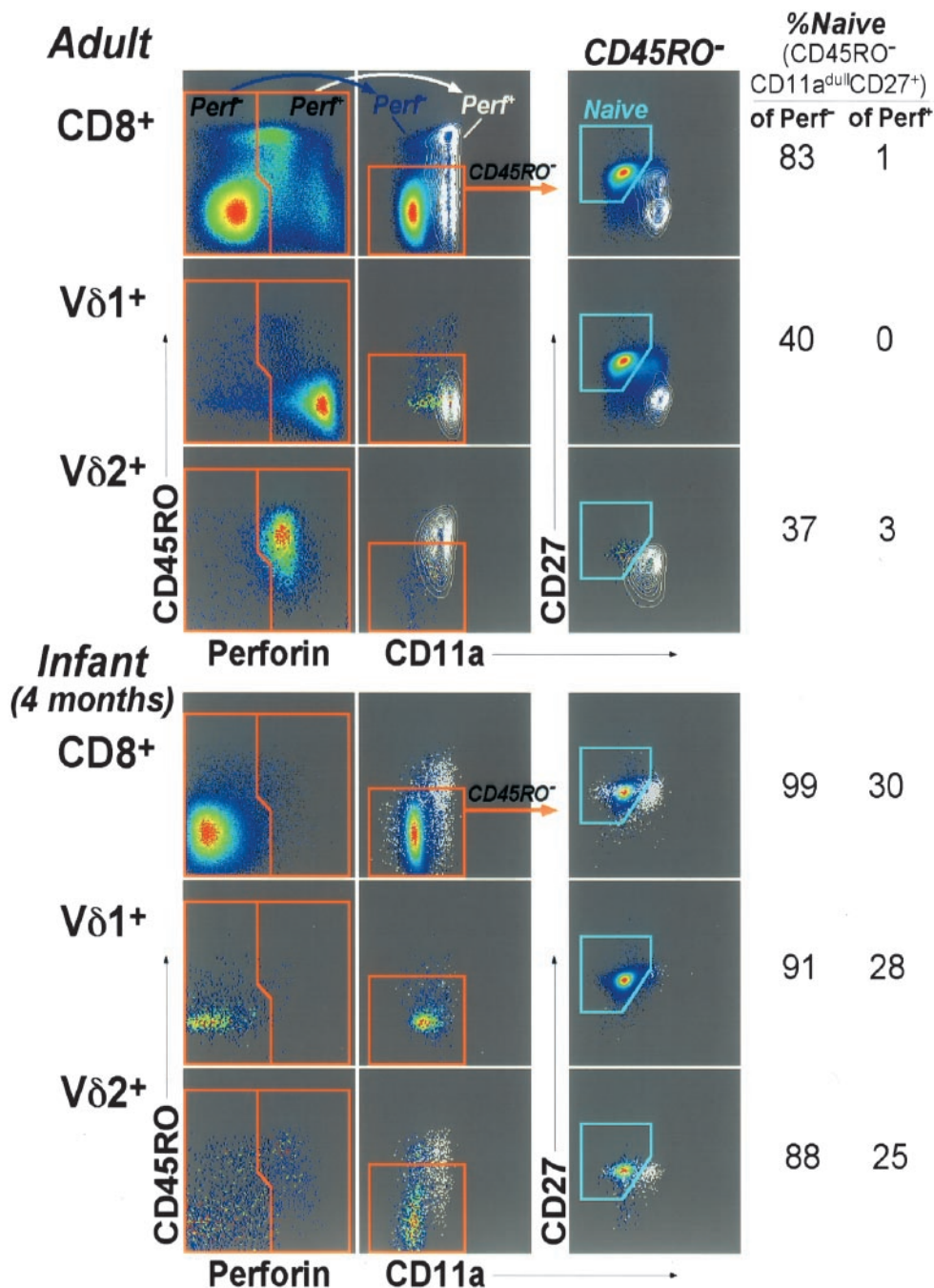
of the stimulation. To determine the relative frequency of cells expressing the Vγ9Vδ2 TCR, the receptor responsive to IPP, the percentage of Vδ2 cells coexpressing Vγ9 was determined by costaining with a mAb to Vγ9. As previously shown, the majority of Vδ2 cells in the adult coexpress Vγ9 (Fig. 8). However, the pairing of Vδ2 with Vγ9 is not the dominant γδ TCR in cord blood and young pediatric samples. Among the pediatric samples analyzed in Fig. 8, the proportion of Vδ2 cells coexpressing Vγ9 increases with age. There is no age-dependent preferential pairing of Vδ1 with Vγ9.

Discussion

This study characterizes the differentiation of human blood γδ T cells in early life. Both the two major types of human γδ T cells, those using either the Vδ1 or Vδ2 TCRs, show signs of prior activation early in life, even before birth. Remarkably, by 1 year of age, almost all Vδ2 T cells in blood have been previously activated and are thus memory T cells. Little is known about the Ag specificities of γδ T cells and about the physiologic roles these cells play. The results of this study provide additional clues as to the nature of these cells, and when interpreted in the context of what has been previously published, help to define the ontogeny of the Vδ1 and Vδ2 γδ T cell subsets in the human.

One difference observed between αβ and γδ T cells is that both Vδ1 and Vδ2 cells include a relatively high proportion of nonnaive cells before birth, as measured in cord blood. Because this activation occurs before environmental exposure, these cells may be responding to self Ags. Consistent with this hypothesis, γδ T cell responses to keratinocytes (25, 26) and stress-induced self Ags have been observed (10, 11), and it has been proposed that Vδ2 cells that recognize phosphoantigens present on foreign pathogens may also recognize homologous self Ags (1).
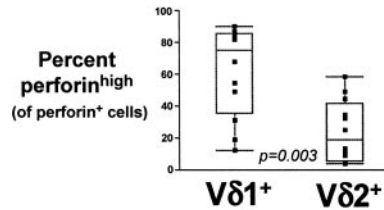
FIGURE 5. When they express perforin, Vδ1⁺ cells tend to express high levels and Vδ2⁺ cells tend to express low levels. Shown are the percentages of perforin-expressing Vδ1⁺ or Vδ2⁺ T cells that express high levels of perforin. Data for adults are shown.

Shortly after birth, within the first year of life, both the Vδ1 and Vδ2 cells show a rapid decline in the proportion of naive cells. The exposure to environmental Ags that occurs after birth is a likely cause for this early activation. The prevalence of γδ T cells at epithelial surfaces, where they represent the first line of defense, may explain the greater extent of γδ T cell activation compared with αβ T cells. There is also a difference in the kinetics of naive T cell loss between the Vδ1 and Vδ2 cells. Although the Vδ1 naive cells drop to below 40%, the Vδ2 cells drop to well below 10%.

To account for this difference between Vδ1 and Vδ2 cells, we propose that during the ontogeny of these subsets, there are developmental windows when either Vδ1 or Vδ2 cells are generated. This would be similar to the so-called waves of T cell development observed in the mouse and in the chicken (27–29). These studies have shown that T cells bearing certain γδ TCRs are only generated during fixed periods and that they then home to certain peripheral sites, where they are maintained by self-renewal. Our data suggest that this may be the case for the majority of Vδ2 cells. If they only develop during a fixed period before or near birth, as they become activated, their numbers are not regenerated by the new generation of naive cells, but instead by proliferation of existing cells.

In contrast, because relatively large numbers of naive Vδ1 cells are found in blood into adulthood, these cells may be regenerated
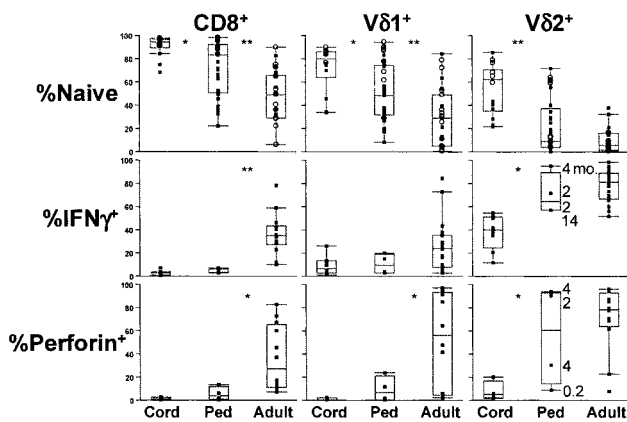
FIGURE 6. Vδ2⁺ T cells show evidence of early activation, as shown by loss of the naive phenotype and staining for IFN-γ and perforin. Data for CD8⁺ (*left panels*), Vδ1⁺ (*middle*), and Vδ2⁺ (*right*) T cells are shown. The percentage of cells that are naive is shown in the *upper row*. Naive cells were gated either with three naive-defining markers (CD45RO⁻ CD11a^dull CD27^bright, ○) or with two naive-defining markers (CD45RO⁻ CD11a^dull, squares). The percentage of cells that produce IFN-γ after 6-h stimulation with PMA and ionomycin is shown in the *middle row*. The percentage of cells that express perforin (either low or high levels) is shown in the *lower row*. The age in months is shown for the four pediatric samples in the *lower rows*. Asterisks indicate significant differences between adjacent groups: *, $p < 0.05$; **, $p < 0.001$.
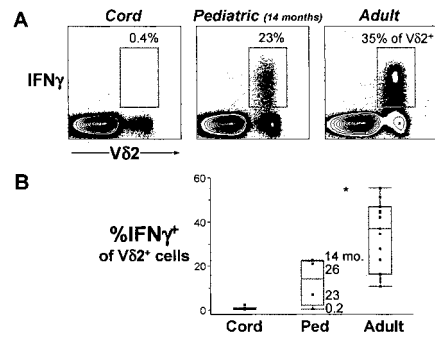

FIGURE 7. After overnight stimulation with IPP, some Vδ2⁺ T cells express IFN-γ in pediatric and adult blood samples. *A*, The expression of Vδ2 (abscissa) vs IFN-γ (ordinate) is shown for samples of cord, pediatric, and adult blood after overnight stimulation with IPP. The percentage of Vδ2⁺ cells expressing IFN-γ is shown. *B*, The percentage of Vδ2⁺ cells that express IFN-γ after overnight stimulation with IPP is shown for cord, pediatric, and adult samples. The age in months is shown for the four pediatric samples. The asterisk indicates a significant difference of $p = 0.03$. For comparison of the cord and pediatric groups, $p = 0.06$.

continuously by de novo development in the thymus. Consistent with this hypothesis, Vδ1 transcripts are prevalent in the postnatal thymus, while Vδ2 transcripts are prevalent in the fetal thymus (30–32). Furthermore, the number of Vδ1 naive cells in blood decreases at a rate similar to that for putatively thymus-derived naive CD4 and CD8 T cells, i.e., they persist through childhood, with gradual loss through adulthood thought to be a consequence of thymic atrophy. Note that it is possible that a small subpopulation of Vδ2 cells is also continually produced by the thymus, because small numbers of naive Vδ2 cells are present in adults.

Based solely on phenotypic analyses, we previously hypothesized that Vδ1 T cells in adult humans are extrathymically derived (22). Although the present more-detailed analysis suggests that they are in fact likely to be thymically derived during early development through adulthood, it is possible that extrathymic differentiation of Vδ1 T cells becomes more prevalent later in life, as thymic output dwindles. Nonetheless, the conclusions of our earlier report are open to new interpretation based on this new data.

Vδ2 cells coexpressing Vγ9 are known to respond to small m.w. phosphoantigens present on the cell surface of some pathogenic bacteria, as well as in human cells (8, 9). In the adult, the large majority of Vδ2 cells coexpress Vγ9. Because the proportion of
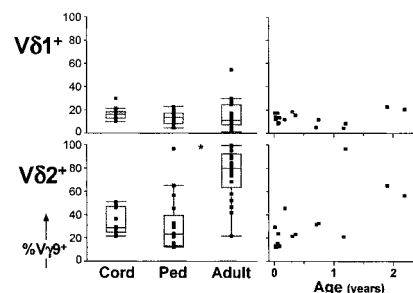
FIGURE 8. The proportions of Vδ2⁺ T cells that express Vγ9 are low in cord and pediatric blood samples, and increase with age. The proportion of Vδ1⁺ (*upper*) or Vδ2⁺ (*lower*) cells that coexpress Vγ9 is shown for the three subject groups (*left panels*). *Right panels*, Show the proportion of cells expressing Vγ9 (ordinate) vs age (abscissa) for the pediatric samples. The asterisk indicates a significant difference $p < 10^{-4}$.

Vδ2 cells coexpressing Vγ9 is low in cord blood and increases with age, it is likely that there is significant positive selection for these cells. There are many different phosphoantigens that are reactive with Vδ2Vγ9 cells, so it may be exposure to a variety of pathogens that results in the selection of these cells. In the macaque, this subset expands in response to mycobacterial infection (33). A study of the life span of $\gamma\delta$ T cells in adult thymectomized mice found that the $\gamma\delta$ cells had a rapid turnover and displayed an activated/memory phenotype, suggesting a chronic response to environmental Ags (34). However, because this cell type is also potentially autoreactive, it may be exposure to self Ags that arise after birth that leads to their increase.

All of the studies reported in this work study T cells in peripheral blood. This is a limitation especially when studying $\gamma\delta$ T cells. Although $\gamma\delta$ T cells are present in low proportions in blood, they are present in much higher proportions in other anatomical sites such as epithelial surfaces in the intestine and skin. In fact, it is likely that $\gamma\delta$ cells are functionally active at these sites. The changes in the frequencies of these cells that we observe in blood may reflect changes at these peripheral sites and/or altered trafficking between the blood and these sites. Clearly, extrapolation of the results from blood cell studies to other immunological sites should be made cautiously. In general, however, the proportion of naive cells in peripheral sites is much lower than that in blood (because of the selective homing of memory/activated cells to those sites). Thus, the dynamics we report in this work, ontologically early loss of naive T cells, must only be more dramatic for these tissues.

Age-related changes in the $\gamma\delta$ repertoire with an expansion of Vδ2 cells and an increase in the expression of CD45RO on Vδ2 cells have been previously reported (12). In this prior report, consistent with our findings, cord blood $\gamma\delta$ cells were largely CD45RO$^-$. The authors also noted an age-related increase in CD45RO expression on Vδ2 cells not observed on Vδ1 cells. They reported that early in life, close to 50% of the Vδ2 cells expressed CD45RO, and that this percentage gradually increased in adults. Because they only measured CD45RO expression, they were unable to identify the nonnaive CD45RO$^-$ cells that we demonstrate appear very early in life. In addition, they could not identify the loss of the large majority of naive Vδ2 cells within the first year of life. The authors also report that most thymic $\gamma\delta$ cells stain for Vδ1, which is consistent with our hypothesis of continual thymic replenishment of Vδ1 cells.

A recent paper reports phenotypic and functional data for naive and memory subsets of Vδ2 cells in blood, lymph nodes, and inflammatory sites (15). The results are consistent with ours, with the exception of reporting a higher proportion of naive Vδ2 cells in adults. This difference is most likely due to the strategy used to identify naive cells. Dieli et al. (15) use only the expression patterns of CD45RA and CD27. As illustrated in Fig. 1A, we found that CD27^{bright} cells are difficult to distinguish from CD27^{low} cells without the use of an additional marker such as CD11a. Therefore, the use of CD27 with CD45RA to identify naive cells can result in nonnaive CD27^{low} cells being classified as naive, leading to a significant overestimation of the frequency of Vδ2 T cells that are naive.

In summary, we describe the developmentally related changes in $\gamma\delta$ T cell subsets that occur early in life. These changes are in many ways similar to those observed for other species, suggesting that the human immune system is also capable of waves of T cell development. A significant difference between the $\gamma\delta$ and $\alpha\beta$ T cell lineages is the much earlier activation and conversion to memory of the $\gamma\delta$ T cells, and illustrates the central role that $\gamma\delta$ T cells have in addressing Ag challenge from birth onward. This is particularly true for Vδ2 T cells, which show the earliest evidence of developmental maturation of any adaptive immune system.

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