Vδ1 and Vδ2 γδ T cells express distinct surface markers and might be developmentally distinct lineages

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Abstract: We report here that the two major types of γδ T cells found in human blood, Vδ1 and Vδ2, were found to have markedly different phenotypes. Vδ2 cells had a phenotype typical of most αβ T cells in blood; i.e., they were $CD5^+$, $CD28^+$, and $CD57^-$. In contrast, Vδ1 cells tended to be $CD5^{-/dull}$, $CD28^-$, and $CD57^+$. Furthermore, although Vδ1 T cells appeared to be "naive" in that they were $CD45RA^+$, they were $CD62L^-$ and on stimulation uniformly produced interferon-γ, indicating that they are in fact memory/effector cells. This phenotype for Vδ1 cells was similar to that of intestinal intraepithelial lymphocytes, a subset that can develop in the absence of the thymus. We suggest that the Vδ1 and Vδ2 T cell subsets represent distinct lineages with different developmental pathways. The disruption of the supply of normal, thymus-derived T cells in HIV-infected individuals might be responsible for the shift in the Vδ2/Vδ1 ratio that occurs in the blood of individuals with HIV disease. *J. Leukoc. Biol.* 70: 518–526; 2001.

Key Words: *intestinal intraepithelial lymphocytes · CD5 · FACS*

INTRODUCTION

Several remarkable changes in T cell subsets occur during HIV disease. The progressive decline in the number of CD4 T cells has been well characterized and is used clinically to monitor disease progress. Although CD4 is a receptor for HIV, the effects of HIV on the immune system extend far beyond its effect on CD4-expressing T cells. For example, we have shown that a subset of CD8-expressing T cells, the naive CD8 T cell subset, declines during HIV disease progression in parallel with the decline in the overall and naive CD4-expressing T cell subsets [1, 2].

Subsets of γδ T cells also change dramatically in frequency during HIV disease. Human γδ T cells use a limited set of variable-region genes (V genes) compared with αβ T cells. In fact, only two Vδ genes, *Vδ1* and *Vδ2*, are commonly found among γδ T cells in humans. In HIV-uninfected individuals, Vδ2 cells are the more prevalent type found in peripheral blood [3], whereas Vδ1 cells are more prevalent among intestinal intraepithelial lymphocytes (IELs) [4]. In HIV disease, there is a dramatic shift in the ratio of these two γδ T cell types in peripheral blood: Vδ1 cells increase and Vδ2 cells decline in number and frequency [3, 5, 6]. The reason for this inversion of the Vδ2/Vδ1 ratio is not known. Studies examining the junctional diversity of Vδ1 and Vδ2 cells in HIV disease have determined that the inversion in this ratio is caused by neither an antigen-driven clonal expansion of Vδ1 cells nor a clonal deletion of Vδ2 cells [7, 8].

To understand the reason for the ratio shift, we used multicolor fluorescence-activated cell sorting (FACS) to study in detail the surface markers expressed by Vδ1 and Vδ2 cells. Unexpectedly, most Vδ1 cells expressed a distinctly different set of surface markers compared with Vδ2 cells, indicating that most Vδ1 and Vδ2 cells belong to very different subsets. In fact, the characteristic phenotype of each subset was not different in HIV-infected adults compared with uninfected adults; i.e., the marked difference in phenotype between Vδ1 and Vδ2 cells was preserved during HIV disease progression.

The phenotype of Vδ1 cells differs from most peripheral-blood T cells, but it is similar to the phenotype of intestinal IELs [9–11], cells that in the mouse develop in the absence of a thymus [12, 13]. In contrast, Vδ2 cells have a phenotype similar to that of the majority of T cells in blood, and the loss of Vδ2 cells in HIV disease is correlated with the loss of the presumably thymus-derived naive CD4 and CD8 T cells. We suggest that most Vδ1 and Vδ2 cells form functionally and developmentally distinct subsets, perhaps representing distinct lineages. The changes in representation of the Vδ1 and Vδ2 subsets might be a consequence of the HIV-induced impairment of typical thymic T cell development. Finally, the correlated loss of naive CD4, CD8, and Vδ2 T cells in HIV disease provides additional evidence for thymic impairment as an important cause of progressive immunodeficiency during HIV disease.

MATERIALS AND METHODS

Study population

Blood samples were drawn from a group of HIV-infected individuals living in the San Francisco area who had initially volunteered for entry into a clinical

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trial [14]. Of 242 subjects screened for entry into the trial, 83 were subsequently enrolled. Blood samples from some of these subjects, including both subjects only screened and subjects enrolled, were used for the studies reported here. Most subjects had CD4 T cell counts below 500/μL and did not have active opportunistic infections, because these were trial entry criteria. Subjects were either untreated or were receiving reverse transcriptase inhibitors only. Protease inhibitors had not been introduced at the time these blood samples were drawn. Most infected subjects were male. Details of the subjects enrolled in the trial and about the larger group of subjects screened for entry into the trial, who were also included as subjects in the study reported here, have been published [14, 15]. The group of HIV-uninfected controls included both male and female laboratory personnel, of approximately the same age range as the infected subjects. HIV antibody status for both groups was determined by ELISA, and positive results were confirmed by Western blot. All participants signed informed-consent agreements.

Flow-cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from 7 mL of heparinized blood by Ficoll-Hypaque centrifugation. Cells were stained with fluorescently labeled antibodies as described previously [16]. Most of the FACS studies reported here used eight-color FACS methods developed in our laboratory. These methods are described in detail elsewhere [17–19]. Described briefly here, cells were stained with combinations of antibodies conjugated to the following eight fluorescent dyes, which were excited by one of three lasers as indicated: fluorescein isothiocyanate (FITC), phycoerythrin (PE), Cy5-PE, and Cy7-PE, excited by a 488-nm argon laser; Texas Red, allophycocyanin (APC), and Cy7-APC, excited by a dye laser tuned to 600 nm; and cascade blue, excited by a 407-nm krypton laser. Samples were analyzed on a modified FACStar Plus (Becton-Dickinson, San Jose, CA) equipped with these three lasers, 10 fluorescence detectors in addition to forward and side scatter, and MoFlo electronics (Cytomation, Fort Collins, CO). Data analysis, including compensation after the data collection, was performed using FlowJo software (TreeStar, Inc, San Carlos, CA).

For each sample, 2×10^5–3×10^5 events were collected. Absolute counts were determined by multiplying the frequency of each subset relative to lymphocytes (determined by scatter gating) by the absolute lymphocyte count obtained from a complete blood count analyzed by a commercial laboratory on a blood sample drawn simultaneously with the blood sample used for FACS analysis. Examples of some of the eight-color combinations used in this study are shown in **Table 1**. The anti-perforin antibody was directly conjugated to Cy5 and collected in the APC channel. Data for Figure 3 (see below) were obtained using the three-color combination FITC CD20, PE CD5, and Cy5-PE CD3.

Cascade blue, FITC, and Texas Red were obtained from Molecular Probes (Eugene, OR). PE and APC were obtained from ProZyme (San Leandro, CA). Cy5 and Cy7 were obtained from Amersham Life Science (Pittsburgh, PA). The antibodies to interferon-γ (IFN-γ), interleukin (IL)-2, CD3, CD4, CD5, CD8, CD16, CD28, CD45RA, CD57, CD62L, TcR γδ, and Vδ2 were obtained from BD/PharMingen (San Diego, CA) and conjugated to the indicated fluorochromes in our laboratory by standard protocols (http://drmr.com/abcon). FITC-conjugated δTCS1 (anti-Vδ1) was obtained from Endogen, Inc. (Woburn, MA). The antibodies to CD8β and perforin (dG9) were kindly provided by E. Reinherz (Dana Farber Cancer Institute, Boston, MA) and E. Podack (University of Miami, FL), respectively.

Intracellular cytokine and perforin assays

A FACS assay was used to determine the frequency of cell staining for IFN-γ and IL-2 after in vitro stimulation [20]. PBMCs (10^6 cells) were stimulated in flat-bottom 24-well plates with phorbol myristate acetate [PMA (50 ng/mL)] and ionomycin (1 μM) in the presence of monensin (1 μM) for 6 h. For staining combinations that included CD62L, a matrix metalloproteinase inhibitor [KB8301 (10 μM); BD/PharMingen] was included during the 6-h incubation to prevent stimulation-induced cleavage of CD62L from the cell surface. PBMCs were stained with the anti-Vδ1 and Vδ2 reagents (for 15 min at room temperature with one subsequent wash) before incubation with PMA and ionomycin, because a nonspecific staining pattern was noted if the PBMCs were stained with these reagents after the stimulation. After stimulation, cells were harvested and washed with phosphate-buffered saline (PBS)-bovine serum albumin (BSA)-azide once and stained for 15 min on ice in the dark to determine their surface phenotypes. Ethidium monoazide bromide [EMA (5 μg/mL); Molecular Probes] was included with the surface stains to label the dead cells. Cells were washed 3× in PBS, exposed to fluorescent light for 10 min to covalently link the EMA, and then fixed with 2% Formalin in PBS for 20 min at room temperature. After washing 3× in PBS-BSA-azide, cells were resuspended in permeabilization buffer (0.5% saponin in PBS-BSA-azide) and kept at room temperature for 10 min. Cells were spun down, resuspended in permeabilization buffer containing the optimal concentration of APC-conjugated anti-IL-2 and either FITC- or cascade blue-conjugated anti-IFN-γ, and incubated at room temperature for 30 min. Finally, cells were washed once with permeabilization buffer and then 3× with PBS-BSA-azide. Cells were stored on ice until FACS analysis (within 24–48 h). A similar assay was used to stain for intracellular perforin, except that fresh PBMCs were used, without any stimulation.

Statistical analysis

JMP software (version 4 for Macintosh) produced by the SAS Institute, Cary, NC, was used for all statistical analyses. Significance values for comparisons between groups were determined by the nonparametric Wilcoxon's rank sum analysis. Pearson correlation coefficients were used to determine significant correlations between Vδ2 counts and CD4 and CD8 subset counts.

RESULTS

Vδ1 and Vδ2 γδ T cells tended to be expressed in distinct subsets identified by the expression of CD5, CD57, and CD45RA

Consistent with previous reports, we found that, although Vδ2 cells normally outnumber Vδ1 in the γδ T cell population in peripheral blood, the majority of the γδ T cells in HIV-infected people expressed Vδ1 (**Fig. 1**). These Vδ1 cells were virtually all $CD5^-$ or $CD5^{dull}$ (**Fig. 2**). This increase in the Vδ1 population did not reflect an overall increase in the frequency of

TABLE 1. Examples of 8-Color Staining Combinations

Cas Blue	FITC	PE	Cy5PE	Cy7PE	Texas Red	APC	Cy7APC
			Surface staining				
CD8β	CD5	TcRγδ	CD3	CD8	CD4	CD57	CD45RA
CD5	Vδ1	Vδ2	CD3	CD8	CD62L	CD57	CD45RA
CD16	Vδ1	Vδ2	CD3	CD8	CD4	CD28	CD45RA
			Intracellular and surface staining				
IFN-γ	Vδ1	Vδ2	EMA[a]	CD8	CD3	IL-2	CD57
CD5	Vδ1	Vδ2	EMA	CD8	CD3	Perforin[b]	CD57

[a] Ethidium monoazide bromide stains dead cells and covalently links to DNA when exposed to fluorescent light (see methods). [b] In this staining combination, perforin is conjugated to Cy5. It is listed in the APC channel because it has similar excitation and emission characteristics and is used here in place of an APC-conjugated reagent.

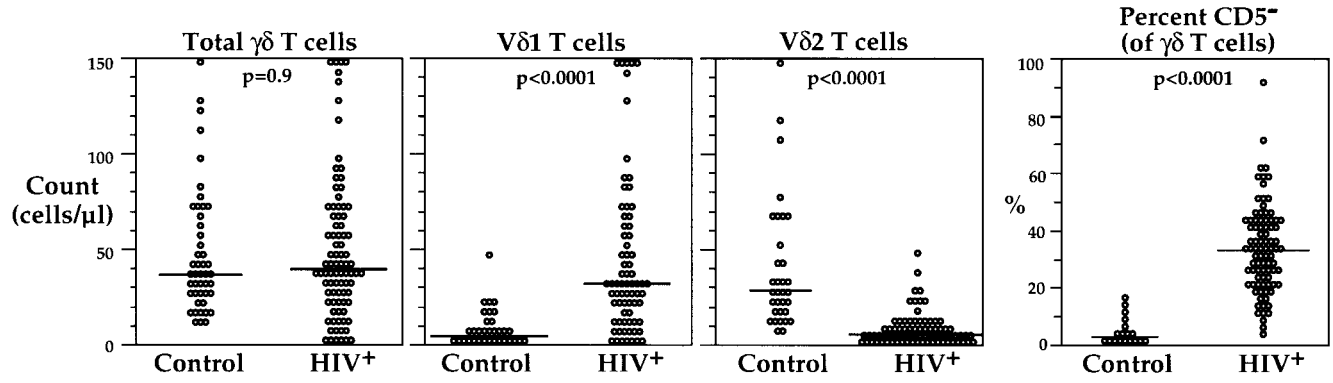

Fig. 1. The γδ T cells that expand in HIV disease often lack expression of CD5. The absolute counts for overall γδ T cells and the Vδ1 and Vδ2 T cell subsets in HIV-infected (n=73) and -uninfected (n=33) individuals are compared. The right panel compares percentages of the γδ T cells that lack expression of CD5. Horizontal bars show the medians. The frequencies for the γδ subsets were determined by FACS from three-color or eight-color combinations including either an anti-γδ monoclonal antibody conjugated to FITC or PE, anti-Vδ1 conjugated to FITC, or anti-Vδ2 conjugated to FITC or PE. Absolute counts were obtained by multiplying this frequency relative to lymphocytes by the absolute lymphocyte count determined from a complete blood count.

γδ cells. Rather it reflected a change in the proportion of γδ cells that expressed Vδ1 and belonged to the $CD5^-$ or $CD5^{dull}$ subsets. Thus, although we found no difference in the overall numbers of γδ T cells between HIV-infected and -uninfected subjects, there was a marked increase in the proportion of $CD5^-$ γδ (Vδ1) cells in the HIV-infected subjects (Fig. 1). Vδ2 cells, in contrast, were rarely $CD5^-$ and tended to express more CD5 than Vδ1 cells (Fig. 2).

Vδ1 and Vδ2 also differed markedly in the expression of CD45RA. Although most Vδ1 cells were $CD45RA^+$, they were not typical naive T cells in that they generally did not coexpress CD62L (Fig. 2). In studies in which naive T cells have been identified only by the expression of CD45RA (or the nonexpression of CD45RO), these γδ cells have been incorrectly counted within the naive population [21, 22].

CD28, the T cell-costimulatory molecule, and CD57, a glycoprotein of unknown function normally expressed on a subset of $CD8^+$ T cells and some natural killer cells, were two other surface markers that showed different expression between these subsets; most Vδ1 cells were $CD28^-$ and $CD57^+$ (Fig. 2). Note that CD57 appeared to show three levels of expression, with Vδ1 cells showing the brightest expression level. Some Vδ2 cells expressed CD57 at intermediate levels (examples shown in Fig. 5 below). Because the demarcation between the $CD57^-$ and $CD57^{dull}$ cells was not always very well resolved, we categorized the $CD57^{dull}$ cells as "negative" in the figures. Few (if any) of either the Vδ1 or the Vδ2 cells expressed CD4, and although a larger proportion of Vδ1 cells expressed CD8 compared with Vδ2 cells, the majority of both Vδ1 and Vδ2 cells were CD4/CD8 double negative (data not shown).

The lack of expression of CD5 was not restricted to Vδ1 γδ T cells. A small population of T cells including both γδ and αβ T cells did not express detectable levels of CD5, and these $CD5^-$ T cells increased in frequency and absolute count in HIV disease. **Figure 3** shows that this subset could be clearly identified as separate in HIV-infected individuals based on the normal or bright level of expression of CD3 and the lack of expression of CD5. The absolute count for this subset became elevated relatively early in HIV disease (CD4 T cell counts near 500/μL) and remained elevated until late in the disease process. Although enriched for γδ T cells compared with the $CD5^+$ population, about half of the $CD5^-$ T cells in HIV-infected subjects and the majority of the $CD5^-$ T cells in HIV-uninfected subjects expressed the αβ T cell receptor (Figure 3).

Representation, rather than the phenotype, of Vδ1 and Vδ2 cells changed in HIV-infected individuals

The Vδ1 and Vδ2 subsets were distinct in both HIV-infected and -uninfected individuals. For the most part, the characteristic phenotypic features of these subsets were not changed; however, there were some differences between infected and uninfected individuals in the proportions of Vδ1 or Vδ2 cells expressing certain markers (see Figure 2). Consistent with the overall expansion of $CD5^-$ T cells in HIV disease, the Vδ1 subset in HIV-infected individuals included a higher proportion of $CD5^-$ cells than did the Vδ1 subset in uninfected controls. Even the Vδ2 subset included a small fraction of $CD5^-$ cells that were rarely found in Vδ2 cells from controls. Similar to the loss of naive CD4 and CD8 T cells in HIV-infected individuals, there were fewer Vδ1 cells in HIV-infected subjects with a naive phenotype ($CD62L^+$ $CD45RA^+$) compared with Vδ1 cells in uninfected controls. This difference was largely caused by the decreased proportion of Vδ1 cells expressing CD62L in HIV-infected individuals.

Vδ1 and Vδ2 T cells showed a similar cytokine profile

Using a FACS method of intracellular cytokine staining, we assessed the cytokine profile of the Vδ1 and Vδ2 T cells. Bulk PBMCs were stimulated for 6 h with PMA and ionomycin and then surface stained with a combination of markers that allow identification of the subset phenotype. Finally, the cells were permeabilized and stained with fluorescently labeled antibodies to the cytokines IFN-γ and IL-2.

The majority of Vδ1 and Vδ2 cells stained for IFN-γ and very few of these cells stained for IL-2 (**Fig. 4** and **Table 2**). This profile was similar to the cytokine profile of typical CD8

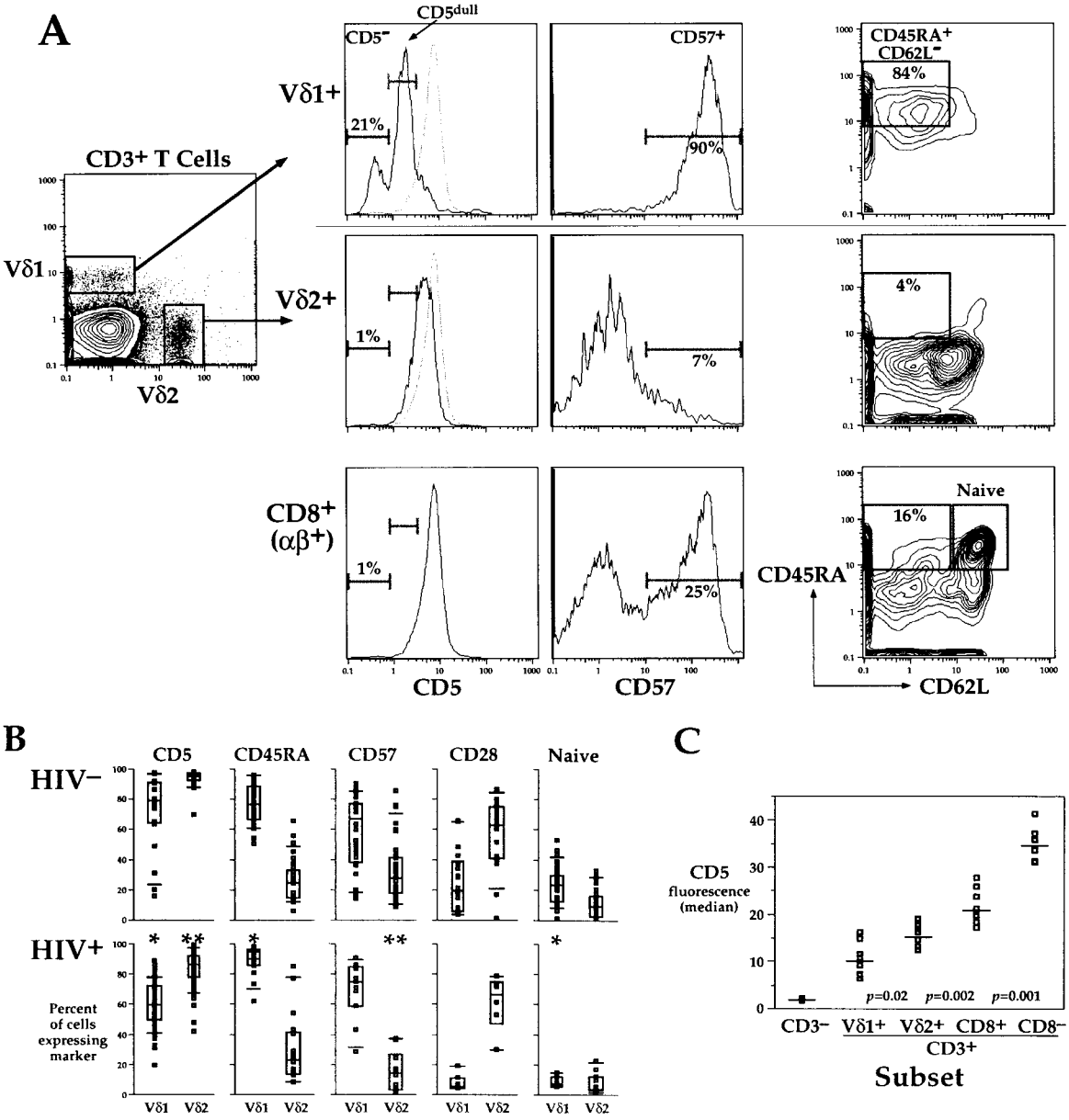

Fig. 2. Vδ1 and Vδ2 cells differ in expression of surface markers, both in HIV-infected and -uninfected individuals. (a) Example of an eight-color FACS analysis of PBMCs from an HIV-uninfected individual. Data are shown either as histograms or as 5% probability plots with or without outliers. PBMCs (2×10^5) were scatter gated on lymphocytes, and then lymphocytes were gated on CD3 to identify T cells (data not shown); T cells were then gated on $V\delta1^+$ cells (top panels) and $V\delta2^+$ cells (middle panels). T cells not expressing Vδ1 or Vδ2 (lower left) and then gated on CD8 (lower panels) are shown for comparison with the γδ T cells. The levels of expression of CD57, CD5, CD62L, and CD45RA are shown on these subsets. The histogram for CD5 expression on $CD8^+$ T cells is reproduced in gray in the upper panels to show the lower level of CD5 expression of Vδ1 cells. Percentages of cells within the subsets that were $CD57^+$, $CD5^-$, and $CD45RA^+CD62L^-$ are listed. (b) The percentages of Vδ1 or Vδ2 cells that expressed each listed marker are shown for HIV-uninfected controls [upper panels ($n=19$–35)] and for HIV-infected subjects [lower panels ($n=7$–62). Various eight-color FACS staining combinations were used to collect these data. The boxes with the intersecting lines represent the interquartile ranges and the medians for each group. The lines above and below the boxes are the 90th and 10th percentiles, respectively. The naive phenotype was defined here as coexpression of CD62L and CD45RA. All the comparisons between Vδ1 and Vδ2 (within HIV-infected or -uninfected subjects) were significant at $P < 0.003$, except for the naive phenotype, which was not significantly different for the HIV-infected subjects. For comparisons between HIV-infected and -uninfected cells, those that were significant are indicated as follows: *, $P < 0.001$; **, $P < 0.02$. (c) The median fluorescence level of expression of CD5 on non-T cells ($CD3^-$), Vδ1 and Vδ2 γδ T cells, and $CD8^+$ and $CD8^-$ (largely $CD4^+$) T cells. Data are shown for eight HIV-infected individuals. Horizontal bars represent the median levels for these eight subjects. *P* values indicate the significance for comparisons between the adjoining groups.

memory T cells. Note that in the examples shown in Figure 4, Vδ1 cells showed a lower level of staining for IFN-γ compared with Vδ2 cells. We observed this staining pattern for most samples analyzed. This difference in staining suggested that the Vδ1 cells might produce less IFN-γ than that produced by Vδ2 cells. However, the relationship between relative brightness of staining and cytokine production has not been definitively determined.

This pattern of cytokine production was similar for γδ cells from either HIV-infected or -uninfected individuals. The only difference we observed that might be associated with HIV disease was that a higher percentage of Vδ1 cells from unin-

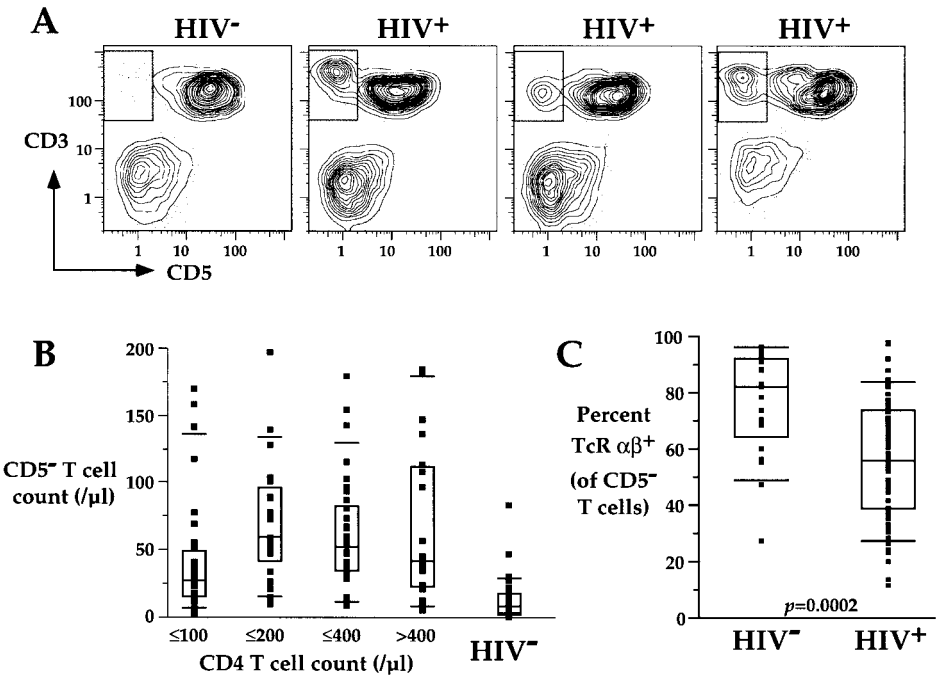

Fig. 3. $CD5^-$ T cells expand in HIV disease and include γδ and αβ T cells. (a) Examples of three-color FACS 5% probability plots showing the expression of CD5 (PE) and CD3 (Cy5-PE) on scatter-gated lymphocytes from a representative HIV-uninfected control and three HIV-infected individuals. The three HIV-infected individuals showed the three patterns of CD3 expression on the $CD5^-$ T cells: bright CD3 expression (second panel), CD3 expressed at the same levels as most $CD5^+$ T cells (third panel), or a mixture of these expression levels (fourth panel). The $CD3^{bright}$ $CD5^-$ cells, as compared with the CD5 cells expressing typical levels of CD3, included a higher proportion of γδ T cells (data not shown). (b) The absolute $CD5^-$ T cell counts are shown for HIV-infected individuals grouped by CD4 count ($n=209$) and for the HIV-uninfected controls ($n=42$). The boxes with the intersecting lines represent the interquartile ranges and the medians for each group. The lines above and below the boxes are the 90th and 10th percentiles, respectively. The count for the infected group as a whole was significantly elevated compared with that of controls ($P<0.0001$). (c) The percentages of $CD5^-$ T cells that were TcR β^+ are shown for HIV-uninfected ($n=21$) and HIV-infected ($n=69$) individuals. As in panel b, the boxes with the intersecting lines represent the interquartile ranges and the medians.

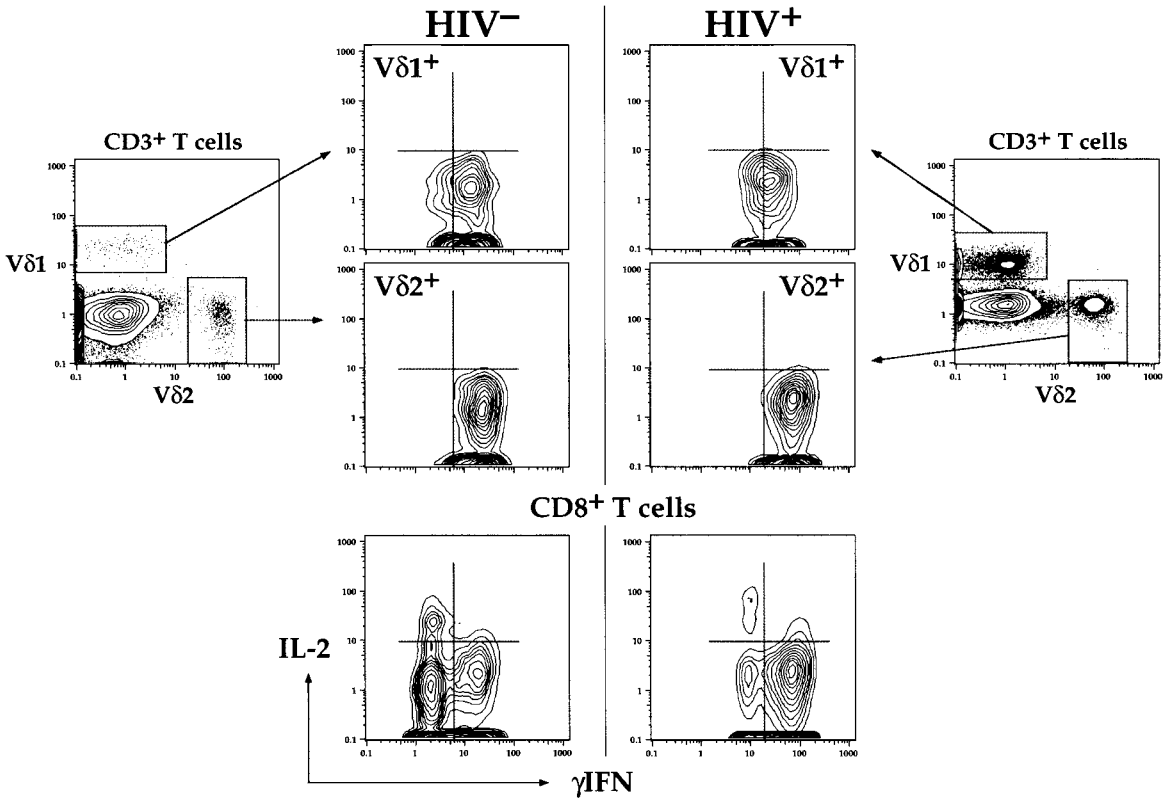

Fig. 4. Intracellular staining for cytokines shows that most Vδ1 and Vδ2 cells stained for IFN-γ but not IL-2. Shown are 5% probability contour plots with outliers for the FACS-determined level of expression of IFN-γ (abscissa) and IL-2 (ordinate) in Vδ1, Vδ2, and $CD3^+$ $CD8^+$ T cells (shown for comparison) from an uninfected individual (left) and an HIV-infected individual (right) after 6 h of stimulation with PMA and ionomycin. Two eight-color combinations of reagents were used, including either Vδ1 or Vδ2, CD3, and CD8, in addition to IL-2 and IFN-γ. PBMCs (5×10^4–2×10^5 collected) were first gated on lymphocytes and then gated on Vδ1, Vδ2, or $CD3^+$ $CD8^+$ cells.

TABLE 2. The Majority of Both Vδ1 and Vδ2 Cells Stain for IFN-γ, but not IL-2

T cell subset	Cytokine	Percent of cells staining for cytokine[a] HIV$^-$	HIV$^+$	p^b
Vδ1 T cells	IFN-γ	77 (55–100)	94 (91–97)	0.2
	IL-2	11 (0–27)	1 (0–2)	0.2
Vδ2 T cells	IFN-γ	94 (84–98)	91 (87–100)	0.7
	IL-2	3 (2–13)	3 (0–13)	0.7

[a] PBMC were stimulated for 6 hours with PMA and ionomycin and intracellularly stained for cytokines as described in the Methods. Median (10^{th} to 90^{th} percentile) for the percentage of cells within the indicated subset staining for the indicated cytokine. For HIV-uninfected, n = 4; for HIV-infected, n = 6–7, depending on adequate representation of the subset. [b] Comparison between HIV-infected and HIV-uninfected, Wilcoxon rank-sum test.

fected individuals stained for IL-2 (Table 2). The Vδ1 cells that stained for IL-2 did not stain for IFN-γ, were CD57$^-$, and were naive in phenotype (data not shown). This was consistent with typical CD5$^+$ αβ T cells, in which naive subsets were IL-2$^+$ and IFN-γ$^-$ [20]. Therefore, the loss of these IL-2-staining cells in the HIV-infected subjects could be accounted for by the loss of "naive" Vδ1 cells. Because only four uninfected individuals have been analyzed for cytokine profile, more must be tested to establish significance.

Both Vδ1 and Vδ2 T cells stained intracellularly for perforin

A similar intracellular staining protocol was used to determine levels of intracellular perforin, one of the proteins released from cytotoxic T cells that participate in target cell killing. For this assay, fresh unstimulated PBMCs were surface stained and then permeabilized and stained intracellularly for perforin. **Figure 5** shows that both Vδ1 and Vδ2 cells stained for perforin. For a small number of samples analyzed, the majority of Vδ1 and Vδ2 cells in both HIV-uninfected and -infected individuals stained for perforin (**Table 3**). There was a higher frequency of Vδ2 cells expressing perforin in the HIV-infected group (P=0.02), but this difference must be confirmed with a larger sample size. Figure 5 also shows that there was some correlation between the expression of perforin and CD57. The Vδ2 cells, which expressed no or lower levels of CD57 compared with the Vδ1 and CD8 cells, also expressed lower levels of perforin.

The decline in Vδ2 cell numbers was correlated with the decline in naive CD4 and CD8 T cells in HIV disease

CD4 and CD8 cells could be subdivided into naive and memory subsets based on the expression of CD62L and CD45RA. Naive T cells were identified as cells coexpressing both of these surface markers, and three memory subsets could be identified based on the lack of expression of either one or both of these markers. The decline in the counts of peripheral blood Vδ2 cells in the group of HIV-infected individuals (n=99) that we studied was loosely correlated with the decline in CD4 T cell counts [r=0.42 (Pearson correlation coefficient);

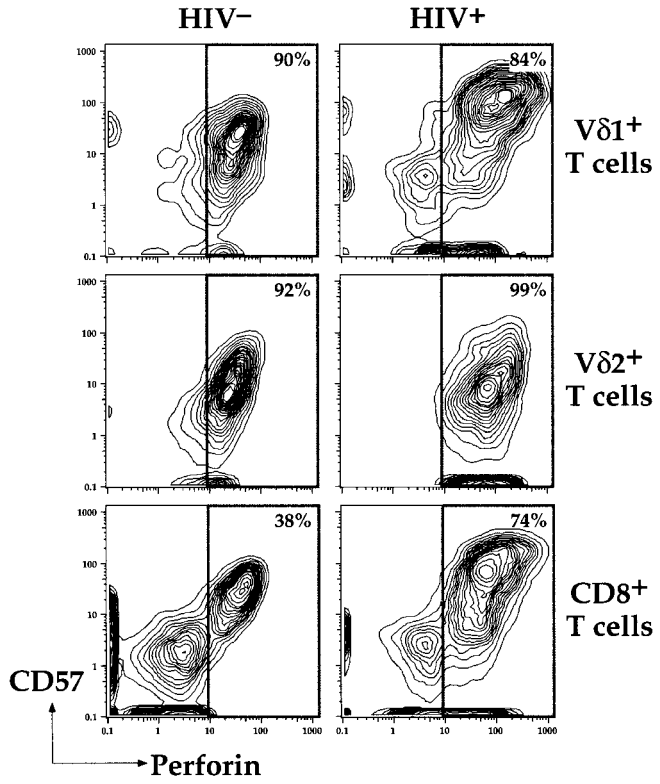

Fig. 5. Both Vδ1 and Vδ2 cells stained intracellularly for perforin. Shown are 5% probability contour plots for the FACS-determined level of expression of perforin (abscissa) and CD57 (ordinate) for Vδ1, Vδ2, and CD3$^+$ CD8$^+$ T cells (shown for comparison) for an uninfected individual (left) and an HIV-infected individual (right). The staining combination used is listed in the last row of Table 1. The percentage of cells in each subset staining for perforin is indicated. Note that these two samples were not stained on the same day, so the absolute intensity of staining for CD57 or perforin between the HIV-infected and the -uninfected samples cannot be compared.

P<0.0001]. This correlation was largely caused by the correlation of Vδ2 cell counts with naive CD4 T cell counts [r=0.48; P<0.0001 (Pearson)]. Of the three memory subsets, only the CD45RA$^-$ CD62L$^+$ memory CD4 subset showed a correlation with the Vδ2 cells [r=0.33; P=0.0009 (Pearson)].

Because CD8 T cell counts showed a pattern of initial expansion with declines noted only in late-stage AIDS, it was not surprising that the Vδ2 cell count was not correlated with the overall CD8 T cell count. However, Vδ2 cells were loosely correlated with the naive CD8 T cell count [r=0.42, P<0.0001 (Pearson)] but were not correlated with any of the CD8 memory subsets. These relationships were consistent with the hypoth-

Table 3. Most Vδ1 and Vδ2 T Cells Stain for Perforin

Subset	Percent staining for perforin[a] HIV$^-$	HIV$^+$	p^b
Vδ1 T cells	71 (22–91)	83 (69–94)	0.2
Vδ2 T cells	77 (50–88)	95 (85–98)	0.02

[a] PBMC were surface stained to identify T cell subsets, and then permeabilized and intracellularly stained for perforin (see Materials and Methods). Median (interquartile range). For HIV-uninfected, n = 5; for HIV-infected, n = 8. [b] Comparison between HIV-uninfected and infected, Wilcoxon rank-sum test.

esis that there is a similar mechanism responsible for the loss of naive T cells and the loss of Vδ2 cells, perhaps caused by HIV-induced impairment of thymic function [23–26].

DISCUSSION

We found that the two types of γδ T cells commonly found in peripheral blood, Vδ1 and Vδ2, expressed distinct sets of surface markers that caused them to be characterized as distinct subsets. Indeed, these markedly different phenotypes suggest that they might be functionally and developmentally distinct lineages. In HIV disease, although there is an expansion of the Vδ1 subset and a decline in the Vδ2 subset [3, 5, 6], the phenotypes and likely the functions of the subsets appear not to change. Therefore, it is the relative representation of these subsets that changes in HIV disease and not the nature of the subsets themselves.

CD5 is one surface marker shown to be expressed at different levels on lineages of T and B cells. For example, CD4 T cells express higher levels of CD5 than CD8 T cells (Fig. 2), and CD5 is expressed at low levels on a subset of B-1 cells but not on conventional B-2 cells [27, 28]. Vδ1 and Vδ2 cells differ in the level of expression of CD5. Many Vδ1 cells lack CD5 expression or express lower CD5 compared with that of Vδ2 cells. This difference is one indication that most Vδ1 and Vδ2 cells might belong to different lineages and that Vδ1 cells might be related to other subsets of T cells (including αβ T cells) that expand in HIV disease and also show lower levels of CD5 expression. Cells that lack CD5 have been shown here (and previously) to expand in HIV disease [29]. $CD5^-$ T cells from HIV-infected adults were previously reported to express only the αβ T cell receptor [29]. We found, however, that γδ T cells represent nearly half of the cells in the enlarged $CD5^-$ T cell subset in HIV-infected subjects.

The use of CD5 expression to distinguish T cell lineages among intestinal IELs has been questioned in one study [30]. In this report, murine $CD5^-$ CD8αβ IELs are shown to up-regulate CD5 surface expression on stimulation; therefore, the authors conclude that $CD5^-$ and $CD5^+$ CD8αβ IELs cannot be considered as separate lineages. However, these data are not inconsistent with our proposal. First, that report studies only αβ and not γδ T cells. In addition, when CD5 was up-regulated, it usually was expressed at intermediate levels, and only infrequently was it expressed at the levels typical of CD8 T cells found in blood. Finally, we analyzed resting blood cells and not activated cells. It is well known that the expression of some surface markers changes on activation. For, example, CD4 is down-regulated on activation [31], and yet these cells remain classified as part of the $CD4^+$ lineage and not the $CD4^-CD8^-$ lineage.

Vδ1 T cells have an overall surface phenotype that is different from that of most peripheral blood T cells but is similar to that of T cells found in the intestinal epithelium. Studies of intestinal IELs from mice show that they include a large proportion of γδ T cells, are $CD5^-$ or $CD5^{dull}$, and often lack CD28 expression [9–11]. In humans, the majority of intestinal IELs use αβ T cell receptors; however, among the γδ T cells, almost all use Vδ1 [4]. Because studies in mice have shown that intestinal IELs can develop in the absence of a functioning thymus [12, 13], the phenotypic similarity between these lymphocytes and Vδ1 cells suggests that these latter cells might also be capable of extrathymic development, perhaps in the intestine.

In human peripheral blood, Vδ2 cells usually outnumber Vδ1 cells [3]. However, in HIV-infected individuals, Vδ1 cells become more prevalent due to a decline in absolute counts of Vδ2 cells and an expansion of Vδ1 cells [3, 5, 6]. The reason for this inversion in the ratio of Vδ1 to Vδ2 cells in HIV disease is not known. An antigen-driven expansion of Vδ1 cells seems unlikely because the junctional diversity of Vδ1 cells from HIV-infected individuals does not differ significantly from that of HIV-uninfected controls [7, 8].

Based on the similar phenotypes of Vδ1 blood cells and cells in the intestinal epithelium, we propose that the source of the expanded Vδ1 cells during HIV disease might be intestinal IELs that migrate into the peripheral blood. This proposed migration of Vδ1 cells from the intestinal epithelium to the blood might be a consequence of HIV-associated intestinal inflammation. Consistent with this hypothesis, expansion of Vδ1 cells in peripheral blood has been observed in other diseases that are associated with intestinal inflammation [32].

The impairment of thymic function that occurs in HIV disease offers another possible reason why the intestine could be the source of the increased number of Vδ1 cells in the blood of HIV-infected individuals. Several studies have indicated that HIV infection is likely to result in impairment of thymic function. Direct histological evidence has demonstrated disruption of the thymic microenvironment by HIV in humans and by simian immunodeficiency virus (SIV) in monkeys [24–26]. In a model where severe combined immunodeficiency mice are implanted with human hematolymphoid tissue (SCID-hu), HIV can infect thymocytes, leading to their destruction and resulting in a disruption of thymic morphology [23]. We have previously reported the progressive decline in naive CD4 and CD8 T cell counts in HIV disease [1, 2], which is likely a consequence of HIV-induced thymic damage because the thymus is the major organ responsible for producing naive T cells.

In HIV disease, there can be increased activity of extrathymic T cell developmental pathways, as a consequence either of loss of thymic function or an increased demand in general for T cell development or both. The intestinal epithelium might be such an extrathymic site where T cells (enriched for Vδ1 γδ T cells and $CD5^-$ αβ T cells) develop and eventually migrate to the periphery.

One of the surface markers that shows differential expression on Vδ1 and Vδ2 cells is CD45RA [21, 22]. This difference has been shown previously; however, the interpretation then was that Vgd1 cells are naive, based on the high level of expression of CD45RA. We confirmed that most Vδ1 cells are $CD45RA^+$, but we found that most are $CD62L^-$ and hence not naive in phenotype. CD45RA alone is not sufficient to distinguish naive from memory cells; an additional marker such as CD62L or CD11a is required to make this distinction. In addition, these cells stain for IFN-γ but not IL-2, a function consistent with the designation of these cells as memory/

effector cells [20]. Perforin expression in both the Vδ1 and Vδ2 cells is also consistent with the designation of these cells as memory/effector cells and is consistent with previous reports showing that most γδ T cells in blood express perforin [33].

In summary, we have found that most Vδ1 and Vδ2 cells have characteristic patterns of antigen surface expression in both HIV-infected and -uninfected individuals, and we suggest that these phenotypic profiles define developmentally distinct lineages. Although this conclusion might not be true for all Vδ1 or Vδ2 cells, because these phenotypic profiles are not exclusive to either subset, it appears to be valid for most cells in either subset, and therefore Vδ1 or Vδ2 might be used as single markers to broadly define these subsets.

Finally, we speculate that the Vδ1/Vδ2 inversion might be a consequence of thymic impairment by HIV disease. The majority of Vδ2 cells could develop in the thymus. They have a phenotype similar to most peripheral blood T cells, which are presumably thymus derived. In addition, the kinetics of the loss of the Vδ2 cells during HIV disease progression parallels the loss of the naive CD4 and CD8 subsets, whose loss likely reflects the decreased ability of the thymus to generate T cells. On the other hand, Vδ1 cells might develop through an extrathymic pathway. They expand in HIV disease and are similar in phenotype to a subset of cells shown to be capable of extrathymic development, intestinal IELs. Finally, the changes in numbers and ratio of Vδ1 to Vδ2 cells during HIV disease might reflect HIV-induced alterations in the activities of thymic versus extrathymic T cell developmental pathways.

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REFERENCES

1. Rabin, R. L., Roederer, M., Maldonado, Y., Petru, A., Herzenberg, L. A. (1995) Altered representation of naive and memory CD8 T cell subsets in HIV-infected children. J. Clin. Invest. 95, 2054–2060.
2. Roederer, M., Dubs, J. G., Anderson, M. T., Raju, P. A., Herzenberg, L. A. (1995) CD8 naive T cell counts decrease progressively in HIV-infected adults. J. Clin. Invest. 95, 2061–2066.
3. Autran, B., Triebel, F., Katlama, C., Rozenbaum, W., Hercend, T., Debre, P. (1989) T cell receptor gamma/delta+ lymphocyte subsets during HIV infection. Clin. Exp. Immunol. 75, 206–210.
4. Deusch, K., Luling, F., Reich, K., Classen, M., Wagner, H., Pfeffer, K. (1991) A major fraction of human intraepithelial lymphocytes simultaneously expresses the gamma/delta T cell receptor, the CD8 accessory molecule and preferentially uses the V delta 1 gene segment. Eur. J. Immunol. 21, 1053–1059.
5. De Paoli, P., Gennari, D., Martelli, P., Basaglia, G., Crovatto, M., Battistin, S., Santini, G. (1991) A subset of gamma delta lymphocytes is increased during HIV-1 infection. Clin. Exp. Immunol. 83, 187–191.
6. De Maria, A., Ferrazin, A., Ferrini, S., Ciccone, E., Terragna, A., Moretta, L. (1992) Selective increase of a subset of T cell receptor gamma delta T lymphocytes in the peripheral blood of patients with human immunodeficiency virus type 1 infection. J. Infect. Dis. 165, 917–919. Erratum, J. Infect. Dis. 166, 950 (1992).
7. Hinz, T., Wesch, D., Friese, K., Reckziegel, A., Arden, B., Kabelitz, D. (1994) T cell receptor gamma delta repertoire in HIV-1-infected individuals. Eur. J. Immunol. 24, 3044–3049.
8. Boullier, S., Cochet, M., Poccia, F., Gougeon, M. L. (1995) CDR3-independent gamma delta V delta 1+ T cell expansion in the peripheral blood of HIV-infected persons. J. Immunol. 154, 1418–1431.
9. Lefrancois, L. (1991) Phenotypic complexity of intraepithelial lymphocytes of the small intestine. J. Immunol. 147, 1746–1751.
10. Lefrancois, L. (1991) Extrathymic differentiation of intraepithelial lymphocytes: generation of a separate and unequal T-cell repertoire? Immunol. Today 12, 436–438.
11. Trejdosiewicz, L. K. (1992) Intestinal intraepithelial lymphocytes and lymphoepithelial interactions in the human gastrointestinal mucosa. Immunol. Lett. 32, 13–19.
12. Kroemer, G., Cuende, E., Martinez, C. (1993) Compartmentalization of the peripheral immune system. Adv. Immunol. 53, 157–216.
13. Lefrancois, L., Puddington, L. (1995) Extrathymic intestinal T-cell development: virtual reality? Immunol. Today 16, 16–21.
14. De Rosa, S. C., Zaretsky, M. D., Dubs, J. G., Roederer, M., Anderson, M., Green, A., Mitra, D., Watanabe, N., Nakamura, H., Tjioe, I., Deresinski, S. C., Moore, W. A., Ela, S. W., Parks, D., Herzenberg, L. A. (2000) N-acetylcysteine replenishes glutathione in HIV infection. Eur. J. Clin. Invest. 30, 915–929.
15. Herzenberg, L. A., De Rosa, S. C., Dubs, J. G., Roederer, M., Anderson, M. T., Ela, S. W., Deresinski, S. C., Herzenberg, L. A. (1997) Glutathione deficiency is associated with impaired survival in HIV disease. Proc. Natl. Acad. Sci. USA 94, 1967–1972.
16. Kantor, A., Roederer, M. (1997) FACS analysis of lymphocytes. In *Handbook of Experimental Immunology*, Vol. 2, 5th ed. (L. A. Herzenberg, D. M. Weir, and C. Blackwell, ed.), Blackwell Science, Cambridge, United Kingdom, 49.1–49.13.
17. Roederer, M., De Rosa, S., Gerstein, R., Anderson, M., Bigos, M., Stovel, R., Nozaki, T., Parks, D., Herzenberg, L. (1997) 8 color, 10-parameter flow cytometry to elucidate complex leukocyte heterogeneity. Cytometry 29, 328–339.
18. Baumgarth, N., Roederer, M. (2000) A practical approach to multicolor flow cytometry for immunophenotyping. J. Immunol. Methods 243, 77–97.
19. De Rosa, S. C., Herzenberg, L. A., Roederer, M. (2001) 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. Nat. Med. 7, 245–248.
20. Mitra, D. K., De Rosa, S. C., Luke, A., Balamurugan, A., Khaitan, B. K., Tung, J., Mehra, N. K., Terr, A. I., O'Garra, A., Herzenberg, L. A., Roederer, M. (1999) Differential representations of memory T cell subsets are characteristic of polarized immunity in leprosy and atopic diseases. Int. Immunol. 11, 1801–1810.
21. Miyawaki, T., Kasahara, Y., Taga, K., Yachie, A., Taniguchi, N. (1990) Differential expression of CD45RO (UCHL1) and its functional relevance in two subpopulations of circulating TCR-gamma/delta$^+$ lymphocytes. J. Exp. Med. 171, 1833–1838.
22. Braakman, E., Sturm, E., Vijverberg, K., van Krimpen, B. A., Gratama, J. W., Bolhuis, R. L. (1991) Expression of CD45 isoforms by fresh and activated human gamma delta T lymphocytes and natural killer cells. Int. Immunol. 3, 691–697.
23. Bonyhadi, M. L., Rabin, L., Salimi, S., Brown, D. A., Kosek, J., McCune, J. M., Kaneshima, H. (1993) HIV induces thymus depletion in vivo. Nature 363, 728–732.
24. Nezelof, C. (1992) Thymic pathology in primary and secondary immunodeficiencies. Histopathology 21, 499–511.
25. Li, S. L., Kaaya, E. E., Ordonez, C., Ekman, M., Feichtinger, H., Putkonen, P., Bottiger, D., Biberfeld, G., Biberfeld, P. (1995) Thymic immunopathology and progression of SIVsm infection in cynomolgus monkeys. J. Acquired Immune Defic. Syndr. Hum. Retrovirol. 9, 1–10.
26. Rosenzweig, M., Connole, M., Forand-Barabasz, A., Tremblay, M. P., Johnson, R. P., Lackner, A. A. (2000) Mechanisms associated with thymocyte apoptosis induced by simian immunodeficiency virus. J. Immunol. 165, 3461–3468.

27. Kantor, A. B., Herzenberg, L. A. (1993) Origin of murine B cell lineages. Annu. Rev. Immunol. 11, 501–538.
28. Hardy, R. R., Li, Y. S., Allman, D., Asano, M., Gui, M., Hayakawa, K. (2000) B-cell commitment, development and selection. Immunol. Rev. 175, 23–32.
29. Indraccolo, S., Mion, M., Zamarchi, R., Coppola, V., Calderazzo, F., Amadori, A., Chieco-Bianchi, L. (1995) A CD3+CD8+ T cell population lacking CD5 antigen expression is expanded in peripheral blood of human immunodeficiency virus-infected patients. Clin. Immunol. Immunopathol. 77, 253–261.
30. Muller, S., Jungo, M., Aichele, P., Mueller, C. (1997) CD5-CD8 alpha beta intestinal intraepithelial lymphocytes (IEL) are induced to express CD5 upon antigen-specific activation: CD5− and CD5+ CD8 alpha beta IEL do not represent separate T cell lineages. Eur. J. Immunol. 27, 1756–1761.
31. Weyand, C. M., Goronzy, J., Fathman, C. G. (1987) Modulation of CD4 by antigenic activation. J. Immunol. 138, 1351–1354.
32. Giacomelli, R., Parzanese, I., Frieri, G., Passacantando, A., Pizzuto, F., Pimpo, T., Cipriani, P., Viscido, A., Caprilli, R., Tonietti, G. (1994) Increase of circulating gamma/delta T lymphocytes in the peripheral blood of patients affected by active inflammatory bowel disease. Clin. Exp. Immunol. 98, 83–88.
33. Nakata, M., Smyth, M. J., Norihisa, Y., Kawasaki, A., Shinkai, Y., Okumura, K., Yagita, H. (1990) Constitutive expression of pore-forming protein in peripheral blood gamma/delta T cells: implication for their cytotoxic role in vivo. J. Exp. Med. 172, 1877–1880.