# Immune Modulating Agents

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# *Immunology of T Cells in AIDS: Dynamics Revealed by Eight-Color Flow Cytometry*

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## I. INTRODUCTION

Individuals infected with the human immunodeficiency virus type 1 (HIV) suffer profound immunological changes. The clinical consequences of these changes include pathological infections by normally "mundane" pathogens, chronic and systemic inflammatory and oxidative stress, and increased incidence of lymphomas. Eventually, the burden on the immune system and the body is too great, and the individual dies.

How HIV affects these widely disparate disorders, however, is still not understood. However, it is clear that HIV disease is accompanied by a wide spectrum of changes in virtually all cell types of the immune system. Even though HIV infection itself is restricted to cells bearing the CD4 molecule (i.e., almost exclusively CD4 T cells and monocytes), dysfunctions have been noted in every other compartment of the immune system. Indeed, historically the first observation of immunological abnormality in patients with acquired immunodeficiency syndrome (AIDS) was a polyclonal B cell activation [1].

Changes in cells of the immune system can be categorized into three partially overlapping types: *representational* (the absolute number of a cell type), *phenotypical* (the collection of proteins expressed by a cell type), and *functional* (the set of responses of a cell type). A single defect may fall into two categories: for instance, a change in the regulation of expression of a cell-surface receptor is a phenotypic change but may also represent a change in the functional responsiveness of the cell.

It is important to note that a change in the representation of a cell type may result in change in the functionality of the overall immune system—even though there is no change in the functionality of any given cell in the system. Therefore, we must determine whether any changes in functional measurements that are associated with HIV disease (or other disorders, for that matter) represent a change in the *intrinsic* functionality of cells in the immune system, or a change in the representation of functionally distinct cells within that system, or both.

In this chapter, we demonstrate that accompanying HIV disease is a profound

change in the *representation* of functionally distinct T cell subsets. Many of these changes may account for the "immunodeficiency" that has been ascribed to nonresponsiveness in in vitro assays or even in vivo. In addition, the changes that we discuss strongly support a model of HIV pathogenesis with the destruction of the thymus as a pivotal event leading to immunodeficiency.

## II. PHENOTYPE CHANGES

Quantitation of cell surface expression by flow cytometry is straightforward but requires rigorous attention to staining conditions. In principle, the amount of antigen expressed on a cell surface will be proportional to the fluorescence obtained when cells are stained with a conjugated monoclonal antibody to that antigen. However, care must be taken to ensure that the staining conditions are reproducible and that the reagent is used at a saturating titer. The requirements for performing quantitative antigen density are discussed in detail elsewhere [2,3].

Many of the molecules expressed on the cell surface play roles in cell function: as either receptors, costimulatory molecules, or other "environmental sensors." Changes in the expression of these molecules within a single cell may herald a change in the functionality of the cell. Furthermore, the quantitation of the expression may reveal regulatory processes effected at the transcriptional level—i.e., changes in the activation state of transcription factors, both enhancers and repressors.

It is important to remember that a change in the expression of an antigen may simply reflect a different frequency (representation) of unresolved underlying cell subsets that differentially express that antigen. For example, consider the expression of CD5, a protein expressed by virtually all T cells. It is well known that CD4 T cells express about two-fold more CD5 than do CD8 T cells. Therefore, the average amount of CD5 per T cell in a mixture of CD4 and CD8 T cells will be weighted by the proportion of each lineage in that mixture [3].

Figure 1 shows that the average expression of CD5 on all T cells is significantly reduced in human immunodeficiency virus- (HIV)-infected adults. However, the loss of CD4 T cells largely accounts for this decrease (Figure 1). Thus, without proper subsetting of the cells to account for the differential representation of CD4 and CD8 T cells, one would incorrectly conclude that the CD5 molecule is downregulated by cells in HIV-infected adults.

We have demonstrated that there are a wide variety of changes in antigen densities of T cell molecules accompanying HIV disease. Many of these occurred on homogeneous cell populations: for instance, the expression of CD62L on naive ($CD62L^+CD45RA^+$) CD8 T cells was reduced by almost 50% (Figure 1)—with no detectable change in the expression of other molecules such as CD45RA [3]. Since CD62L is involved in cell adhesion processes, this result may signify a change in the ability of naive T cells in HIV-infected adults to perform certain homing functions. CD62L is efficiently removed from the cell surface on cell stimulation (through protein kinase C); the reduction in CD62L expression may be caused by the systemic inflammatory stress, manifesting as increased serum levels of molecules like tumor necrosis factor (TNF), that accompanies HIV disease.

The expression of the same antigen can be differentially affected on various leukocyte populations. In HIV-infected adults, CD16 is expressed at lower levels on

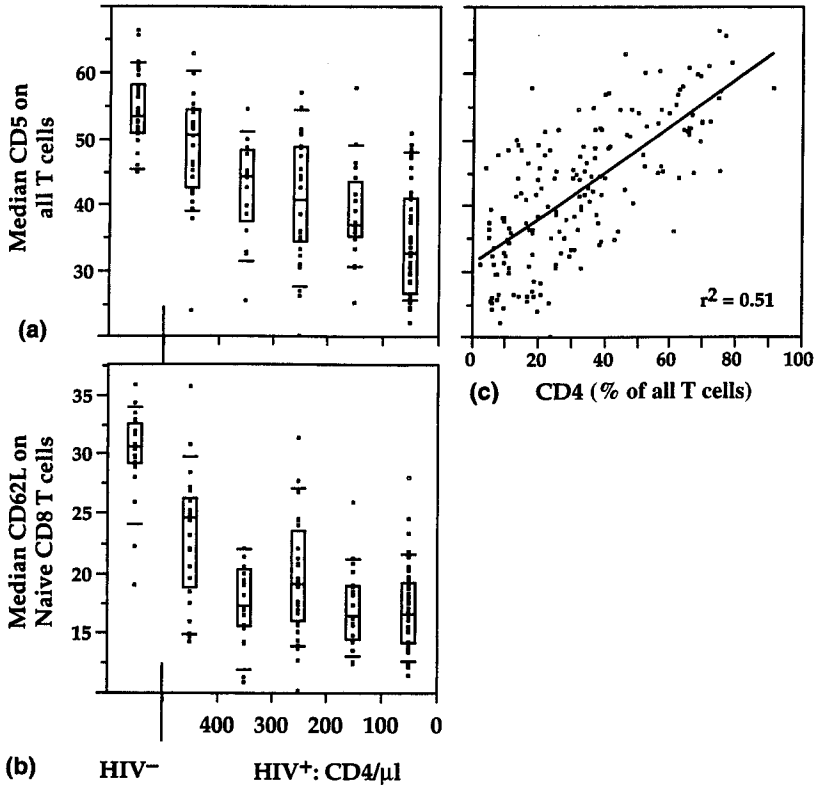



**Figure 1** Antigen density changes in HIV disease. (a, b) Antigen density changes in HIV disease may be potential surrogate markers of disease states. The expression of CD5 on all T cells (a) or CD62L on CD8 naive T cells (b) is correlated with disease progression. Both antigens show a progressive decline. In the case of CD5, this decline is primarily due to the loss of CD4 T cells (discussed later). However, for CD62L on the homogeneous naive CD8 T cells, this change may represent a change in the regulation of surface expression of this adhesion molecule on a cell-by-cell basis. Different antigen density changes occur at different stages of disease; here, CD62L exhibits the greatest change during the early stages. (c) Changes in antigen density may reflect changes in the representation of underlying subsets. CD5 expression is approximately two-fold higher on the surface of CD4 T cells than it is on CD8 T cells. Thus, measuring the average CD5 expression on T cells weights CD4 T cells greater than it does CD8 T cells. As is shown here, the average expression of CD5 is well correlated with the representation of CD4 T cells among the total T cell subpopulation. This concept extends to other functional measurements as well: for instance, a change in the secretion of a cytokine may reflect no more than a change in the representation of the subset(s) of cells normally producing that cytokine—even if there is no change in the regulation of the production of that cytokine on a cell by cell basis. Thus, the extrinsic change in representation of functionally distinct subsets overshadows any intrinsic variation in cell functionality that may or may not be present. (Adapted from Ref. 3.) HIV, human immunodeficiency virus.

natural killer (NK) cells, at higher levels on monocytes, and unchanged on granulocytes [3]. This kind of differential regulation underscores the necessity for subsetting cells before quantitating antigen density measurements.

## III. PROGNOSTIC SIGNIFICANCE OF ANTIGEN DENSITY MEASUREMENTS

We found more than a dozen different antigen density changes that were significant in HIV disease. Some of these changes occurred early in disease; some only occurred late. Thus, it is possible that these measurements have useful prognostic value for HIV disease.

Indeed, Giorgi and colleagues have elegantly demonstrated the power of antigen density measurements for predicting progression to AIDS. They quantitated the expression of an activation marker, CD38, on CD8 T cells and found that higher expression was correlated with faster progression to clinical AIDS [4]. In fact, the CD38 density measurement was considerably more powerful than either CD4 counts or viral load measurements. Even in combination with these two standard measurements, CD38 antigen density is a powerful predictor for rate of progression to AIDS. We have recently extended Giorgi and associates' findings by examining a cohort of patients that is relatively advanced in disease (CD4 $< 200/\mu l$). We found that CD38 is a powerful predictor for progression to death in relatively advanced-stage patients (Figure 2).

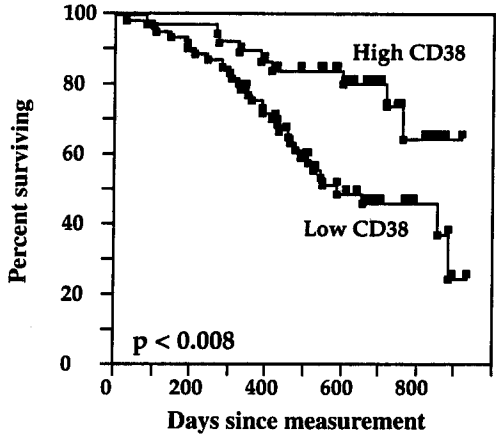



**Figure 2** Antigen density measurements: prognostic value. The expression of CD38 on CD8 T cells has been shown to be the most powerful predictor of progression to AIDS by Giorgi and colleagues. Here, we show that this parameter is also a powerful predictor of progression to death in individuals who are already below 200 CD4/$\mu$l. The survival rate (Kaplan–Meier analysis) of those who have high or low CD38 expression on day 0 is plotted; mortality rate is twice as great for those with high CD38 expression. AIDS, acquired immunodeficiency syndrome.

## IV. COMPLEXITY OF THE T CELL COMPARTMENT

One of the earliest markers for HIV disease progression is the absolute number of CD4 T cells in the periphery—i.e., accompanying HIV disease is a progressive decline in peripheral CD4 T cell counts. While the mechanisms accounting for this loss are still disputed, the loss itself serves as a relatively powerful staging tool. Contrary to the CD4 T cells, number of CD8 T cells is elevated early in disease and remain elevated until relatively late.

CD4 and CD8 T cells are not homogeneous subsets, however. They can be broadly classified into naive and memory T cells—functionally distinct stages of differentiation that can be identified phenotypically (Figure 3). The memory subsets themselves can be further divided into several subsets.

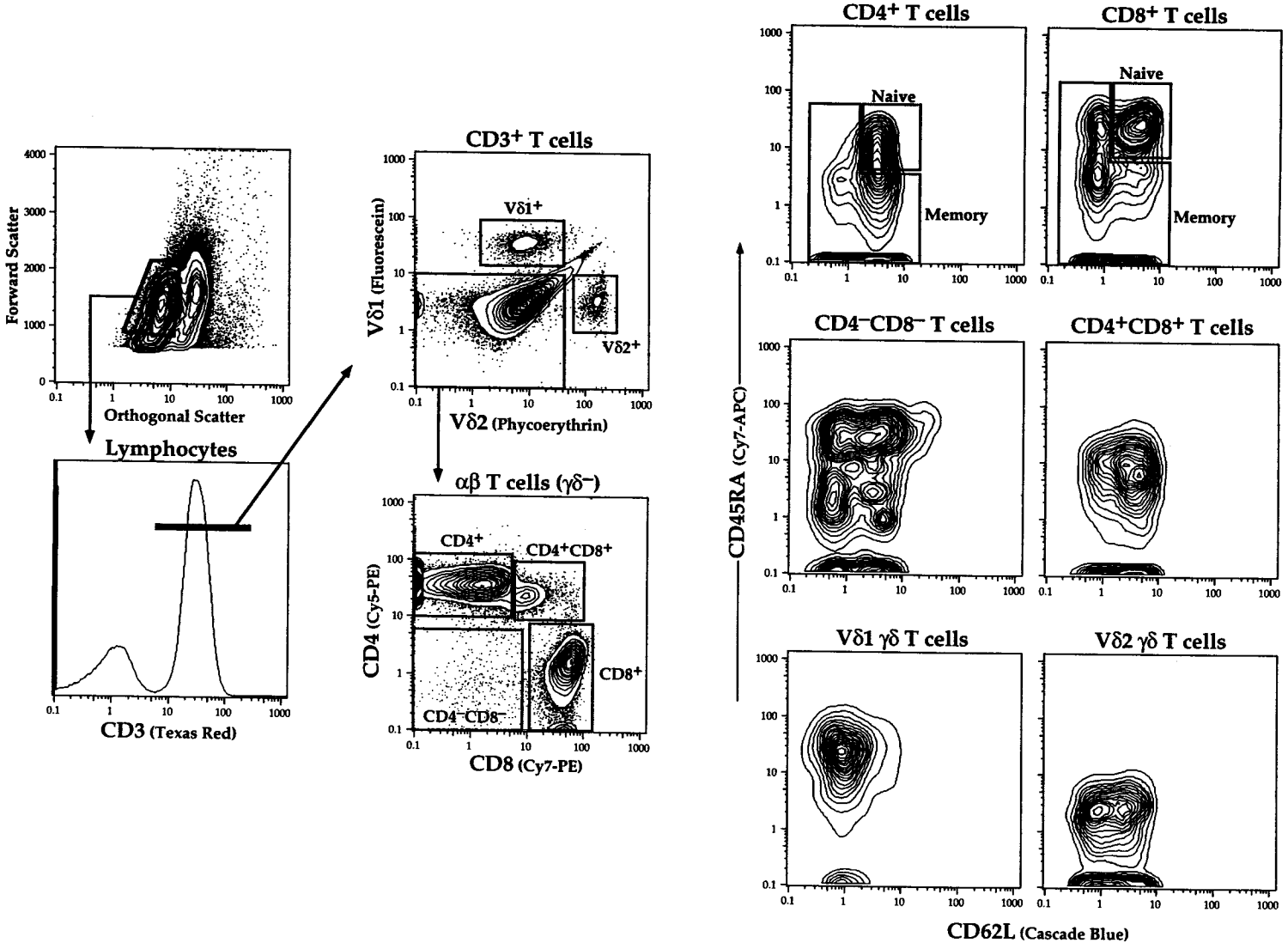
Indeed, the complexity of the T cell compartment is such that it cannot be resolved by measuring only three (or even four) surface antigens simultaneously (see Figure 3). For instance, unambiguous identification of just the CD4 T cell compartment requires three measurements: CD3 (to identify T cells), CD4, and CD8. The latter is necessary so that the $CD4^+CD8^+$ population can be excluded from analysis. Since identification of differentiation stages (naive/memory) requires at least two more measurements (CD45RA and CD62L), it is apparent that a minimum of five antigens must be simultaneously measured to approach homogeneous populations.

It is important to remember that there is a significant population of $CD4^+CD8^+$ (double positive [DP]) and $CD4^-CD8^-$ (double negative [DN]) T cells in the periphery. The DP and DN cells are mature T cells that have functional profiles distinct from those of either of the CD4 or CD8 lineages—for instance, a large fraction of DP cells produce interleukin 4 (IL-4) in response to stimulation (Mitra and Roederer, in preparation). DP response can cause significant artefact in functional experiments that use only one or two-color phenotyping to identify T cell lineages: DP cells will be perforce included in a typical CD8 gate (e.g., $CD3^+CD8^+$). This may lead to the erroneous conclusion that CD8 T cells produce IL-4 (whereas only the DP cells are contributing the cytokine). The complement of functions that DP and DN cells carry out remains to be determined—requiring at minimum three-color identification to identify those lineages uniquely.

In order to resolve these populations, we recently developed eight-color fluorescence-activated cell sorter (FACS) analysis (for example, Figure 3), which gives us the capability to determine the phenotype of T cell subsets with a precision previously unattainable. The primary goal in developing this technology was to achieve the ability to resolve functionally and phenotypically homogeneous subsets of T cells. In addition, we will be able to combine (for example) five-color phenotypic identification of T cell subsets together with three-color functional analysis (intracellular cytokine measurements, apoptosis measurements, etc.).

## V. T CELL DYNAMICS IN AIDS: LOSS OF CD4 AND CD8 T CELLS

When we examined the fine T cell subsets in our cohort of HIV-infected adults, we were surprised to find a selective loss of CD8 naive T cells [5] despite an overall increase in CD8 T cell numbers (Figure 4). Indeed, the loss of the CD8 naive T cells


Forward Scatter
Orthogonal Scatter
Lymphocytes
CD3 (Texas Red)
CD3+ T cells
Vδ1 (Fluorescein)
Vδ2 (Phycoerythrin)
Vδ1+
Vδ2+
αβ T cells (γδ−)
CD4 (Cy5-PE)
CD8 (Cy7-PE)
CD4+
CD4+CD8+
CD8+
CD4−CD8−
CD4+ T cells
CD8+ T cells
Naive
Memory
CD4−CD8− T cells
CD4+CD8+ T cells
Vδ1 γδ T cells
Vδ2 γδ T cells
CD45RA (Cy7-APC)
CD62L (Cascade Blue)

seemed to occur at roughly the same rate as the loss of the total CD4 population. Longitudinal analysis has confirmed this hypothesis (Figure 5). Indeed, the rate of loss of naive CD8 T cells is virtually identical to the rate of loss of naive CD4 T cells in the same individual over time.

The loss of naive CD8 T cells predicates that the memory CD8 compartment is expanding—since total CD8 counts are elevated and remain relatively stable during most of the disease. It was found over a decade ago that there is a significant increase in CD8 T cells bearing an activation phenotype ($CD38^+$ $HLA\text{-}DR^+$) [4]. Presumably, these cells are involved in immune responses in the individual (against HIV or other pathogens). What about the remaining CD8 T cells, those not involved in responses to HIV and concurrent pathogens? These cells, after all, underlie the basic immunological memory that protects against future pathogenic infections.

At this time, there is no way to identify these cells uniquely. Thus, we took the approach of enumerating unactivated CD8 T cells as a measure of the representation of the repertoire that is not directed against HIV or concurrent pathogens. As shown in Figure 6, the absolute number of resting CD8 T cells declines progressively during disease—much as the total CD4 count and the naive CD8 count do. On the basis of these data, we conclude that all T cells which were present at the time of HIV infection are progressively lost during disease and that these cells are not replaced through de novo thymopoiesis.

Another lineage of T cells disappears progressively with disease: a subset of $\gamma\delta$ T cells. In healthy $HIV^-$ individuals, a vast majority of peripheral $\gamma\delta$ T cells bear the $V\delta2$ variable region gene; in $HIV^+$ individuals, however, this condition changes such that the $V\delta1$-expressing cells become the majority [6–8]. Indeed, in absolute terms, they disappear at a rate similar to that of CD4 T cells, naive CD8 T cells, and unactivated memory CD8 T cells (Figure 6).

These findings have significant implications for immunopathogenesis of HIV disease. The primary conclusion is that HIV causes cell loss through mechanisms other than direct cytolysis as a result of infection. Therefore, there must be other mechanisms, such as bystander killing, homeostatic dysregulation, or loss of the ability to generate cells.

In addition, the observation that *all* T cell subsets are lost at the same rate is

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**Figure 3** Phenotypic identification of T cell lineages and differentiation states: identification of more than a dozen phenotypically distinct subsets. T cell subsets in a single healthy individual are shown by progressive gating of a single sample stained simultaneously with antibodies against CD3, CD4, CD8, CD45RA, CD62L, Vδ1, and Vδ2. Lymphocytes are identified by forward and side scatter (top left); T cells are selected on the basis of CD3 expression (bottom left). Within the CD3 T cells, Vδ1 and Vδ2 T cells are identified. The CD4 and CD8 subpopulations from $\delta^-$ ($\alpha\beta^+$) T cells are shown in the middle bottom. Here, CD4 and CD8 single-positive T cells, as well as $CD4^+CD8^+$ (DP) and $CD4^-CD8^-$ (DN) T cells, can be identified. For each of these four T cell subsets and the δ-expressing subsets, the expression of CD45RA and CD62L is shown (right). For the single-positive T cells, functional studies have identified that the $CD45RA^+CD62L^+$ cells are naive T cells; all others are memory T cells (as shown in the outlined areas). The functional capacity of the subsets defined by CD45RA and CD62L for the DP, DN, and δ subsets has not been determined.

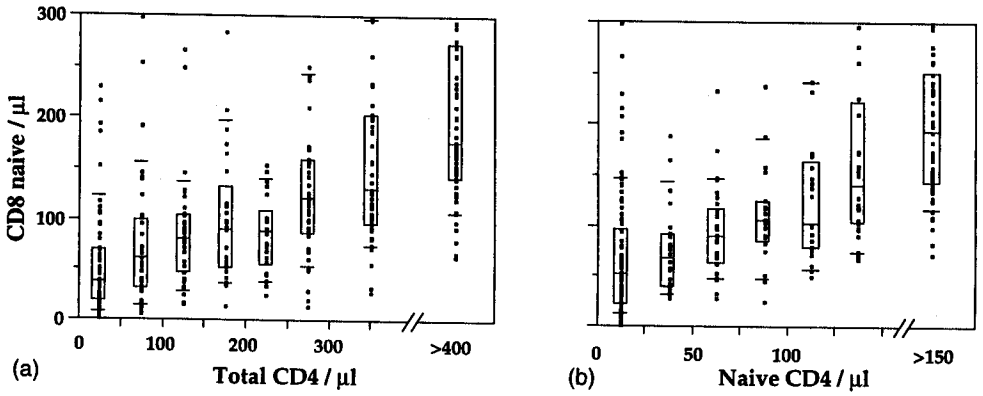


**Figure 4** Correlated loss of naive CD4 and CD8 T cells. Cross-sectional analysis of almost 300 HIV-infected adults, showing the correlation between naive CD8 T cell counts and total (a) or naive (b) CD4 counts. The cross-sectional analysis suggests that CD8 and CD4 subsets are lost at similar rates during the progression of HIV disease. HIV, human immunodeficiency virus.

highly suggestive that the same mechanism accounts for their loss. Thus, the major cause of the loss of CD4 cells is not due to a mechanism specific to CD4 cells—ruling out hypotheses such as infection and cytolysis, gp120-mediated "inappropriate" signaling through the CD4 molecule, or superantigenlike stimulation by an HIV protein.

## VI. EXPANSION OF UNUSUAL T CELL SUBSETS IN HIV DISEASE

It is well established that the proportion of "activated" CD8 T cells is increased significantly in HIV disease. Why is it, though, that the CD8 memory T cells expand whereas CD4 memory T cells do not? For the most part, these CD8 T cells express CD38—however, they do not express CD25, CD69, nor CD71, the classical markers of T cell activation. In addition, these cells are primed to apoptose, and once put into culture do not survive more than 1 or 2 days (data not shown). Perhaps the expanded CD8 subset represents an unusual type of cell, even a different lineage, than the "normal" thymically derived CD8 or CD4 T cells.

Another unusual population of CD8 T cells that increase in HIV-infected adults are $CD57^+CD28^-$ cells [9]. These cells are thought to be terminally differentiated effector (CTL) cells, being large granular lymphocytes with detectable intracellular perforin expression. The expansion of these two cell types is thought to be a product of the constant hyperactivation of the immune response to HIV. However, it is noteworthy that these kinds of cells are also expanded in the early stages of immune reconstitution (with allogeneic bone marrow), only to decline once the "normal" T cell population has recovered (Watanabe and Roederer, in preparation).

Recently, we have investigated several unusual T cell phenotypes that are significantly expanded in HIV disease. These phenotypes include $CD5^-$ T cells [10], and Vδ-bearing γδ T cells. Examples of these cell types are given in Figure 7.

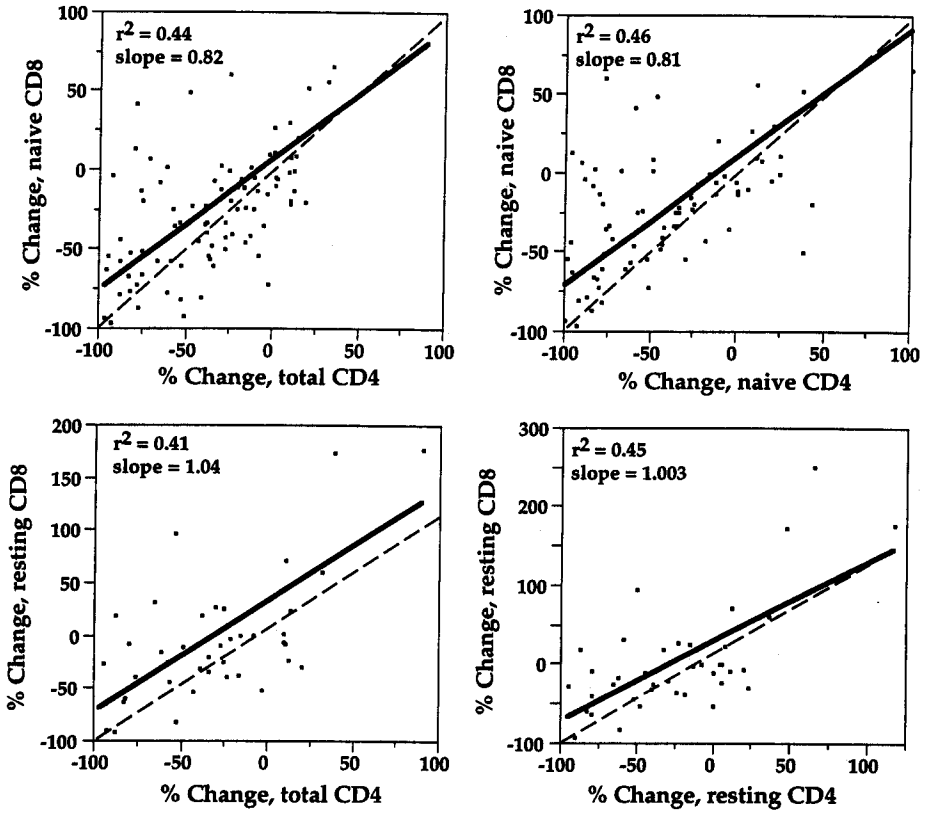


**Figure 5** Longitudinal analysis of T cell loss. For individuals who were analyzed two to four times over a 2-year period, the rate of loss of various T cell subsets was calculated and expressed relative to the starting value. The percentage change in these subsets is shown in the bivariate plots; linear regression is shown (dark line). The linear fits are remarkably close to that expected by equivalent rates of loss (dashed lines). This confirms that hypothesis based on the cross-sectional data that all of these T cell subsets disappear at similar or identical rates in HIV-infected adults. HIV, human immunodeficiency virus.

Unlike the disappearance of the "normal" Vδ2 cells (Figure 6), the Vδ1 T cells expand in HIV disease. These two kinds of γδ T cells have completely different phenotypes: Vδ2 are predominantly $CD5^+CD45RA^-CD57^-CD28^+$, whereas Vδ1 are $CD5^-/^{dull}CD45RA^+CD62L^-CD57^+CD28^-$. This latter phenotype is similar to that described for intestinal intraepithelial lymphocytes (iIELs). The iIEL T cells are educated and differentiate in the gut, in a thymus-independent fashion. The Vδ2 T cells, on the other hand, have a phenotype that is similar to that of "normal," thymic-derived T cells.

Another unusual population of T cells that increases in HIV disease are those that do not express CD5. These cells are quite rare in healthy $HIV^-$ individuals. While they were originally thought to be primarily αβ T cells [10], we find that roughly half express the γδ receptor. Thus, this population is only partially overlapping with the (increased) Vδ1 subset noted.

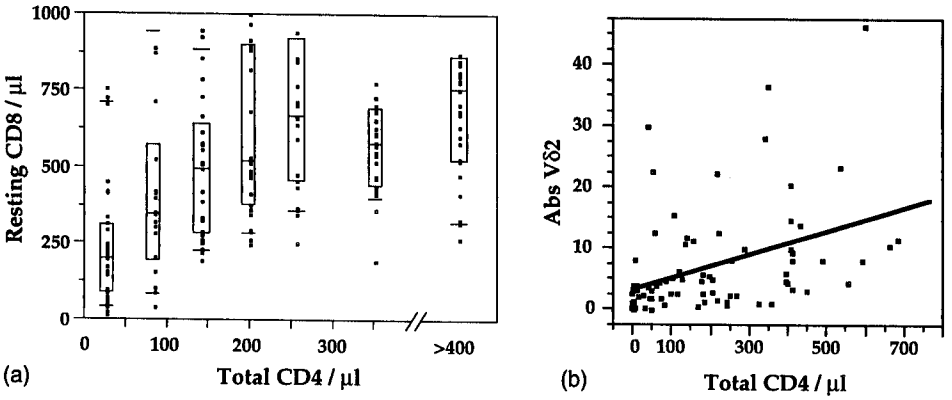


**Figure 6** All "normal" T cell subsets disappear progressively in HIV disease. (a) The absolute number of unactivated ($CD38^-HLA\text{-}DR^-$) CD8 T cells is plotted against the absolute number of CD4 T cells. During middle to late stages of disease (CD4 counts dropping below 400), the resting CD8 compartment disappears at the same rate as CD4 T cells (see also the longitudinal analysis in Figure 5). (b) The γδ T cells that are most prevalent in healthy $HIV^-$ adults express the Vδ2 gene. These cells are also progressively lost during HIV disease, at a rate that is correlated with the loss of naive CD8 T cells. HIV, human immunodeficiency virus; HLA, human leukocyte antigen.

## VII. T CELL DYNAMICS IN HIV DISEASE: A HYPOTHESIS

The emergence of these unusual T cell subsets in HIV disease suggests a hypothesis to account for many of the observed changes in T cell subsets: that extrathymic differentiation is (partially) compensating for the loss of thymic-derived T cell lymphopoiesis. This hypothesis is more completely stated in three parts as follows: (1) Accompanying HIV disease at an early stage is an insult to the thymus that shuts off the release of any new naive T cells. There is histological evidence that the thymus is functionally destroyed in infected individuals; as well, the loss of all naive T cells supports the lack of regeneration of this compartment. (2) Both naive and memory T cells that are not involved in anti-HIV-mediated immunity turn over with normal kinetics: probably on the scale of many months for memory cells and years for naive T cells. While there may be nondifferentiational division occurring during this time, there must be a stochastic loss of clones over time which underlies the turnover of the population. In healthy adults, the output of the thymus is sufficient to balance this slow loss of cells. The loss of memory T cells leads to progressive immunodeficiency (susceptibility to opportunistic infections); the inability to replenish the naive compartment renders this immunodeficiency permanent.

The combined loss of thymic activity and normal turnover leads to progressive declines in all thymic-derived T cell populations. This may be an underlying cause of (3) an expansion of extrathymically derived T cell education that attempts to compensate for the loss of thymically derived cells. The full functional capacity of these cells is unknown—are they capable of mediating the complex cascade of responses that are associated with normal T cell immunity? It is likely that the answer to this is no—i.e., that these T cells are capable of only a limited set of

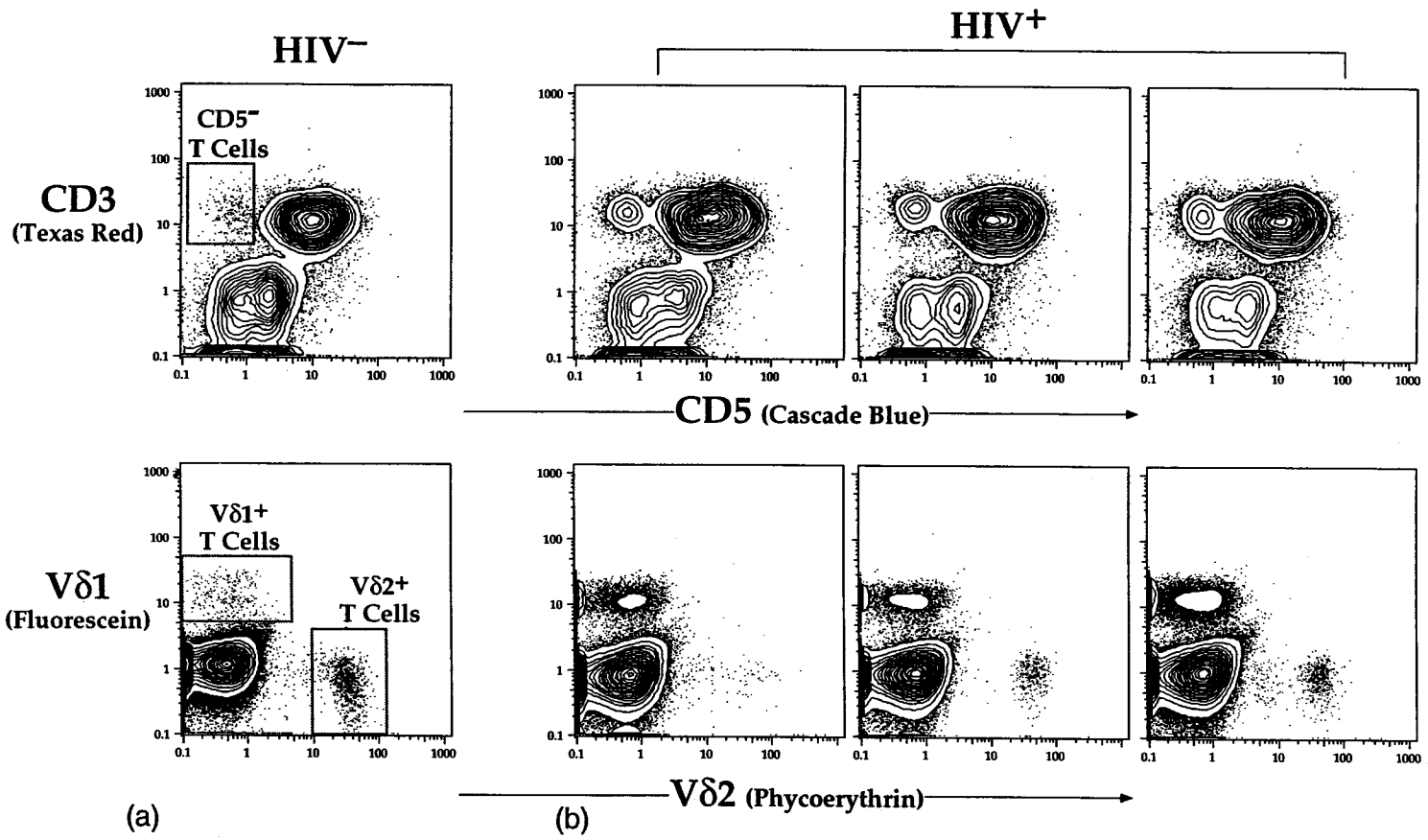


**Figure 7** "Unusual" T cell subsets expand in HIV-infected individuals. The phenotypes of cells for an uninfected healthy control (a) and three different HIV-infected adults (b) are shown. The top panels show plots of CD5 vs. CD3 for all lymphocytes. The $CD5^{-}CD3^{+}$ population (boxed) is expanded in both frequency and absolute number (not shown) in the infected adults. The bottom panels are the phenotypes of CD3 gate lymphocytes (i.e., only T cells) with regard to Vδ1 and Vδ2. In healthy individuals, Vδ2 T cells predominate; the opposite condition prevails in HIV-infected adults, who progressively lose Vδ2 T cells with a concomitant expansion of Vδ1 T cells. Note from Figure 3 that the Vδ1 and Vδ2 T cells have entirely different phenotypes (based on expression of CD45RA and CD62L), suggesting that they represent completely different lineages or at least different developmental programs. HIV, human immunodeficiency virus.

responses. The limited immunity imparted by these cells may explain why there is only a limited set of opportunistic infections typically found in late-stage AIDS: other pathogens are sufficiently suppressed by the "unusual" T cell subsets.

## VIII. CONCLUSIONS

Understanding the basis for the changes in the T cells in HIV disease is crucial for the evaluation of potential therapies. Already there are several clinical trials planned or under way in which gene therapy of stem cells is carried out with the intention of rendering the resulting T cells resistant to HIV infection. But if there is no thymic activity, such gene therapy is destined to fail, since no mature T cells will be generated. Further, because all lineages of "normal" T cells disappear during HIV progression—CD4, CD8, and a subset of $\gamma\delta$—it is not at all evident that protecting cells from HIV infection (i.e., CD4 T cells) will correct the basic defect leading to this cell loss.

Therefore, we must consider therapies that restore thymopoiesis if we are to restore immune function in HIV-infected individuals. Such therapy may include thymus transplantation, an exciting prospect that is now being vigorously pursued by several groups. Alternatively, it may be possible to stimulate extrathymic differentiation to an even greater degree and attempt to regenerate more of the T cell responses. Such reconstitution is important even despite new, advanced antiretroviral therapies (such as protease inhibitors combined with reverse transcriptase inhibitors) which are extremely effective at reducing or even removing virus from the periphery. The preliminary evidence is that these therapies effect, at best, a mild increase in immune function—underscoring the need to develop effective immunomodulatory therapy for this insidious disease.

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