Dynamics of fine T-cell subsets during HIV disease and after thymic ablation by mediastinal irradiation

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The T-cell compartment is considerably more complex than just CD4 and CD8 T cells. Indeed, we can identify dozens of functionally and phenotypically distinct subsets within the peripheral blood of humans. These subsets are differentially affected in diseases which may underly some of the functional defects attributable to the disease. In HIV disease, all thymic-derived T-cell populations are gradually lost at identical rates during late-stage disease progression, while unusual, perhaps extrathymically-derived T cells expand. This expansion may reflect an attempt on the part of the immune system to compensate for the significant insult of HIV infection to the host: the abrogation of normal thymopoiesis and T-cell homeostasis.

Key words: Hodgkin's lymphoma / memory T cells / naive T cells / T-cell dynamics / thymus

THE T-CELL COMPARTMENT in humans is remarkably diverse, both functionally and phenotypically. Much of this diversity has come to light only recently, with the advent of cell identification and separation technologies sufficiently advanced to resolve this heterogeneity. The vast majority of work on T-cell subsets has been performed on peripheral blood T cells (isolated from peripheral blood mononoclear cells, or PBMC), primarily because of the ease of obtaining a sample. Lymph node biopsies (and other peripheral sites) are rapidly becoming a more commonly-obtained tissue, but are still the domain of only a few laboratories. Analysis of peripheral sites is also made complicated by the presence of an increased proportion of concurrently activated cells: cells which have a morphology and surface phenotype that is not necessarily characteristic of any particular subset or type of cell.

While the use of PBMC as a window on the immune system is easy, it is also not necessarily accurate. The T cells in PBMC represent only a fraction (2%?) of the T cells in the body, and certainly the representation of various subsets is considerably different than in peripheral compartments. Thus, we must temper hypotheses derived from the analysis of peripheral blood with the understanding that this compartment may not be in equilibrium with the majority of the immune system.

Nonetheless, a great deal has been learned and continues to be learned from analysis of PBMC; and, ultimately, any common prognostic and diagnostic tests will necessarily be performed with blood cells.

T-cell complexity

The T-cell compartment is commonly divided into several lineages based on the broad functionality of cells contained within the lineage. The major lineages are the $CD3^+CD4^+CD8^-$ (helper, or T_h) and $CD3^+CD4^-CD8^+$ (cytotoxic/suppressor, or T_c) T cells. These cells arise through thymic education and constitute about 95% of the T cells in the PBMC from healthy adults. Another, quantitatively minor, lineage consists of T cells expressing the $\gamma\delta$ receptor; these can be divided on the basis of expression of CD4 and CD8 as well (and these probably represent distinct lineages). In addition, there are mature $\alpha\beta$ receptor-expressing T cells that express neither CD4 nor CD8 (double negative, or DN); these are functionally distinct from either the T_h or T_c cells and probably constitute a separate lineage as well. Finally, it has recently become apparent that there are additional lineages that are derived from extrathymic education, in sites such as the intestine (or even in the bone marrow itself). These cells have a distinct phenotype and functional capacity from thymic-derived T cells; however, these qualities overlap with some mature T cells and it can be difficult to uniquely identify these cells.

Cells leaving the thymus are termed naive, since they are mature T cells which have not yet encountered their cognate antigen. These cells, upon encounter with antigen, undergo significant expansion and differentiation; at the resolution of the immune response, a vast majority die through apoptosis. The surviving memory cells have a 'resting' (unactivated) phenotype and recirculate through the immune compartments carrying out a constant surveillance for antigen. Upon re-exposure, these memory cells proliferate, but can also carry out direct effector functions (like cytokine secretion, cytotoxicity, etc.), resulting in a more rapid clearance of antigen. Figure 1 summarizes the simplified life cycle of a T cell.

Based on functional studies, naive T cells have been distinguished from memory cells by surface immunophenotyping as shown in Figure 2. Unique indentification of naive T cells requires the simultaneous measurement of at least two antigens in addition to the lineage markers (e.g. CD4, CD8, or $\gamma\delta$ receptor). Naive T cells express CD45RA; the second antigen can be either CD62L[1] (naive T cells are positive) or CD11a[2] (naive cells are dim). In addition, naive T cells uniformly express CD28, and do not express CD16, CD38, CD45RO, CD56, CD57, HLA-DR (data not shown).

Memory T cells are heterogeneous in that many antigens (such as those listed above) can subdivide the T-cell population into distinct subsets. Indeed, by
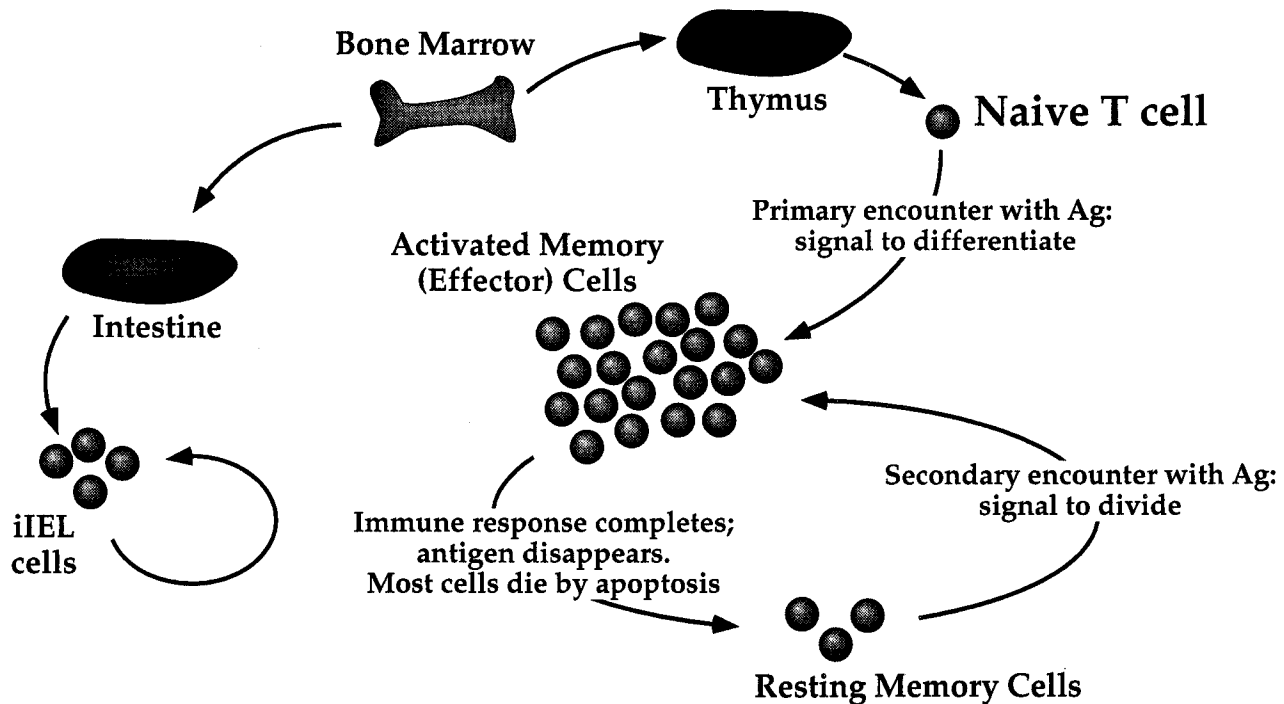

Figure 1. Life cycle of peripheral T cells. The majority of peripheral T cells undergo education (positive and negative selection) in the thymus. Those few that survive enter the blood as mature naive T cells. Naive T cells upon stimulation, produce essentially no cytokines (other than IL-2 and GM-CSF) and have no effector functions such as cytolysis; however, they have an enormous proliferative response. Proliferation is accompanied by differentiation into effector populations that carry out the immune response. At the resolution of the immune response (when antigen disappears), most of the cells undergo apoptosis; a few remaining memory cells, which are differentiated but resting T cells, will continue to circulate through the body. Upon activation, some of these cells immediately produce effector cytokines such as IL-4 and γIFN, and (in the case of T_c) can effect cytolysis. The proliferative response is significantly lower than that of naive T cells. There are alternative differentiation pathways for the generation of T cells that occur extrathymically. The dynamics of the iIEL-type of cells is poorly understood; for instance, what is their lifespan, what recirculation patterns (including the blood) do they exhibit, and can they regenerate with de-novo production from stem cells?

simultaneously measuring eight different surface antigens, we are able to recognize at least two dozen distinct subsets of cells.[3] These subsets all have distinct functional profiles and undoubtedly carry out specific functions within the body.

When phenotypically homogeneous subsets of memory cells are examined in isolation, there is much less functional heterogeneity than for the whole T-cell compartment. Indeed, we are optimistic that functionally homogeneous subsets of T cells can be
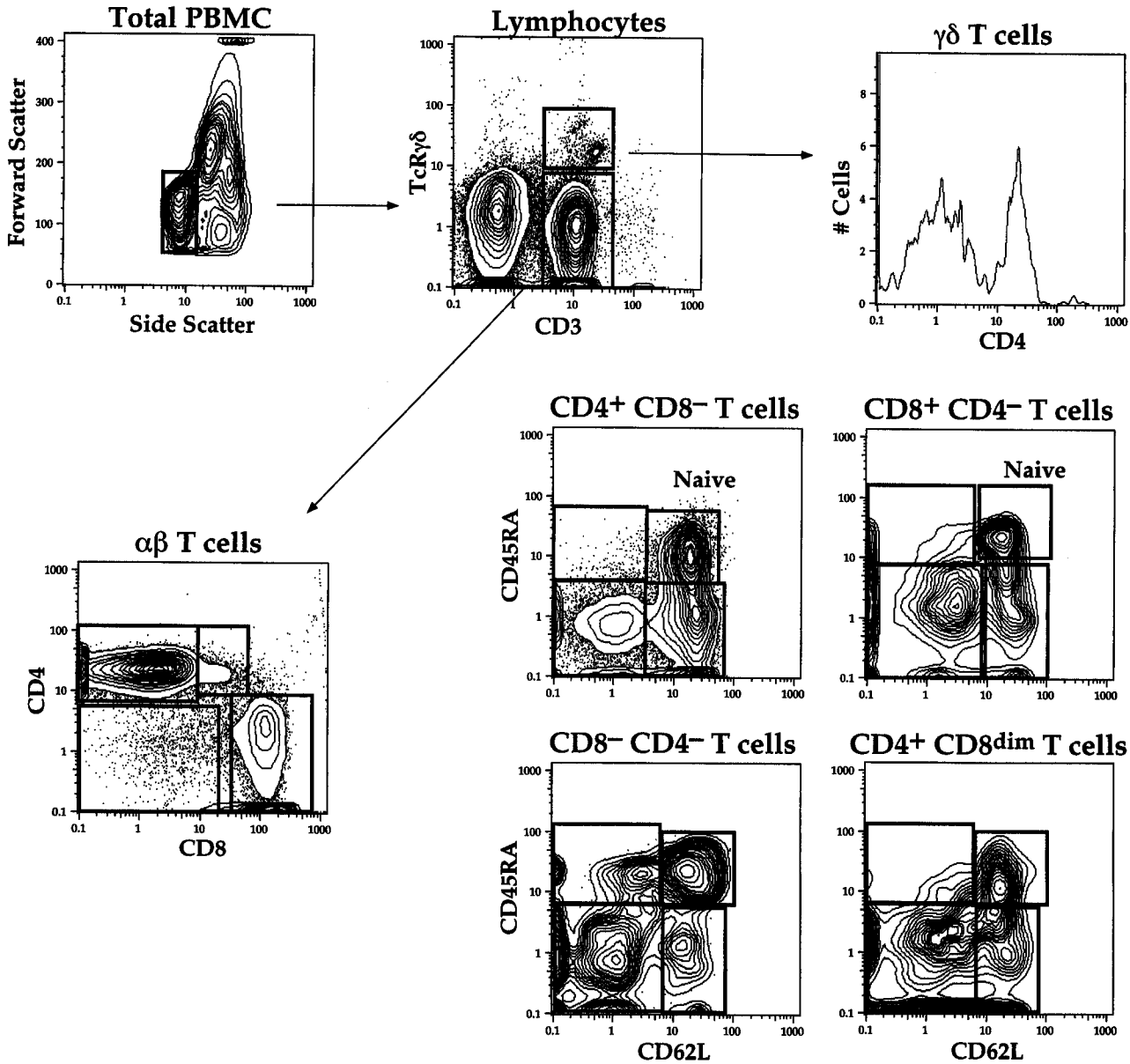

Figure 2. Subsets of peripheral T cells. PBMC were simultaneously stained with conjugated antibodies to CD3, TcRγδ, CD4, CD8, CD45RA, and CD62L, and analysed by eight-color FACS.[3] Progressive gating from the top left is shown: lymphocytes (based on light scatter); δ T cells versus αβ T cells. CD4 expression further divides the γδ T cells; also, CD45RA and CD62L can further subdivide them into populations akin to the αβ T cells.[9] The αβ T cells are divided into four separate lineages, T_h, T_c, DN, and $CD8^{dim}$; for each of these lineages, the CD45RA versus CD62L expression pattern is shown. For at least the T_h and T_c cells, naive T cells are identified as the cells co-expressing CD45RA and CD62L; it is likely that differentiation stages within the other lineages can be similarly distinguished.

identified by sufficiently detailed surface immunophe-notyping.[4] Should this be the case, it may be possible to supplant many of the in-vitro functional assays by simply determining the representation of phenotypic subsets,. Table 1 summarizes some of the functional characteristics of major differentiation stages within the T-cell lineages.

The meaning of pathological changes in the T-cell compartment

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 bulk 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 pathologies, for that matter) represent a change in the intrinsic functionality of cells in the immune system, or a change in the representation of function-ally-distinct cells within that system, or both.

In this manuscript, we will describe several profound long-term changes in representation of T-cell subsets that occur in HIV disease and in adults treated for Hodgkin's lymphoma. While these are very different pathologies, they share some very interesting changes in the T-cell compartment: and from these changes, we hope to derive a better understanding of T-cell dynamics.

In addition, it is certain that many of these changes by themselves underlie the functional defects ascribed to these individuals, bringing up the question: are there any functional defects at the cellular level? Answering this question is important to the consideration of therapeutic intervention: if the answer is 'no', then therapies should be designed which change the generation of T cells, or change the homeostatic regulation of T-cell levels, in order to bring about functional restoration of the immune compartment.

T-cell changes in HIV disease

One of the earliest markers for HIV disease progression is the absolute number of T_h cells in the periphery—i.e. accompanying HIV disease is a progressive decline in peripheral T_h 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 T_h cells, T_c cells are elevated early in disease, and remain elevated until relatively late.

However, there are profound changes that occur within each of these lineages that significantly complicate this picture. Indeed, when the subsets of T cells are enumerated using CD45RA and CD62L (as defined in Table 1), it becomes apparent that there is a significant insult to the T_c compartment as well as

Table 1. Summary of functional differences between T differentiation stages*

Stage	Phenotype	Ca†	Prolif‡	PCD§	Cytokines¶			HIV‖	Perf**
					IL-2	IL-4	γIFN		
Naive	CD45RA⁺CD62L⁺	++	++	−	+	−	−	−	−
	CD45RA⁻CD62L⁺	+	+	+	+++	+	+§§	+	−
Memory	CD45RA⁻CD62L⁻	+	+	+	+++	−	+§§	+	+
	CD45RA⁺CD62L⁻	+/−	−	+++	+	+/−	++	nt	++

*Only the stages defined by CD45RA and CD62L are defined. The functional comparisons are identical within both CD4 (T_h) and CD8 (T_c) subsets. While the qualitative differences between differentiation stages are similar for the T_h and T_c lineages, there are significant quantitative differences between the lineages.[4] For instance, T_c cells produce very little IL-4, but the cells that do produce IL-4 are CD45RA⁻CD62L⁺. Likewise, in T_h cells perforin is found only in a very small minority of cells; however, these are CD45RA⁺CD62L⁻.
†Relative ability to mobilize intracellular calcium after cross-linking of CD3.[15]
‡Proliferative response after stimulation with mitogens such as PHA, or CD3⁺CD28 crosslinking.[16,17]
§Relative apoptotic potential (programmed cell death) after placing cells in culture.
¶Cytokine profile as determined either by intracellular cytokine staining after stimulation with phorbol ester and ionomycin, or by quantitating mRNA in purified subsets after stimulation with PHA, CD3, or CD3 + CD28.[4,17]
‖Relative ability of HIV to replicate in these cells (CD4 subsets only).[17]
nt, not tested.
**Relative expression of intracellular perforin (CD8 subsets only).
§§γIFN production is primarily found in the CD11a^bright cells within these subsets.[4]

the T_h compartment. Specifically, despite the overall increase in total T_c number, there is a loss of naive T_c cells that appeared to occur at the same rate as the loss of total T_h cells. Longitudinal analysis has confirmed this hypothesis (Figure 3). Indeed, the rate of loss of naive T_c cells is virtually identical to the rate of loss of naive T_h cells in the same individual over time.

This change in representation of functionally distinct subsets has important implications for functional assays performed on PBMC or bulk T-cell populations (Table 2). For instance, comparing the cytokine profile of PBMC from patients with AIDS with that of PBMC from healthy controls is totally confounded by the change in T-cell representation. The PBMC from AIDS patients will have a profile characteristic primarily of memory CD8 T cells — whereas that from healthy individuals will represent the balance between T_h and T_c, and between naive and memory cells.

The loss of naive T_c cells predicates that the memory T_c compartment is expanding–since total T_c 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 T_c cells bearing an activation phenotype ($CD38^+HLA-DR^+$).[5] Presumably, these cells are involved in immune responses in the individual (against HIV or other pathogens). What about the remaining T_c 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 uniquely identify these cells. Thus, we took the approach of enumerating unactivated (i.e. not expressing CD38 or HLA-DR) T_c cells as a measure of the representation of the repertoire that is not directed against HIV or concurrent pathogens. As shown in Figure 3, the absolute number of unactivated memory CD8 T_c cells declines progressively during disease–much like the total T_h count and the naive T_c count. On the basis of this 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 changes such that the $V\delta1$-expressing cells become the majority.[6-8] Indeed, in absolute terms, $V\delta2$ cells disappear at a similar rate
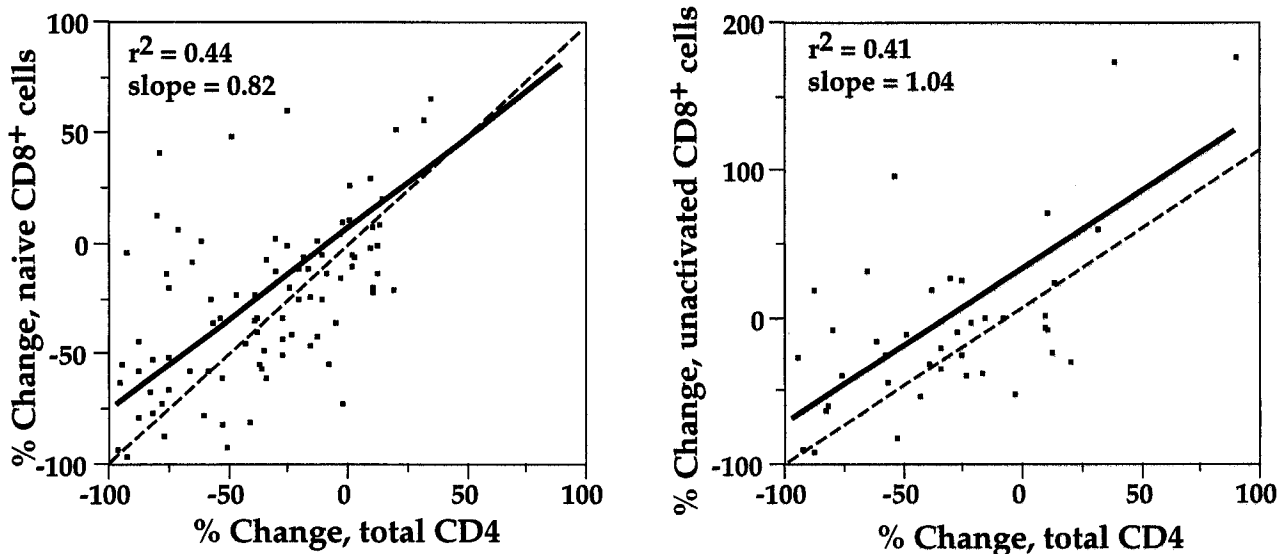

Figure 3. Longitudinal analysis of T_c populations in HIV-infected adults. A cohort of HIV-infected adults was followed for a period of 1 or 2 years with at least annual measurements of absolute cell counts and relative proportions. Naive T cells were identified as in Figure 2; unactivated T_c cells are identified as those CD8$^+$ T cells which do not express CD38 or HLA-DR (which includes both naive and memory subsets). For these populations as well as the total CD4 count, the rate of loss of cells was computed over the time-span that each individual was followed. The percentage change in the T_c subsets is shown against the percentage change in the T_h count; the solid line is the best-fit linear regression. The dashed line is the relationship expected for identical loss rates.

as do T_h cells, naive T_c cells, and unactivated memory T_c cells (data not shown). Of the γδ T cells, the Vδ2 T cells found in healthy individuals (and which disappear in HIV disease) most closely resemble thymically-educated T cells by phenotype: the γδ T cells that become prevalent in late HIV disease resemble intestinal intra-epithelial lymphocytes (iIEL) and may be extra-thymically derived.[9]

The observation that all (thymically-derived) T-cell subsets are lost at the same rate is highly suggestive that the same mechanism accounts for their loss. Thus, the major cause of the loss of T_h cells is not due to a mechanism specific to T_h cells–ruling out hypotheses such as infection and cytolysis, gp120-mediated 'inappropriate' signaling through the CD4 molecule, or superantigen-like stimulation by an HIV protein. Indeed, it is tempting to pinpoint the progressive destruction of the thymus in late-stage disease as the primary cause of the loss of these cell types.

Long-term T-cell changes in patients treated for Hodgkin's disease

In order to address the potential role of the thymus in maintaining (or regenerating) T-cell populations we examined a cohort of adults treated for Hodgkin's disease (HD).[10] HD is known to encompass thymic involvement; in addition, these patients were treated with mediastinal irradiation that presumably destroys any residual thymic activity.

Figure 4 shows that these patients have a long-term defect in the representation of naive T cells, for both the T_h and T_c lineages. The total number of memory T cells returns to normal (T_h) or even slightly above-normal (T_c) within 2.5 years. Indeed, the previously-observed deficit in total T_h counts, after treatment of

Table 2. Representation of T-cells subsets in HIV disease

		Frequency (% of T cells)	
Cell type		HIV⁻	HIV⁺ (CD4<200/µl)
CD4	Naive	33	2
	Memory	33	7
CD8	Naive	19	10
	Memory	15	81

The fraction of total T cells of each of the cell types was computed and averaged for cohorts of 34 HIV⁻ healthy adults and 74 HIV⁺ adults with CD4 counts under 200 per µl of blood. Native T cells are defined as CD45RA⁺CD62L⁺; memory T cells are defined as all other subsets.

these patients, is solely due to the inability of the naive compartment to regenerate.

Expansion of unusual T-cell subsets

In both HIV-infected adults and HD treated adults, there is often a significant increase in 'unusual' T-cell subsets. These are subsets that are extremely rare in healthy adults. We have commonly observed three different types of subsets.

First are T cells which do not express CD5. In healthy adults, a vast majority of CD3⁺ T cells co-express CD5. In HIV-infected adults, there is a significant expansion of this lineage, with some individuals having as many as 200 CD5-negative T cells/µl blood.[9] About half of these cells express the αβ receptor and half the γδ receptor.

Second are CD8^dull T cells. These cells express low levels of CD8 compared to T_c cells; in fact, they express CD8αα homodimers rather than CD8αβ heterodimers. The CD8^dull T cells can be either CD4⁺ or CD4⁻; a significant fraction of these cells express intracellular perforin,[10] suggesting that they are cytotoxic effector cells.

Finally there are the Vδ1 T cells. Unlike the 'normal' Vδ2 cells which disappear in HIV-infected individuals, the Vδ1 T cells expand. 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⁻.

These unusual T-cell subsets, which are distinct from each other, have a phenotype similar to that described for intestinal intra-epithelial lymphocytes (iIEL).[11-14] iIEL T cells are educated and differentiate in the intestine, in a thymus-independent fashion. We hypothesize that in the case of depleted (or absent) thymic activity, as is the case for treated HD patients and for HIV-infected adults in relatively late stage disease, the immune system attempts to compensate by increasing the activity of alternative T-cell differentiation pathways. In the patient, this is manifested by an increase in the number of extrathymically-derived T cells in the blood, where they are typically very rare.

Accompanying HIV disease is also an expansion of so-called 'activated' T cells–bearing CD38 and /or HLA-DR.[5] However, it is not clear that these cells have been activated by normal pathways, or that they are really activated T cells. These cells do not express the obligate markers of T-cell activation such as CD25,

CD69, or CD71; they do not appear to be in cell cycle; they do not secrete cytokines unless stimulated; and they are not as large in size as one would expect for activated T cells. It is quite possible that these cells are derived from a different lineage of T cells than either the T_h or T_c cells, and are expanded in HIV disease in a compensatory mechanism to offset the loss of the 'normal', thymically-derived T-cell subsets.

Summary

The changes in the T-cell compartment accompanying HIV disease are profound and encompass virtually every subset. In particular, it is apparent that there is a progressive obliteration of all T-cell subsets that are normally found in healthy adults. This broad defect was obscured by a concomitant expansion of unusual T-cell subsets, recognized only by detailed immuno-
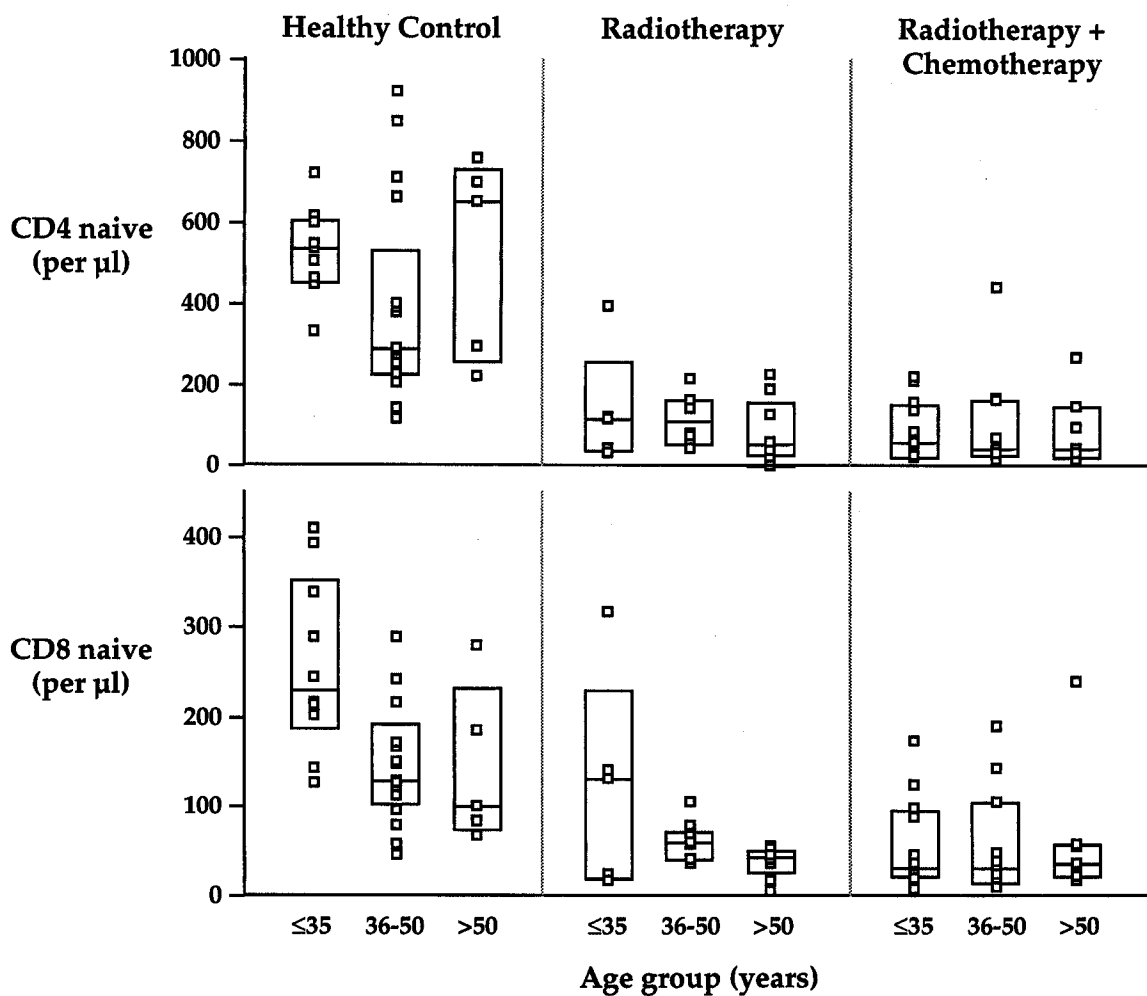

Figure 4. Long-term deficit in naive T cells in patients treated for Hodgkin's lymphoma. Patients were treated with mediastinal irradiation; the combination of the irradiation and the original disease is expected to obliterate thymic activity. In these patients there is a long-term deficit in the naive T-cell counts irrespective of the age at analysis, and irrespective of the time since therapy,[10] including some patients analysed more than 20 years after the initiation of therapy. In general, total T-cell counts returned to near-normal within 2.5 years.[10] The 'box plots' identify the median and interquartile range for each distribution.

phenotyping and functional analysis. This expansion may reflect an effort on the part of the immune system to compensate for the insult to the thymic-derived populations.

While there are similar changes in the immune system in both HIV disease and in patients treated for HD, there is a profound difference clinically: HD patients are generally healthy and not subject to most opportunistic infections. Clearly HIV is having a significant impact on the functionality of the immune system outside of the insult to T-cell homeostasis. However, with the advent of extremely effective antiviral therapies, we must now turn our attention to the regeneration of a normally-functioning T-cell compartment.

The immunocompetence of the unusual T-cell subsets remains to be determined. If they have a broad protective activity that can successfully replace that provided by the normal T-cell compartment, then we should endeavor to find therapies which can stimulate the further expansion of these cells. Otherwise, we must focus on therapies that can restore normal thymopoiesis and, ultimately, normal T-cell homeostasis.

References

1. Picker LJ, Treer JR, Ferguson DB, Collins PA, Buck D, Terstappen LW (1993) Control of lymphocyte circulation in man. I. Differential regulation of the peripheral lymph node homing receptor L-selectin on T cells during the virgin to memory cell transition. J Immunol 150:1105-1121
2. Okumura M, Fujii Y, Takeuchi Y, Inada K, Nakahara K, Masuda H (1993) Age-related accumulation of LFA-1^{high} cells in a CD8$^+$ CD45RA^{high} T-cell population. Eur J Immunol 23:1057-1063
3. Roederer M, Rosa SCD, Gerstein R, Anderson MT, Bigos M, Stovel R, Nozaki T, Parks DR, Herzenberg LA, Herzenberg LA (1997) 8-Color, 10-parameter flow cytometry: elucidation of complex leukocyte heterogeneity. Cytometry (in press)
4. Mitra DK, Rosa SD, Luk A, Balamurugan A, Khaitan BK, Tung J, Mehra NK, Terr AI, O'Garra A, Herzenberg LA, Herzenberg LA, Roederer M (1997) Differential representations of memory T cell subsets are characteristic of polarized immunity in Leprosy and atopic diseases. Submitted
5. Giorgi JV (1996) Phenotype and function of T cells in HIV disease, in Immunology of HIV Infection (Gupta S, ed) pp 181-199. Plenum Press, New York
6. De Maria A, Ferrazin A, Ferrini S, Ciccone E, Terrangna 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 [published erratum appears in J Infect Dis 1992 Oct; 166:950]. J Infect Dis 165:917-919
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 ML (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. De Rosa SC, Mitra D, Watanabe N, Herzenberg LA, Herzenberg LA, Roederer M (1997) Vδ1 and Vδ2 γδ T cells may represent developmentally distinct lineages: the Vδ1 to Vδ2 shift in HIV disease signals thymic impairment Submitted
10. Watanabe N, Rosa SCD, Cmelak A, Hoppe R, Herzenberg LA, Herzenberg LA, Roederer M (1997) Long-term depletion of naive T cells in patients treated for Hodgkin's disease. Blood in press
11. Lundqvist C, Baranov V, Hammarstrom S, Athlin L, Hammarstrom ML (1995) Intra-epithelial lymphocytes. evidence for regional specialization and extrathymic T cell maturation in the human gut epithelium. Int Immunol 7:1473-1487
12. Morrissey PJ, Charrier K, Horovitz DA, Fletcher FA, Watson JD (1995) Analysis of the intra-epithelial lymphocyte compartment in SCID mice that received co-isogenic CD4$^+$ T cells. Evidence that mature post-thymic CD4$^+$ T cells can be induced to express CD8 alpha in vivo. J Immunol 154:2678-2686
13. Reimann J, Rudolphi A (1995) Co-expression of CD8 alpha in CD4$^+$ T-cell receptor alpha beta + T cells migrating into the murine small intestine epithelial layer. Eur J Immunol 25:1580-1588
14. Latthe M, Terry L, MacDonald TT (1994) High frequency of CD8αα homodimer-bearing T cells in human fetal intestine. Eur J Immunol 24:1703-1705
15. Roederer M, Bigos M, Nozaki T, Stovel RT, Parks DR, Herzenberg LA (1995) Heterogeneous calcium flux in peripheral T cell subsets revealed by five-color flow cytometry using log-ratio circuitry. Cytometry 21:187-196
16. Rabin RL, Roederer M, Maldonado Y, Herzenberg LA, Herzenberg LA (1995) Altered representation of naive and memory CD8 T cell subsets in HIV-infected children. J Clin Invest 95:2054-2060
17. Roederer M, Raju PA, Mitra DK, Herzenberg LA, Herzenberg LA (1997) HIV does not replicate in naive CD4 T cells stimulated with CD3/CD28. J Clin Invest 99:1555-1564