

## Chapter 182

# Flow Cytometric Evaluation in AIDS

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Flow cytometric analyses can provide an enormous amount of information about the state of the immune system. In a disease of the immune system, such as the acquired immunodeficiency syndrome (AIDS), such information is critical for prognosis, disease staging, as well as monitoring of therapeutic interventions. However, it has also been central to the development of an understanding of the immunopathogenesis of the diseases caused by the human immunodeficiency virus (HIV).

A vast majority of flow cytometric analyses have involved enumeration of simple subsets of peripheral blood mononuclear cells (PBMC), including T cells (both CD4<sup>+</sup> and CD8<sup>+</sup>), natural killer (NK) cells, B cells, and monocytes; a few studies have dealt with neutrophils. Most of these studies involved the use of only one or two simultaneous immunofluorescence measurements, and were able to distinguish only broad subsets of cells.

More recently, the advent of practical three-color flow cytometric analyses have brought about significant advances in the understanding of the immunopathogenesis accompanying HIV disease, in terms of the specific changes of subsets within T cells, B cells, etc. In particular, the T cell lineage, which contains at least a dozen identifiable phenotypic subsets, has significant changes in the representation of quasi-activated cells, memory and naive cells, as well as other functionally-defined subsets.

Detailed understanding of these changes will be of significant help to the understanding and treatment of AIDS. By identifying the changes in all the subsets of leukocytes, we can begin to ascribe the mechanisms by which HIV damages the immune system. These may lead us to specific immunotherapies that can restore immune function, the basic defect caused by HIV disease. Likewise, identification of important changes in subsets will provide us with surrogate markers for disease that may be useful for prognosis, diagnosis, and monitoring studies.

*Overview.* In this chapter, we present a comprehensive review of the phenotyping studies that have been performed on HIV-infected individuals since the early 1980's. Some mention of functional relevance of these subsets is provided to demonstrate relevance of the measurements; however, the literature on function is vast and is beyond the scope of this review.

The review of phenotypic studies is grouped by subset: first T cells (CD4<sup>+</sup> and CD8<sup>+</sup>), then  $\gamma\delta$ -T cells, B cells, NK cells, monocytes, and neutrophils. Most studies reported only subset enumerations in terms of frequency (such as percent of lymphocytes) or in terms of absolute numbers (cells per  $\mu$ l of whole blood), or both. A few studies also measured antigen density, quantitating how many antigen molecules per cell are present (in relative terms). These types of measurements are specifically identified.

This chapter is organized in two sections. The first is the review of the phenotypic studies performed by flow cytometry for AIDS. This section is grouped by cell type, starting with T cells (CD4 and

CD8), then NK and B cells, and finally monocytes and granulocytes. The second section attempts to address the complexities of the flow cytometric analyses which have led to considerable confusion, as noted in the review. Some examples of the problems are given, along with suggestions for resolving and avoiding these problems in future immunophenotyping studies.

### T cells

The earliest (1982–1984) immunologic defect noted in HIV-infected adults, identified phenotypically, was the decrease in representation of CD4 T cells and increase in CD8 T cells [1–6]. These studies were first performed before HIV was identified, on homosexual men presenting otherwise rare syndromes. The findings were soon confirmed in HIV-infected hemophiliacs [7], who received blood products concentrated from thousands of donors prior to HIV-screening of blood.

Soon after, it was noted that HIV-infected people had a significant increase in the proportion of T cells that had an activated phenotype. Increased HLA-DR expression on T cells was first noted by Mildvan et al [2]; increased CD38 expression was first noted by Schroff et al [8]. These T cells actually express a "quasi-activated" phenotype, since they do not also express either CD25 or CD71, both of which are prototypical markers of activation of T cells.

T cells can also be subdivided into naive cells (those which have not encountered antigen, and have most recently arisen from development), and memory cells (those which have undergone an antigen-induced differentiation). While the precise phenotype of naive T cells is still being elucidated, markers that are differentially expressed on the subsets include CD11a (LFA-1), CD11b, CD28, CD29 (4B4), CD62L (Leu8), CD45RA (2H4), and CD45RO. None of these markers by itself uniquely identifies the subsets. CD11a is weakly expressed on naive, and strongly expressed on memory (an epitope of CD11a detected by S6F1 appears to be negative on naive T cells). CD28 is expressed on most T cells, although there is a population of CD28<sup>-</sup> cells (which generally express CD11b). CD29 is a marker expressed predominantly on memory cells. Finally, CD45RA and CD45RO are generally expressed in a mutually exclusive fashion (except on activated cells), with CD45RO never appearing on naive T cells (however, some memory cells are CD45RA<sup>+</sup>CD45RO<sup>-</sup>). Recent three-color experiments have shown that naive cells can be more accurately identified by the combined expression of CD62L and CD45RA, as well as CD11a-dim [9–11].

The majority of circulating T cells express the  $\alpha\beta$  T cell receptor; a minor subset expresses the  $\gamma\delta$  T cell receptor. Various studies have attempted to define the T cell V $\beta$  repertoire for both CD4 and CD8 populations in HIV disease [12–25]. The goal of the majority of these studies was to determine whether a deletion of a specific V $\beta$  subset could be detected, predominantly among

the CD4 T cell populations (i.e., superantigen effect). In addition, it was proposed that certain  $V\beta$  populations might be expanded among the CD8 T cells: such an observation might identify an important functional subset that provides protective immunity in this disease. The results of these studies have been highly variable, showing numbers of different  $V\beta$  alleles being reduced among the CD4 T cells and expanded among the CD8 T cells. One interesting study suggested that  $V\beta$ -12 CD4 T cells may be preferentially infected with HIV [13], thereby adding credence to the hypothesis that HIV might act as a superantigen. Overall, no clear-cut pattern has emerged from these studies.

## CD4 T cells

### Introduction

The hallmark immunologic feature of infection with the human immunodeficiency virus (HIV) is the depletion of the CD4<sup>+</sup> T cell population [26, 27], since the CD4<sup>+</sup> T cell serve as the major target for HIV by the binding of HIV surface gp120 glycoprotein with the CD4 antigen [28]. This interaction leads to infection of the CD4 T cells and results in the gradual decline of CD4 cell numbers with the ultimate result being the development of an AIDS-defining condition. Demonstration of this loss of CD4 cells associated with an immunodeficiency syndrome in homosexual males is one of the earliest observations in the AIDS epidemic [27]. This marker served as an important parameter early on in this epidemic by which patients who were infected could be evaluated, before we identified that this disease was due to a retrovirus, and before an antibody test (ELISA) was available. The importance of CD4 cells in HIV disease pathogenesis has been recently recognized by the inclusion of a CD4 count less than 200/ $\mu$ l in the definition of AIDS [29]. This highlights the important role for monitoring CD4 T cell numbers in HIV disease. In addition, it has been recognized that at a CD4 level of 200/ $\mu$ l, HIV<sup>+</sup> individuals will develop *Pneumocystis carinii* pneumonia [30]. This information has allowed us to utilize this laboratory measure as a means of implementing prophylactic therapy against this and other life-threatening opportunistic infections.

The use of CD4 counts in staging and prognosis of HIV disease is ubiquitous. Nevertheless, there are substantial and complex problems with the quantitation of this cell type. Issues that are of consequence to this quantitation include instrumentation, experimental (sample handling, preparation, and staining), biologic variables (including diurnal or even seasonal variations in lymphocyte counts), and data analysis [31-44]. The discussion of these details is beyond scope of this review.

In addition to monitoring CD4 cell numbers, the function of the CD4 cell is clearly of central importance to both the host's cellular and humoral immune responses. It has been shown in numerous studies that CD4 cell function is severely compromised in the HIV-infected host [45-49]. However, little work has been done to try to establish whether these changes are due, either wholly or in part, to the significantly changed representation of subsets within the CD4 T cells (see below).

Many of the *in vitro* functional assays have been performed using cumbersome assays that are difficult to reproduce from laboratory to laboratory. Current work is underway utilizing flow cytometric technology to perform such functional assays. In the future, these types of studies will provide powerful tools for both

phenotypic and functional evaluation of the CD4 cell by FACS technology in HIV disease.

### Activation markers

Activation markers on CD4 T cells have not been as well characterized as for CD8 T cells, but it is clear that there is a significant increase in the representation of activated CD4 T cells. Typically, the activation markers on CD4 T cells only become prominent at much later stages of disease than for CD8 T cells. There is an increased representation of CD4 T cells expressing HLA-DR [50-57]. While CD38 appears to be increased as well [57], one group found no change [50]. (Note that in children, a great majority of CD4 T cells express CD38; HIV-infected children show a decrease in the expression of CD38 [58]). Finally, there is an increase in CD57<sup>+</sup> CD4 T cells [59], which are almost all CD7<sup>-</sup> [60]. However, this is not the "classical" activation phenotype associated with mitogenically stimulated CD4 T cells, since there is no increase (or perhaps even a decrease) in the expression of CD25 [61-63].

Unlike CD8 T cells, few studies have evaluated three-color staining of activation markers on CD4 T cells. The increased expression of HLA-DR and CD38 on CD4 T cells are concordant, since there is an increase in HLA-DR<sup>+</sup>CD38<sup>+</sup> cells [58, 64], but only a slight increase in HLA-DR<sup>+</sup>CD38<sup>-</sup> cells, and no change in HLA-DR<sup>-</sup>CD38<sup>+</sup> cells [57].

*Memory/Naive markers.* Changes in naive and memory markers on CD4 T cells are probably the least agreed-upon measurements. In particular, the proportion of CD62L-expressing cells has been shown to increase [65, 66], remain stable [67-70], or decrease [50, 66, 71, 72]. Likewise, CD45RA-expressing CD4 T cells have been shown to increase [59, 73-77], remain stable [47, 57, 66, 67, 69, 70, 76, 78-81], or decrease [56, 71, 72, 82]. Some of these conflicts might be explained by differential expression of HIV antigens [73]; or by the observation that there may be an early increase in CD45RA expression, followed by a decrease at the onset of AIDS [52, 81]. CD45RO, which is expressed mutually exclusively with CD45RA (except for co-expression on activated cells), was shown to decrease in one study [83], but was significantly increased in several others [56, 78, 84]. Indeed, the increase in CD45RO cells was seen concomitantly with an increase in CD45RA cells, suggesting an increase in the double-positive (activated) cells. Indeed, most HLA-DR<sup>+</sup> cells are also CD45RO<sup>+</sup> [56, 64].

CD29 is also a putative memory marker. The proportion of CD29-expressing CD4 T cells has been shown either to increase (in children only) [72], decrease [59, 74, 77, 80, 85], or to remain stable [47, 63, 67, 69, 72, 76, 81]. The proportion of CD4 cells expressing either of CD29, CD45RA, or HLA-DR were all higher in patients with Kaposi's Sarcoma or opportunistic infections, compared to asymptomatic individuals [86]. Three-color staining revealed a decrease in CD45RA<sup>-</sup>CD29<sup>-</sup> cells, but an increase of CD45RA<sup>+</sup>CD29<sup>+</sup> cells [55].

Using three-color immunofluorescence to more accurately identify naive cells (i.e., naive cells are CD45RA<sup>+</sup>CD62L<sup>+</sup>; all other combinations are memory T cells), a preferential depletion of these cells over time can be identified. While CD45RA<sup>-</sup>CD62L<sup>+</sup> cells increase [58], CD45RA<sup>+</sup>CD62L<sup>-</sup> cells (which are activated and probably co-express CD38 and/or HLA-DR) increase in late stage AIDS [87]. However, there is a significant, proportionate loss of the CD45RA<sup>+</sup>CD62L<sup>+</sup> (naive) CD4 T cells [58, 87].

**Other markers.** In HIV<sup>-</sup> individuals, a vast majority (>95%) of CD4 T cells express CD28. However, in HIV<sup>+</sup> individuals, the CD28<sup>-</sup> cells are significantly increased [53, 56, 88–91]; these cells express CD57 [89] and HLA-DR, CD38, and are CD45RO<sup>+</sup> [56], and probably express CD11b, which is also increased [53].

Of 61 different monoclonal antibodies tested for the Fifth Workshop, 17 showed different reactivity to CD8 T cells between HIV-infected and uninfected individuals [91]. The proportion of (dim) CD21-expressing cells is decreased [92], as well as the proportion of CD26<sup>+</sup> cells [93] and CD101<sup>-</sup> cells [91].

**Functional correlates.** Phenotypic functional correlates among CD4 cell subsets have focused on the potential differential functional role that CD4 cells have with regard to cytokine production. The two broad classes of functional subsets based on cytokine activity have been proposed to involve Th1 and Th2 cells. The Th1 cells are responsible primarily for IL-2 and IFN- $\gamma$  production and promote cellular immune responses, while Th2 cytokines including IL-4, IL-5, IL-6, and IL-10 are responsible for antibody mediated responses. Recent studies have suggested that among CD4 cells, the CD7<sup>-</sup> cells are expanded in HIV disease, and may be associated with a Th2-like phenotype. Delineation of the phenotypic and functional associates between CD4 and other cell subsets for cytokine activity will be advanced by utilizing single cell assays to measure cytokine production by flow cytometry [94].

The changes in the naive and memory subsets of CD4 T cells may directly influence viremia. HIV preferentially infects memory CD4 T cells *in vitro* [95]. Consistent with this observation, HIV replicates preferentially in memory CD4 T cells from infected patients [96]; there are different requirements for HIV replication in naive versus memory CD4 T cells [97]. This differential replication has been suggested to account for some of the selective functional defects observed in HIV-infected individuals.

### CD8 T cells

**Introduction.** In addition to the characteristic loss of CD4 cells, there is a concomitant increase in the CD8 cell population. The CD8 antigen is known to be expressed on not only the T cell subset but also on a subset of natural killer (NK) cells as well. Many of the earlier studies evaluating CD8 cells in HIV disease did not rely on reagents that clearly defined these two subsets. More recently, it has been recommended in several national guidelines to combine the CD8 marker with a T cell lineage marker (such as CD3) to more accurately evaluate the CD8 T cell subset.

The rise in CD8 T cells represents an important host immune response to the virus [63, 98–105]. Numerous functional studies evaluating both CD8 CTL and antiviral suppressor activity have demonstrated the important role of the CD8 cell in natural host defense against HIV disease. This type of response is not unique to HIV infection and can be seen in many other viral syndromes (including CMV and other herpes virus infections). As opposed to the progressive loss of CD4 cells over the course of HIV infection, the CD8 cell population remains elevated throughout the course of disease until the very final stages of HIV infection.

As is described below, numerous CD8 markers have been defined in this disease which may provide important prognostic information for following a patient over the course of this disease. These include activation markers, those that identify naive and memory cells, and those that are associated with cell function. To date, there have been no conclusive studies demonstrating the

utility of CD8 T cell counts in monitoring therapeutic responses, but current studies are underway to evaluate these cells in antiretroviral and immune-based therapeutic protocols. In addition, clinical trials to expand CD8 cells *ex vivo* for reinfusion are in progress, demonstrating the important role of this cell type in controlling HIV infection.

**Activation markers.** Perhaps the single most agreed-upon phenotype change in HIV-infected adults is the increase in activation markers on CD8 T cells. The increased frequency of CD8 T cells bearing HLA-DR was noted in 1984 [50], and has been noted by virtually every study on CD8 T cells [51, 52, 54, 56, 66, 69–72, 75, 76, 78–80, 89, 101, 106–118]. In addition, there is increased expression of the activation markers CD57 [51, 58, 59, 66, 70–75, 78, 79, 108, 109, 111, 112, 115, 119–124] and CD38 [53, 55, 56, 66, 69–71, 75, 79, 89, 101, 106, 107, 112–116, 118, 125]. Originally, the presence of CD38 and HLA-DR on the CD8 T cells was thought to indicate that these cells were immature, and that HIV was inhibiting T cell development [106]; however, we now know that these markers are present on cells that have an activation profile and give memory responses. It is interesting to note that in healthy children not infected with HIV, most CD8 T cells are positive for CD38, while HIV-infected children exhibit a *decrease* in the representation of CD38<sup>+</sup> CD8 T cells [58].

In general, the increase of HLA-DR expression on CD8 T cells is seen very early in disease. In fact, it is detectable prior to seroconversion [113]. While HLA-DR is increased in the asymptomatic phase, increases in CD38 and CD57 occur progressively throughout disease, with HLA-DR declining in late-stage AIDS [57, 70, 112, 115].

Three- and four-color immunophenotyping has further resolved these populations. In particular, the elevated expression of CD57 is found only on CD38<sup>+</sup> or HLA-DR<sup>+</sup> cells, and only on CD45RO<sup>+</sup> cells [111]. On the other hand, the increase in HLA-DR expression occurs equally on the CD57<sup>+</sup> and CD57<sup>-</sup> cells [108]. For the CD8 cells, unlike CD4 T cells, the expression of CD38 and HLA-DR is not correlated; that is, there are four subsets expressing the various combinations of these markers. The studies using three-color analysis have universally shown a decline in the representation of the HLA-DR<sup>-</sup>CD38<sup>-</sup> cells; and an increase in the HLA-DR<sup>+</sup>CD38<sup>+</sup> cells [57, 58, 101, 111, 116, 118]. However, changes in the single positive cells are more variable. The HLA-DR<sup>-</sup>CD38<sup>+</sup> cells were seen to increase [101, 118] or decrease [111, 116]; the HLA-DR<sup>+</sup>CD38<sup>-</sup> cells likewise were seen to increase [116], decrease [101], or remain stable [58, 111, 118]. A prevalence of the HLA-DR<sup>+</sup>CD38<sup>-</sup> cells is associated with a good prognosis for progression of the disease [118].

However, the activation phenotype seen on these cells is unusual or only partial, because the presence of HLA-DR, CD38, and CD57 is not accompanied by activation markers such as CD25 [61–63], CD69, or CD71 [66].

**Memory/Naive markers.** For CD8 T cells, there is general agreement that a shift towards the memory phenotype is observed. Since there is a significant increase in activated cells, this is not surprising (naive T cells are probably only activated after encounter with antigen, after which they become memory cells). Consistent with a shift towards memory, two-color studies have demonstrated a decrease in the proportion of cells expressing CD62L [50, 65, 68–70, 101, 114, 115], an increase in the CD11a-bright cells [63, 117, 126, 127], a decrease in CD45RA<sup>+</sup> cells [47, 56, 58, 63, 79, 101, 111, 117, 128], and a commensurate increase in

the CD45RO<sup>+</sup> cells [56, 71, 83, 84, 101, 112, 117, 128]. Interestingly, the changes in CD29 expression are far less marked [47, 63, 117], suggesting that expression of CD29 is lost upon activation *in vivo*.

Some activation markers have been found to be expressed on CD45RA<sup>+</sup> cells [111]: specifically, there were increases in CD45RA<sup>+</sup>HLA-DR<sup>+</sup> and CD45RA<sup>+</sup>CD38<sup>+</sup> cells, but CD57<sup>+</sup> cells were only CD45RO<sup>+</sup>. On the other hand, another study found that the CD45RA<sup>+</sup>HLA-DR<sup>+</sup> cells decreased [55]. In any case, these cells are not necessarily naive T cells, since some memory cells can express CD45RA. This will only be resolved with appropriate four- or five-color immunofluorescence staining.

More recently, three-color studies that more accurately identify the naive CD8 T cells demonstrate a loss of these cells continuously throughout HIV disease, even when the total CD8 count has increased [87, 129]. Even in children, in whom a vast majority of T cells are of the naive phenotype, there is a significant progression of the compartment towards memory phenotype [58, 71, 84, 129, 130].

**Other markers.** The CD28 molecule provides important costimulatory signals for T cell activation. The proportion of cells expressing CD28 decreases significantly with HIV disease [56, 88–91, 101, 124], suggesting that there is a large increase in cells which have very different activation profiles (and may even be anergic [124]). Although the CD28<sup>-</sup> cells are typically CD11b<sup>+</sup>, there is also a decrease in the proportion of CD11b<sup>+</sup> cells [66, 75, 82, 101]. (A decrease in the proportion of these cells occurs whether the absolute number of CD11b<sup>+</sup> cells increases [51, 53, 73, 131] or remains stable [74, 77]). The CD28<sup>-</sup> cells that increase appear to be CD38<sup>+</sup> and/or HLA-DR<sup>+</sup>, as well as CD45RO<sup>+</sup> [89]. Finally, the increased CD28<sup>-</sup> cells are predominantly CD57<sup>-</sup> [124]. This loss of CD28 may be associated with increased Th2-like cytokines, including IL-4 and IL-10.

Of 61 different monoclonal antibodies tested for the Fifth Workshop, 22 showed different reactivity to CD8 T cells between HIV-infected and uninfected individuals [91]. There was increased expression of CD99, but decreased expression of CD101 and CD31 [91]. The relationship of these markers to activation and/or the naive/memory phenotypes still needs to be established. The proportion of cells expressing CD26 is decreased [93], and there were no changes observed in the proportion of CD8 which dimly express CD21 [92]. Finally, an increase in the expression (antigen density) of CD11c was noted [126], and one group noted that the activated CD8 T cells expressing CD38 or HLA-DR had increased expression of the CD8 molecule itself [107].

**Functional correlates.** Most of the functional studies attempting to correlate phenotypic changes in CD4 and CD8 subsets with functional alterations have focused on the CD8 effector cell population. These studies have attempted to develop correlates of phenotypic expression with either CTL or anti-HIV suppressor activity [110, 112, 116, 132]. The major phenotypic marker associated with cytotoxic T cell activity is HLA-DR. However, it has been demonstrated that CD8 T cells expressing HLA-DR (but lacking CD25) have reduced clonogenic potential, may be expanded by chronic immune activation, and are ineffective in mounting functional immunity [110]. Further evidence of immune suppression comes from studies of a subset defined as being CD8<sup>+</sup>CD57<sup>+</sup> that may inhibit cytotoxic effector cell activity. In addition to CTL functional studies, CD8 antiviral activity has been extensively evaluated; cells with the phenotype of CD28<sup>+</sup>HLA-

DR<sup>+</sup> have been found to be associated with this particular antiviral function.

Giorgi and colleagues have evaluated the anti-HIV functional capacity of the CD38 and HLA-DR expressing CD8 T cells by sorting these subsets to purity [116]. The CD38<sup>+</sup>HLA-DR<sup>+</sup> cells showed significant anti-HIV CTL activity; the single positive cells were also active, but activity was reduced about 50% on a per-cell basis compared to the double positive. The CD38<sup>-</sup>HLA-DR<sup>-</sup> population had little activity. These results suggest that the quasi-activation phenotype is a direct consequence of antigen-specific immunologic activity. Interestingly, expression of CD38 on CD8 T cells was correlated with poor prognosis, whereas expression of HLA-DR in the absence of CD38 was associated with good prognosis [118].

#### γδ T cells

The fraction of T cells bearing the γδ receptor is typically very low (1 to 5%). Therefore, it is more difficult to assess changes in the representation of these cells amongst the HIV-infected population. Indeed, there are conflicting reports about the absolute numbers of these cells, most of which found no change [72, 133, 134], but some reported an increase [135, 136], and one report of a decrease [137]. There appears to be some HIV-infected individuals who have a selective expansion of a subset of γδ T cells that are normally very infrequent in uninfected individuals [133, 134].

#### B cells

In general, the absolute number of B cells in the blood is decreased [52, 63, 72, 73, 76, 127, 138–140], although some reports found no change [66, 80, 141] or an increase [54]. Likewise, the percentage of B cells among lymphocytes is unchanged [51, 138] or is decreased mildly [78, 142].

Activation of B cells was one of the first immune dysregulations noted for HIV disease; it was described in 1983 as a polyclonal B cell activation [143]. Several observations confirm the activated state of B cells in HIV-infected individuals, including hypergammaglobulinemia, elevated expression of B cell activation markers, increased frequency of B lymphomas. Phenotypic activation is evidenced by an increase in the proportion of B cells expressing CD71 [75, 141] and CD38 [101]. Akin to the T cells, this activation may not be "classical", in that the proportion of B cells expressing CD25 is unchanged [62] or decreased [61]. B cells which have lost expression of CD62L may also be activated; the proportion of CD62L<sup>-</sup>B cells seems to increase [70, 73, 141], although this was not always observed [54, 139]. Finally, activation of B cells is evidenced by the increased expression (antigen density) of CD20 [144] and CD21 [145], although the fraction of B cells expressing CD21 is unchanged in adults [52, 76] (and decreased in children [76]).

B cells can be divided into two lineages, the CD5<sup>+</sup> "B1" cells, and the CD5<sup>-</sup> "B2" cells. The proportion of B1 cells seems to be increased [72, 139, 140, 142, 146], although one report found no difference [147]. Interestingly, the increased B1 representation correlated with hypergammaglobulinemia, autoimmune phenomena, and correlated negatively with total CD4 counts [140]. Since expression of CD5 on B1 cells is relatively weak, differences in reagents or instrument sensitivity can affect the detection of these cells and may account for the variations reported. In HIV<sup>+</sup> children over two years of age, a fraction of B cells are CD10<sup>+</sup>

[139], and most of these are CD5<sup>+</sup>; HIV<sup>+</sup> adults also had increased proportions of CD10<sup>+</sup> B cells [146] but decreased CD23<sup>+</sup> B cells [146]. Finally, the phenotype of the spontaneous immunoglobulin secreting B cells in the periphery is consistent with that of "preplasma cells", that is, CD38<sup>+</sup> 4F2<sup>-</sup> [148].

#### NK cells

Much less attention has been paid to NK cells than to other lymphocyte subsets. Early studies on phenotype have artefactual errors due to the use of markers to identify these cells that are also expressed on some T cells (CD8, CD16). Likewise, many phenotypes associated with CD8 T cells are artefactually affected by the CD8<sup>+</sup> NK cells. In healthy individuals, the best phenotypic marker for NK cells is CD56; however, recent evidence indicates that the proportion of NK cells expressing CD56 is decreased in HIV-infected adults [149].

On the other hand, the defect in the functionality of NK cells has been measured for many years [122]. Consistent with the functional defect, absolute numbers of NK cells are decreased [76, 101, 112, 123, 127, 135, 150], although some groups found no change [66] or even an increase [80]. The fraction of NK cells expressing CD8 is more strongly decreased than the CD8<sup>-</sup> NK cells [150], and the fraction of NK cells expressing CD57 may decline in AIDS [123].

#### Monocytes

A number of studies have reported changes in monocytes in HIV-infected individuals, including altered subsets of monocytes and altered expression of markers. However, there is quite a bit of disagreement. Some of this disagreement arises from the method used to identify the monocytes by flow cytometry. These methods can include simple scatter gates (high forward and orthogonal scatter), dim CD4 expression, or CD14 expression. Indeed, one group found a decreased representation of monocytes that were CD14<sup>+</sup> [151].

The absolute number of monocytes is reported to remain unchanged [76, 152], or decrease [52, 153, 154]; the percentage of monocytes among PBMC is unchanged [78, 154] or increased [51].

Expression of HLA-DR on monocytes is necessary for antigen presentation to CD4 T cells; in HIV-uninfected individuals, most monocytes express HLA-DR. In view of the CD4 defect noted earlier, several groups have carefully analyzed the expression of MHC class II on monocytes. Two reports noted a decrease in the proportion of monocytes expressing HLA-DR [155, 156]; however, most reports show no change [52, 76, 153, 157, 158], or an increase in the proportion [55, 78, 159]. In addition, the proportion of CD4<sup>-</sup>HLA-DR<sup>+</sup> monocytes increased [55]; however, the proportion of CD4<sup>-</sup> monocytes did not change [55, 157, 158] or decreased [152, 160]. The amount of HLA-DR expressed per monocyte was either unchanged [157], or increased [151, 153, 156, 159]. It has been suggested that the increase in HLA-DR on monocytes (both in percentage and expression) is an adaptive response to the loss of CD4 T cells.

Other subsets of monocytes change as well. The proportion of CD13, CD33, and CD11b-expressing monocytes all declined in HIV-infected individuals, and also showed a greater decrease in HIV-antigen-positive (p24<sup>+</sup>) individuals compared to p24<sup>-</sup> individuals [151, 158] (one report found no change in the proportion of CD11b-expressing monocytes [161]). The proportion of monocytes expressing CD25 also increased [159]; those expressing

CD71 or CD11c did not change [157]. For the Fc receptors, the proportion of monocytes expressing CD16 remained stable [161] or increased [151, 154, 162], and those positive for CD64 increased [163] or remained the same [151, 157, 161]. Finally, no change in the proportion expressing CD32 was noted [161].

Expression of several markers on the monocytes also changed with HIV infection, suggesting that these cells may be somewhat activated. In particular, increased expression (antigen density) of CD11a [151, 164], CD11b [164], CD11c [126, 164], CD14 [154], CD18 [164], and CD64 [163] were all demonstrated. However, others found no difference in the expression of CD18 [151] or CD11b [157]. In addition, there were no changes in the expression of CD4, CD71, CD32, CD54, and CD16 [151, 157]. Changes in expression of these molecules on a cell-by-cell basis, when observed, were found even in the absence of a change in the representation of these populations, indicating that there was an intrinsic change in the functionality of the monocytes.

#### Neutrophils

Since most flow cytometric analyses have been performed on Ficol-processed blood, there is much less information available about neutrophils. Outside of functional analyses, there have been a few reports about the phenotype of neutrophils. The absolute number of neutrophils is decreased [165–167]. The proportion of neutrophils expressing CD16 (FcR III) is decreased, whereas the proportion expressing CD32 (FcR II) and CD11c is unchanged [168]. CD64 (FcR I)-expressing cells were either unchanged [168] or slightly increased [163]. Activation of neutrophils was deduced by the observations of increased expression (antigen density) of CD11b and CD18 [164, 165], and decreased expression of CD62L [165].

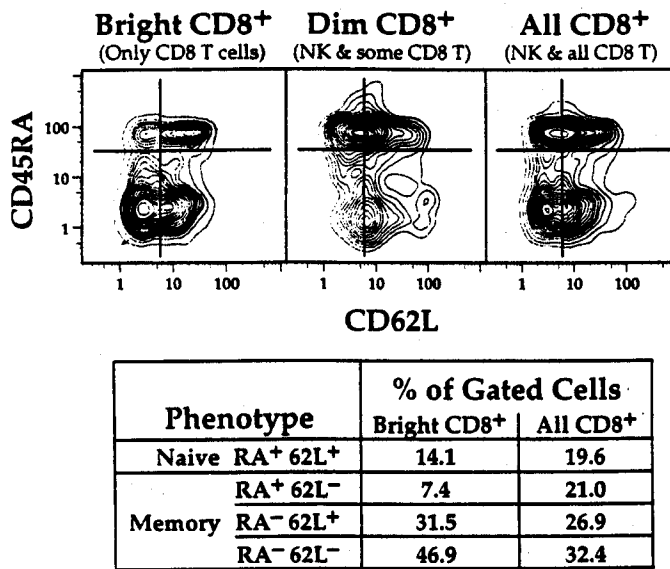
#### Why is there so much confusion?

There are several reasons for the confounding results presented above. These include both methodologic and analytical factors. We discuss these briefly, showing examples of how they may arise, and provide potential solutions for each of the problems. Ideally, by addressing these problems, the extant confusion can be reduced significantly, and avoided completely in the future.

1. *Unique identification of subsets.* Many analyses have relied on inadequate identification of the subset of interest. A very common example comes from the study of CD8 T cells. Some NK cells express low levels of CD8; and therefore, the use of isotype-based gates to define CD8 cells will include NK cells in the analysis. Since NK cells express a different complement of surface markers, this can lead to considerable confusion.

An example of this is given in Figure 182.1. This figure represents an attempt to determine the representation of naive and memory subsets within CD8 cells, using three-color flow cytometry. Since naive/memory determination requires two colors (CD45RA and CD62L), CD8 T cells must be identified solely on the basis of expression of CD8 (instead of the superior combination of CD3 and CD8). The use of a CD8 "anchor gate" that is too low (for instance, set by the isotype staining controls) thereby includes NK cells significantly alters the apparent representation of CD45RA/CD62L subsets.

In HIV disease, the problem of misidentification of subsets is particularly difficult, since many kinds of cells present at very low frequencies in healthy HIV<sup>-</sup> adults become prevalent in infected adults. This is true not only in the T cell subsets, but all PBMC



**Fig. 182.1.** Inappropriate "anchor gates" for CD8 can affect subset frequencies. The three plots show the distribution of CD45RA and CD62L expression on cells gated for CD8 expression; this stain was performed on an HIV-infected adult. The left plot shows the distribution only for cells which have high CD8 expression (consisting only of CD8 T cells); the middle plot, for cells which have dim CD8 expression (consisting of both NK cells and CD8 T cells); the right plot, for all cells which express CD8 above the isotype control (i.e., the combination of the first and second plots). Since NK cells are predominantly CD45RA<sup>+</sup> and CD62L<sup>-</sup> (middle plot), the inclusion of these cells in frequency measurements significantly affects the representation of naive and memory CD8 T cells. This is quantitated in the table, which shows the percentage of cells in the plots for each of the quadrants. While the CD8 T cells are almost 50% CD45RA<sup>-</sup> and CD62L<sup>-</sup> cells, inclusion of the NK cells in the gate makes the apparent representation less than 33%. Note that the inclusion of the NK cells also completely masks the location at which the distinction between naive and memory CD45RA<sup>+</sup> CD8 T cells can be made, making it impossible to accurately quantitate naive CD8 T cells.

The plot on the left represents only the bright CD8 cells, which are CD8 T cells (as determined by a parallel stain using CD3 in combination with CD8). However, this plot necessarily does not include all CD8 T cells—it excludes those which are dim for CD8. However, the representation of the CD45RA and CD62L subsets within CD8 can still be determined from this gate, as a percentage of CD8 T cells—and the absolute number of these subsets can be determined by multiplying this percentage by the absolute number of CD8 T cells determined by the CD3-CD8 stain. Of course, the most accurate method for calculating these cell numbers would be obtained from 4-color stains, using CD3, CD8, CD45RA, and CD62L.

subsets. Indeed, even granulocytes can show alterations in physical properties such as density, which causes them to co-separate with PBMC on Ficoll gradients.

Another example, which may underlie some of the controversy regarding CD45RA expression on CD4 T cells, is shown in Figure 182.2. In HIV<sup>-</sup> adults, there are very few CD45RA<sup>+</sup> memory cells (i.e., CD62L<sup>-</sup>). Thus, CD45RA expression alone is adequate for distinguishing between naive and memory cells. However, many HIV-infected adults have significant proportions of CD45RA<sup>+</sup>CD62L<sup>-</sup> (memory) cells (see middle panels of Fig. 182.2), but essentially no naive T cells. In this individual, CD45RA alone cannot distinguish naive and memory T cells. In fact, this individual has a normal representation of CD45RA<sup>+</sup> cells amongst CD4 T cells, but very few naive CD4 T cells.

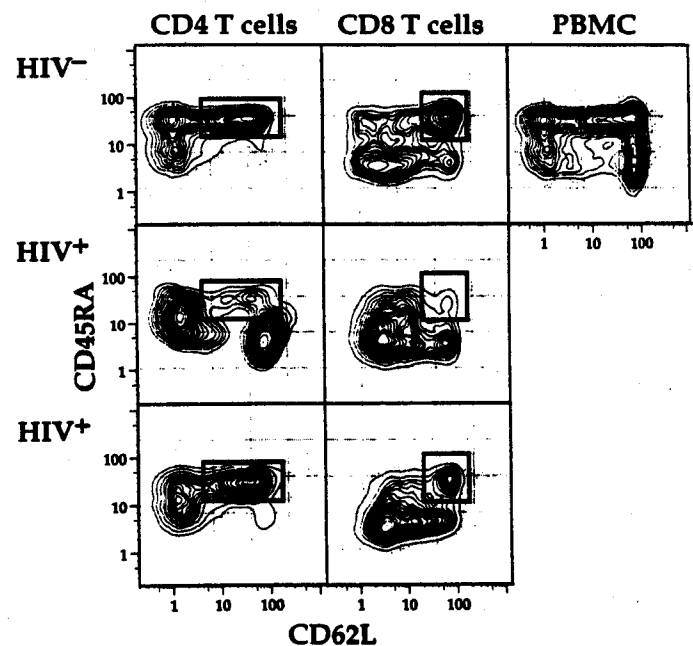
There is no easy solution to these problems. First, we must

recognize that the phenotypes of cells in HIV-infected adults can be very different that uninfected adults in unpredictable ways. This means that we cannot necessarily rely on evaluation of HIV<sup>-</sup> PBMC as "training" for evaluation of HIV<sup>+</sup> PBMC. From an experimental standpoint, altered phenotypes (and their functional correlates) must be confirmed by the use of multiple markers. Furthermore, it may be necessary to confirm changes using different reagents identifying the same marker. For example, different (brighter) conjugates, different clones used to detect the same marker. Therefore, three- (or more) color flow cytometry is essential to the precise determination of changes within subsets.

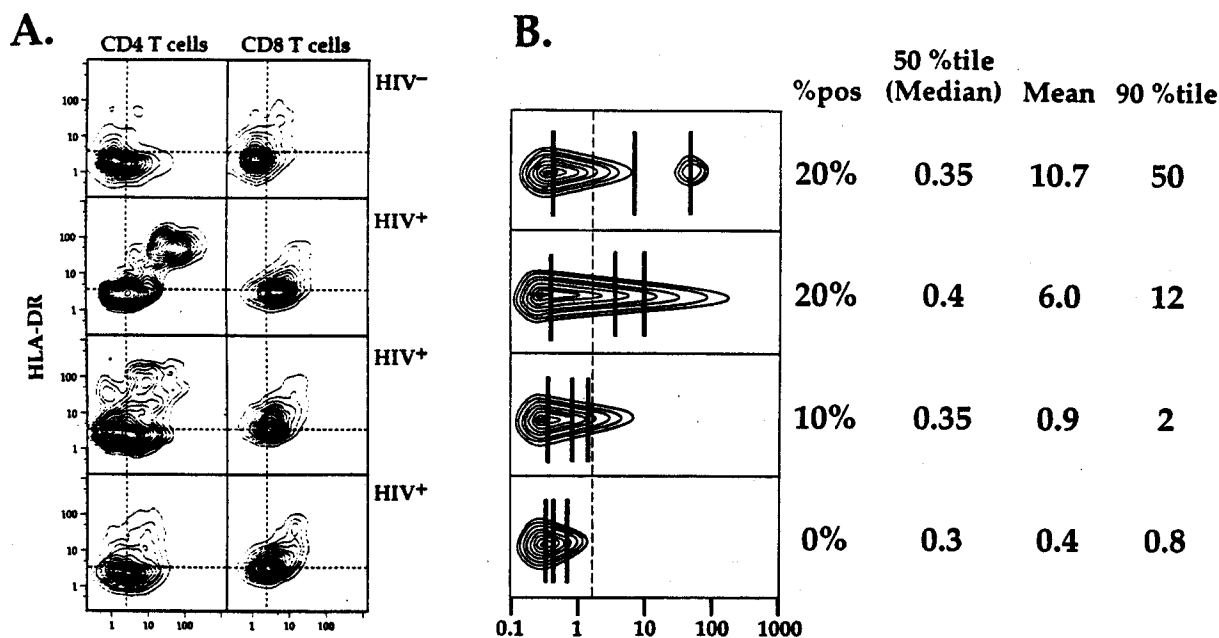
2. *Complexity of antigen expression: antigen density.* The expression of many antigens is relatively simple. They are either absent, or present at the same level on all expressing cells. This makes identification of cells expressing the antigen straightforward. However, the expression of many antigens is considerably more complex in two ways: there may be a continuum of expression from negative to positive, or at least a very broad expression pattern; and, the expression may be different on various subsets within a lineage.

Broad expression patterns are commonly found for antigens associated with activation, such as CD38 and HLA-DR. Four examples of the expression pattern of these two antigens on CD4 or CD8 cells is shown in Figure 182.3A. For the most part, there is no clear delineation between negative and positive for either of these antigens.

Quantitation of expression of these antigens has been typically reported by the percentage of cells expressing levels above the isotype control. However, this suffers from the artefactual contribution of reagent brightness: that is, a very bright reagent (for example, phycoerythrin conjugates) will reveal more of the dim cells than would a dull reagent. (This phenomenon is discussed in detail in Chapter 49 of this Handbook, using CD5 expression on



**Fig. 182.2.** Complexity of antigen expression. The expression of CD45RA and CD62L on CD4 and CD8 T cells is shown for one uninfected adult and 2 HIV-infected adults, and on total PBMC for the uninfected adult. Naive T cells are phenotypically defined as those T cells which are CD45RA<sup>+</sup> CD62L<sup>+</sup> (boxes).



**Fig. 182.3. Quantitating antigen density.** (A) Examples of the distribution of CD38 and HLA-DR on CD4 or CD8 T cells from one HIV<sup>-</sup> healthy adult and three HIV<sup>+</sup> adults. The distributions are often continuous, with no easily separable positive and negative population. Further, as shown in the bottom right, the centroid of the population can shift but still be below the isotype control: suggesting that a significant fraction of cells express very low levels of the antigens. (B) Examples of methods to quantitate the expression. For each hypothetical distribution, the fraction of cells above the isotype control (dotted line) is given. The position of the median, mean, and 90th percentile of fluorescence is shown by the dark bars and the values are given in the table. The different distributions illustrate the dependence of these parameters upon the types of distributions obtained.

Each type of measurement illustrated suffers from its own disadvantages; perhaps the most sensitive method to quantitate the expression of these antigens is by using the 90th percentile of fluorescence. (The mean fluorescence determination can often be biased by a few extremely bright outlying cells, especially in these logarithmically scaled distributions).

In cases such as the expression of CD38 and HLA-DR, the distribution is sufficiently complex that it cannot be accurately described by a single number. Ultimately, it is desirable to compute a small set of parameters which can describe the extent and variability of expression of such antigens, and to use this set as potential surrogate markers.

B cells as an example). Examples of different methods for quantitating antigen expression are shown in Figure 182.3B.

**3. Complexity of expression: variations among subsets.** Again, the expression of an antigen can be simple, in that all subsets of a lineage express it to the same degree (for example, CD4 is expressed equivalently by memory and naive CD4 T cells). However, underlying variation in antigen expression can lead to complexities that confuse analysis.

As an example, we show the expression of CD45RA and CD62L on CD4 and CD8 T cells in Figure 182.2. The complexity of expression of these antigens makes it impossible to accurately quantitate naive CD4 and CD8 T cells without using at least two three-color stains: CD45RA, CD62L, and CD4 or CD8. Both B cells and NK cells express CD45RA and CD62L to different extents than T cells; thus, they contribute variably to the different subsets. This is evidenced by the distribution shown for PBMC, and is shown in detail for NK cells in Figure 182.1.

Furthermore, the same gates cannot be used for either CD4 or CD8 T cells. These two different kinds of T cells express significantly different amounts of CD45RA. A significant number of naive CD4 T cells express less CD45RA than do some memory CD8 cells. This implies that naive and memory counts must be determined independently for each of the CD4 and CD8 lineage cells.

Another complexity is exemplified by the appearance of a new population of cells (in the middle panel): CD4<sup>+</sup> CD45RA<sup>+</sup> CD62L<sup>-</sup> cells. These cells are very infrequent in HIV-uninfected

adults, but can represent as high as 75% of CD4 T cells in HIV-infected adults (averaging over 15% of CD4 T cells). These cells are activated T cells, in that they express CD38 and HLA-DR; they also express higher amounts of CD45RA than do the naive (CD62L<sup>+</sup>) T cells. By "training" on HIV-uninfected adults, we might decide that CD45RA expression was sufficient to distinguish naive CD4 T cells and use that marker alone to enumerate them.

Finally, while there are clearly-defined subsets of T cells based on the expression of CD45RA and CD62L, they are not "positive" and "negative". This is easily seen for CD62L, in that even the "negative" cells express enough CD62L as to be brighter than isotype control-stained cells. Therefore, quantitating these subsets cannot be accurately performed by using gates derived from isotype controls. Indeed, the best way to compare subset representations between different individuals is to use the same antibody reagents, prepared at saturating concentrations, for an entire study: this allows the use of the same gating statistic to be used for all individuals and reduces the subjective quality of the analysis. (The use of saturating reagents, a crucial part of any FACS experiment, is discussed in detail in Chapter 49 of this Handbook).

**4. Presentation of numerical analyses.** A difficulty in comparing the results from different groups arises when the presentation of lymphocyte subset representation measurements is as a percentage (such as of lymphocytes, or of CD4 T cells, etc.) for one and as an absolute number for another. Of course, for single sample

analyses, presentation of absolute numbers only is sufficient to calculate frequencies, though presentation of frequencies alone cannot be used to calculate absolute numbers. However, when analyses for many individuals are grouped, this no longer becomes possible because the mean average number of CD4 T cells for 100 individuals cannot be divided by the mean average number of lymphocytes for those individuals to obtain the mean percentage. The optimal solution is to always present *both* pieces of information in any data presentation.

**Summary.** In many of the studies that were reviewed here, a specific question was being addressed and answered. Such a focused analysis is important in scientific presentations; however, this focus often excluded data or information which can allow comparison across analyses. In other words, it becomes nearly impossible to reconcile differences between manuscripts, because we cannot ascertain if the discrepancy arose from reagents (such as one group used a very bright reagent, the other a dull reagent), from methodology, or from more complex underlying aspects such as differences between study populations.

There are no perfect solutions to these problems, as noted above. However, we should strive to present "control" data—in other words, any new analysis such as the presentation of a novel surrogate marker, should include sufficient additional analyses as to make the presentation comparable to current literature. This is most important in antigen density measurements, where a great deal of variation can arise from instrumentation, reagent choice, staining protocols, and so forth.

As an example, consider an hypothetical study that measures the antigen density of "CD999" on CD8 T cells in HIV-infected adults, and its correlation with disease progression. At a minimum, such a study should endeavor to include antigen density measurements of CD38 on CD8 T cells—and, optimally, using the same fluor on the two antibodies (i.e., if PE CD999 is used, then the antigen density as revealed by PE CD38 should also be measured). In this study, the antigen density of CD38, known to increase during HIV progression, would serve as a "normalization" value for comparison between studies. Indeed, *any* publication of novel immunophenotyping studies should provide an appropriate analysis of previously-published data that can serve in this regard.

Perhaps the most important lesson to be learned from the plethora of conflicting data is that single-color analyses are no longer adequate for immunophenotyping studies. Even two-color analyses have become inadequate for resolving the fine subpopulations that are now of interest. Three-color analyses have now become routine in many laboratories; four- to six-color analyses will likewise become routine in the next ten years. This increased power is not without its cost: the complexity of analyses increases geometrically with the number of independent measurements. Routine multicolor experiments will require a strong commitment on the part of the flow cytometry manufacturers to produce and actively support easy-to-use instrumentation coupled with powerful analysis software, as well as the reagent manufacturers to provide well-controlled, high-quality conjugates of monoclonal antibodies.

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