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Good Cell, Bad Cell: Flow Cytometry Reveals T-cell Subsets Important in HIV Disease

Pratip K. Chattopadhyay* and **Mario Roederer**
ImmunoTechnology Section, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health

Abstract

Flow cytometry is a key technology in the study of HIV disease. In this article, we review various cellular markers that can be measured in the setting of pathogenesis or vaccination studies, including markers of activation, differentiation, senescence, immune suppression, and function. In addition, we discuss important considerations for making these measurements. Finally, we examine how flow cytometry studies have taught researchers about the disease process, and the potential for flow cytometry technology to guide treatment decisions and evaluate vaccine candidates in the future.

Keywords

flow cytometry; HIV; T-cells

Introduction

With 33 million existing cases, and 7000 cases diagnosed daily, the worldwide scope of the HIV/AIDS epidemic is immense (1). Accordingly, the scientific resources directed at the problem are enormous (more than $10 billion within the last decade alone) (2). In particular, significant effort is devoted to the clinical monitoring of CD4 T-cell counts (3), which is used to guide treatment decisions (4). However, beyond the clinic, a myriad of other cell subsets are studied in HIV+ populations, with the aim of predicting disease prognosis, developing new targets for therapy, finding surrogate markers for vaccine efficacy, or understanding disease pathogenesis.

Flow cytometry has emerged as the primary technical tool for these endeavors, in part because of its historical application defining immune cell subsets (5). Indeed, many of the T-cell subsets altered by HIV disease were first identified by flow cytometry. The volume of literature in this area is a testament to the power of flow cytometry; it is ideally suited for understanding the cellular changes associated with HIV because it measures protein expression at the single-cell level. This characteristic allows researchers to define, with exquisite specificity, the immune cells and functions that play a role in HIV disease.

Cellular Parameters Studied in HIV Disease

Over the past 25 years of HIV research, flow cytometry has revealed a host of molecules modulated over the course of disease. Early studies used flow cytometry to describe changes in the peripheral blood that led to immunodeficiency (6),(7), and confirmed that the loss of

*Correspondence should be directed to: Pratip K. Chattopadhyay, ImmunoTechnology Section, VRC, NIH, Room 5509, 40 Convent Drive, Bethesda, MD – 20892, (301) 594-8491, pchattop@mail.nih.gov.

CD4+ T-cells was a hallmark of infection. A great deal of work since has sought to define the reasons for this loss and further describe the biology of infection. This work has helped define many new T-cell subsets, thereby demonstrating the power of flow cytometry, and such efforts continue today (8). When designing new studies, researchers typically must choose a subset of these molecules to examine. The decision is best made with an understanding of each marker's biology and what changes indicate about disease progression. In this review, we first examine fundamental aspects of the biology and measurement of various markers. In later sections, the role of these molecules in disease pathogenesis will be described.

Markers of activated cells

The activation of T-cells is a key aspect of HIV pathogenesis (8). It accelerates viral replication (9),(10),(11), and is a strong predictor of disease progression (even independently of CD4+ T-cell counts) (12),(13),(14),(15). Notably, low levels of activated cells may even be associated with resistance to infection (*i.e.*, in highly exposed, persistenly seronegative women (16)), while high levels of immune activation before seroconversion (17), and during the first eight months of infection (15), may be linked to rapid progression. Finally, failure of highly active anti-retroviral therapy (HAART) is associated with ongoing CD4+ T-cell activation (18).

The importance of T-cell activation in HIV was first recognized in the late 1980s, through seminal studies of HLA-DR and CD38 by Giorgi and colleagues (19). HLA-DR is constitutively expressed by antigen-presenting cells (APCs), and is involved in the presentation of antigen to CD4+ T-cells. Although it is not expressed by most T-cells, a subset of activated T-cells becomes HLA-DR+ during an immune response. The reason for, and consequence of, HLA-DR up-regulation by T-cells is not well understood, but there is clear evidence that this phenomenon is a hallmark of HIV disease progression (19), (20), (21). In contrast, CD38 is constitutively expressed by naïve T-cells, down-regulated in resting memory cells, and then elevated again in activated cells (22). These aspects of HLA-DR and CD38 expression indicate that precise analysis requires multicolor flow cytometry (*e.g.*, 6+ colors). For studies of HLA-DR, T-cell markers must be included in the staining panel (particularly since the prognostic significance of HLA-DR differs between CD4+ and CD8+ T-cells (12)) and markers of APCs may be useful as exclusion (or "dump" channel) markers. For studies of CD38, markers that define naïve and memory T-cell subsets (described below) are necessary to discriminate those cells expressing CD38 as a consequence of T-cell activation. Notably, the density of CD38 expression on individual cells is informative as well (23), (13), (24), though such measurements require additional experimental parameters, so that the number of molecules expressed can be accurately derived from the fluorescence intensity (25).

The cell surface glycoprotein CD69 is another marker of T-cell activation that is commonly studied. Though the precise function of this molecule is not known, the distribution and kinetics of its expression have been investigated extensively (26). These studies indicate that CD69 is one of the earliest markers up-regulated upon T-cell activation, that it is widely expressed in inflamed tissue (by a broad array of cell types), and that it is usually transient in peripheral blood cells (as it is diluted with proliferation) (27). The early kinetics and broad distribution of CD69 expression make it an ideal means to identify antigen-responsive T-cells in *ex vivo* stimulation assays, and viably purify these cells. However, because it is diluted with cell proliferation, it can not be used for long-term stimulation assays. Moreover, when studied directly *ex vivo* (without *in vitro* re-stimulation), it can severely underestimate the proportion of activated cells *in vivo* (which tend to be HLA-DR+ CD38+ CD69−).

Finally, markers of cellular proliferation can be used as surrogates for T-cell activation (since activation is required for proliferation). Indeed, the measurement of one such molecule, Ki-67, correlates with expression of HLA-DR, CD38, and CD69 in HIV+ individuals (28). Compared to other markers of activation, however, Ki-67 has an important advantage: it provides a measure of T-cell turnover. Specifically, since the loss of CD4+ T-cells occurs gradually over years of chronic HIV disease, the level of T-cells over short periods of time (*i.e.*, days or weeks) is relatively constant. During this time, the proportion of proliferating cells essentially reflects the proportion of destroyed cells (*i.e.*, steady-state kinetics) (29), so high levels of Ki-67 expression reflect rapid T-cell destruction (and replacement) in HIV disease. In theory, the measurement of activation markers does not provide this information, since not all activated cells are destined to die. However, since Ki-67 is a nuclear antigen, measurement can only be performed after fixation and permeabilization; therefore, viable proliferating cells cannot be purified for further analysis. In this regard, measurement of other cell surface molecules associated with cellular proliferation might be preferred (such as CD71, the transferrin receptor).

Markers of T-cell senescence

In healthy individuals, T-cell activation is tightly regulated, requiring an initial signal delivered by T-cell receptor engagement and a second signal provided by the binding of costimulatory proteins on the cell surface. During chronic HIV infection, CD8+ T-cells expressing CD28, one of these costimulatory molecules, are progressively replaced by CD28− cells (30). The CD8+ CD28− cells that accumulate exhibit characteristics similar to cells that have repeatedly undergone antigen-driven proliferation *in vitro*: cell cycle arrest, short telomeres, and inhibition of telomerase activity (31). This phenotype is shared by CD8+ T-cells in other settings of chronic antigenemia, such as cytomegalovirus (CMV) infection (32); however, it should be noted that these cells are not anergic. In fact, IFNγ production (33), proliferation (34), and high cytolytic activity (34), (35) are observed within the CD28− subset of CD8+ T-cells. Still, these cells (called effector or terminal effector cells) certainly exhibit reduced proliferative potential (36); thus, the buildup of CD28− cells in HIV disease may severely inhibit the homeostatic replacement of lost T-cells.

Similarly, although its precise function on T-cells remains unknown (32), the expression of CD57 also defines a subset of senescent cells expanded in HIV infection (37), (38). These cells are largely contained within the CD28− population; however, they can be found even in central memory cell populations (which are CD28+). Interestingly, CD57 expression tracks closely with the cytolytic enzyme perforin, thereby defining a subset of cells (also containing granzyme A and B) that is highly cytotoxic (39). In this way, highly cytotoxic senescent cells can be purified from the T-cell compartment for further analysis using just a single cell surface marker.

Markers like PD-1 and CTLA-4 provide negative costimulatory signals to T-cells that inhibit activation (40), (41). Both are elevated in untreated HIV infection, suggesting a role for these markers in the immune dysregulation associated with disease (42), (43), (44), (45). This is further supported by the observations that 1) HIV-specific CTL expressing PD-1 have increased susceptibility to spontaneous and Fas-mediated apoptosis (46), and 2) blockade or knockout of CTLA-4 increases inflammation (40). Notably, the distribution of these molecules among T-cell subsets is not uniform. Both are up-regulated upon stimulation, but constitutive expression can be observed in central memory T-cells (in the case of PD-1, (47), (48)) or regulatory T-cells (for CTLA-4, (40)). Thus, when studying these markers, researchers must consider whether direct, *ex vivo* analysis or *in vitro* re-stimulation best reflects what is occurring *in vivo*. Unfortunately, there is little data available to guide this decision.

Finally, a common feature of HIV pathogenesis is the appearance of cells expressing markers of apoptosis, such as CD95 (Fas) and Fas ligand (FasL). It is believed that viral proteins, immune activation, and/or cytokine dysregulation can induce the pathways associated with these markers (49); however, the relative importance of each mechanism remains unclear. In any case, recent studies describe elevated levels of CD95+ (50) and FasL+ (51) cells even in the earliest phases of infection. Moreover, cells described as senescent by other markers (CD28−. CD57+, PD-1+, CTLA4+; see above) often express markers associated with the CD95/FasL apoptotic pathway. Thus, senescence of T-cells in HIV disease involves multiple factors, including the loss of costimulatory molecules, accumulation of terminally differentiated cells, expression of inhibitory receptors, and induction of apoptosis.

Markers of early T-cells

The accumulation of activated and/or senescent cells in HIV+ individuals suggests that less differentiated cells are progressively lost. Indeed, there is evidence that naïve (52), (53), central memory (15), and IL7 receptor alpha (CD127) positive cells are depleted in HIV+ individuals (15). It is important to note that, among the many cell types described in this review, naïve and memory subsets may be the most difficult to define; there is little consensus regarding which cell surface markers best discriminate the various stages of T-cell differentiation (54).

The markers most commonly considered for human studies are CD45RA, CD45RO, CD11a, CCR7, CD27, CD28, CD57, and CD62L. When measured together, cell subsets expressing nearly every possible combination of these markers can be found, suggesting great phenotypic heterogeneity among T-cell subsets. However, since it is usually impractical to measure all simultaneously, a balance must be struck between technologic limitations and identification of “pure” subsets. This decision is easiest for cells that have not yet encountered antigen (naïve cells), since this population is wholly CD45RA+ CD45RO− CD11a^{dim} CCR7+ CD27+ CD28+ CD57− CD62L+ (54). Importantly, a combination of any three of these markers can distinguish naïve cells from memory cells with high specificity (55). For memory cell subsets, it becomes harder to select a panel of markers to discriminate subsets, as protein expression is more heterogeneous than naïve cells. Moreover, it is often not known which phenotypically-distinct subsets are functionally different. Nevertheless, broad distinctions can be drawn based on a wide variety of attributes. Central memory populations are long-lived (40), traffic through lymph nodes (using molecules like CCR7 (56) and CD62L (54)), exhibit longer kinetics for cytokine production than other memory cell subsets (57), (58), and are enriched for IL2-producing cells (59). The phenotype of these cells is typically CD45RA− CD45RO+ CD11a^{high} CCR7+ CD27+ CD28+ CD57− CD62L+. However, cells lacking expression of CD27, CD28, or CD62L are observed frequently, and, in the absence of functional data describing these cells, it remains unclear if these are truly central memory cells or some transitional phenotype. Interestingly, cells exhibiting markers of senescence (CD57+) or exhaustion (PD-1+) are present among CCR7+ “central” memory cells as well (37), (46). Finally, effector cells (known for rapid production of cytokines and cytolytic enzymes) can be grouped together roughly by the absence of CCR7, CD28, and CD62L from their cell surface. However, again, the discrimination of “pure” subsets of effector cells is complicated by their varying degrees of CD45RA, CD45RO, CD27, and CD57 expression.

In summary, a number of markers can be used to measure naïve and memory T-cell subsets in HIV+ individuals. Unfortunately, the expression of particular combinations cannot be related precisely to the nomenclature that typically describes maturational states (naïve, central memory, effector, senescent/terminal). Still, regardless of which markers are employed, a consensus has emerged that untreated HIV infection is characterized by the loss

of less mature cells and the accumulation of highly-differentiated cells. This can be restated in terms of regenerative potential as well: early cells, capable of homeostatic proliferation (naïve and central memory, CD127+ cells), are progressively replaced with terminally differentiated late-stage cells. In this regard, the measurement of only a couple of key markers (*e.g.*, CD127, CD57) may be sufficient to describe the maturational status of T-cells in HIV+ individuals. Similarly, the measurement of certain markers may be of little value, because they are lost upon cryopreservation (CD62L, (54)) or are inversely expressed (CD45RA and CD45RO, (54)). In the case of CD45RA and CD45RO, only one of these markers is needed when other markers of T-cell differentiation are examined.

Markers for Regulatory T-cells

Recently, much attention has been focused on defining and quantifying a population of T-cells capable of suppressing T-cell function. These regulatory T-cells were first described by Gershon and Kondo (60) through studies of transferred cells in mice, and were later defined on the basis of flow cytometric measurements of CD25 expression (61). Recently, the identification of these cells was refined through measurements of forkhead transcription factor p3 (FoxP3) (62). In theory, the accumulation of regulatory T-cells in HIV infected individuals may contribute to immunodeficiency and hasten progression to AIDS (63), (64). As such, these cells could be a therapeutic target or a correlate of poor vaccine efficacy. However, the examination of regulatory T-cells is particularly complicated in HIV+ individuals due to the abundance of activated cells, which can also express CD25 and FoxP3 without producing suppressive cytokines (such as IL10) (65). Thus, better ways of identifying regulatory T-cells are needed, and may be possible using combinations of CD127 (66), CD39 (67), and/or glucocorticoid-induced tumor necrosis factor receptor (GITR) (68). Additionally, the measurement of immunosuppressive cytokines (*e.g.*, IL10, TGFβ) may be informative, although the proportion of cells expressing these cytokines after *in vitro* re-stimulation is usually very low and therefore long stimulation periods are required for detection.

Markers of T-cell function

Measurements of T-cell function are central to understanding HIV disease, since they reveal how HIV dysregulates immunity, and indicate which T-cell functions are necessary for natural or vaccine-induced control of the virus. These measurements may be performed to examine the state of global T-cell function (using polyclonal stimulants, like staphylococcus enterotoxin B (SEB) or phorbol myristate acetate (PMA)/ionomycin) or the nature of various antigen-specific T-cell responses (using peptides or proteins derived from HIV or other pathogens). When examining antigen-specific T-cell responses, it is often necessary to compare responses to HIV against other viruses (*e.g.*, CMV, Epstein-Barr Virus (EBV)), in order to distinguish deficits in HIV-specific immunity from general deficits in T-cell function (69). Regardless, flow cytometric assays are typically used to measure three types of T-cell function: cytotoxicity, proliferation, and cytokine secretion.

In the setting of viral infections, cytotoxicity is an important means of eliminating infected cells and preventing cell-to-cell spread of viral particles. Two pathways are known to mediate this function. The first involves the engagement of death receptors (such as CD95 or the TNFα receptor), while the second involves the release of cytolytic enzymes (such as granzyme (grz) A, grz B, or perforin) (70). Differential expression of these molecules is observed (*e.g.*, grz A+ grz B− perforin− and grz A+ grz B+ perforin+ subsets co-exist), suggesting that cells can differ in their intrinsic cytotoxic capacity (39). However, the cyotoxic capacity of various subsets has never been compared, because cytolytic enzymes must be measured after fixation and permeabilization (which prevents isolation of viable cells for *in vitro* cytotoxicity assays). Isolation of live CD95+ or TNFα receptor+ cells is

possible, but *in vitro* apoptosis assays are not sufficiently sensitive to detect differences in the apoptotic ability of cells expressing various combinations of these markers. Moreover, this pathway may not be restricted by antigen-specificity (CD95 and TNFα receptor are not strictly associated with antigen stimulation *in vitro*); therefore, it is difficult to establish how the capacity to induce apoptosis *in vitro* reflects what occurs during an *in vivo*, pathogen-specific response. Still, there is a substantial body of literature that describes dysregulation of cytotoxic pathways in HIV+ individuals (71). Notably, within HIV+ individuals, perforin levels are lower within HIV-specific CD8+ T-cells than other antigen-specific cell populations (72), (39).

When assessing cytotoxic potential of cells within functional assays, it is important to consider the kinetics with which cytolytic enzymes are released and regenerated upon antigenic stimulation. Specifically, cytolytic enzymes can be secreted immediately upon *in vitro* re-stimulation, and although intracellular stores of enzymes subsequently regenerate, the rate at which they are replaced may differ by molecule or cell type. This complicates efforts to measure cytolytic enzymes within *in vitro* functional assays, because the ideal stimulation period (six hours? overnight?) cannot be determined easily. Furthermore, it remains unclear whether a recently stimulated cell regenerates the same combination of cytolytic enzymes that it had with previous antigen exposure (*i.e.*, does a stimulated grz A+ grz B− perforin− regenerate only grz A, or does it acquire intracellular stores of grz B and perforin?). Thus, the plasticity of phenotypes may complicate efforts to define correlates of vaccine efficacy, since cell types deemed important by *in vitro* functional assays may have little resemblance to the *in vivo* cell responsible for vaccine-induced protection.

Proliferation of T-cells can be measured in a variety of ways. As described earlier, direct staining of T-cells for Ki-67 can indicate the proportion of proliferating cells *in vivo*. When peptide-MHC Class I or II multimers (“tetramers”) are included in the staining panel, *in vivo* proliferation can be assessed by antigen specificity. However, there are important limitations to this method. First, the use of tetramers requires *a priori* knowledge of HLA type (and tetramers for all HLA types may not be available readily). Second, it is not clear that all Ki-67+ cells complete the proliferative process, since HIV proteins may induce cell cycle arrest in some CD4+ cells, and some Ki-67+ CD8+ cells may die before completing division (73). Third, Ki-67 does not reveal the proliferative capacity of cells that are not concurrently exposed to antigen *in vivo*. For example, in people with chronic HIV infection, the proliferative capacity of resting T-cells directed against opportunistic antigens might not be reflected by Ki-67 expression, since these antigens are not likely to be present. Thus, in many cases, the only way to assess proliferative potential is through *in vitro* stimulation assays.

To assess cell proliferation *in vitro*, a common stimulation assay employs carboxy fluorescein succinimidyl ester (CFSE) to label cells (74). For this method, cells are loaded with an acetylated form (CFDA-SE), which crosses cellular membranes and reacts with intracellular esterases. These enzymes remove acetate groups and convert CFDA-SE to CFSE, which is fluorescent. Subsequently, CFSE couples covalently to intracellular proteins, forming a stable complex that cannot leak out of the cell. In proliferation assays, PBMC are loaded with CFDA-SE for 5–10 minutes, and (after a wash step) the loaded cells are then cultured in the presence of antigen for 4–7 days. With each round of proliferation, CFSE is equally divided among daughter cells, so that the fluorescence (on a per cell basis) is reduced by half. In flow cytometry experiments, this is usually visualized as a series of peaks on univariate histogram. The brightest peak represents cells that have not proliferated, while the dimmest represents cells that have proliferated the most. Using mathematical models, the number of rounds of proliferation can be estimated, along with the proportion of cells that participated in each round of proliferation. The assay becomes more powerful

when combined with cell sorting (to isolate and further characterize proliferating cells) or multicolor staining (to assess other phenotypic or functional characteristics). However, three limitations of the CFSE should be noted. First, when cells are overloaded with CFSE, proliferation is reduced (perhaps due to CFSE toxicity). Second, the fluorescence of CFSE is brighter and the spectrum is broader than fluorescein-conjugated antibodies; therefore, compensation must be performed with CFSE-labeled cells. Third, the assay does not quantify terminal or effector cells well, since these cells may release cytokines or kill other cells without dividing.

Finally, measurements of cytokine production are also an important means of assessing T-cell function in HIV+ individuals. Intracellular cytokine staining (ICS) is the most common method for performing these measurements on a flow cytometer (75). Briefly, this technique involves stimulating cell samples *in vitro* for a period of 6–18 hours with HIV proteins or peptides (typically representing commonly circulating strains of HIV), alongside chemicals that trap cytokines intracellularly (*e.g.*, Monensin or Brefeldin A, inhibitors of protein secretion). Cells are then stained with antibodies against cell surface proteins, fixed, permeabilized, and incubated with antibodies against cytokines. By using this method in the context of multicolor flow cytometry, cytokine production can be assessed within individual cells, and related to other T-cell phenotypes (such as T-cell senescence or activation). The primary disadvantage of this technique is that it does not allow for isolation of viable cells (since fixation/permeabilization is lethal). Still, the assay has an important advantage over classical, bulk secretion assays (such as ELISAs), which cannot discriminate cells secreting a single cytokine from those secreting multiple cytokines simultaneously.

In fact, recent data suggests that cells capable of secreting multiple cytokines simultaneously may play an important role in the immune response against HIV. In these studies, four cytokines (MIP1β, IFNγ, IL2, and TNFα) were measured together with the degranulation marker CD107a, to provide an indication of the quality of the immune response (5). Cells secreting multiple cytokines were more frequently observed in those individuals who maintained low levels of virus and normal levels of CD4+ T-cells for many years (long-term non-progressors, LTNP) (76). Moreover, these cells were also frequently observed in individuals infected with HIV-2 (77), a less pathogenic disease than HIV-1. These data suggest that the presence of polyfunctional cells (expressing 3+ functions) is associated with reduced disease. However, this association could simply be a consequence of reduced viremia in those with less pathogenic disease, rather than a direct cause of viral control. To address this issue, prospective experiments were performed in the setting of murine Leishmania, where an effective vaccine was available. In that model, vaccinated mice generated more polyfunctional cells than sham-injected mice, and subsequently experienced a reduced disease burden upon Leishmania challenge (78). This suggests, then, that the presence of polyfunctional cells is a cause, not merely an effect, of antigen control. Similar results have since been observed in monkeys vaccinated against - and then challenged with – SIV, providing more evidence for the importance of polyfunctional cells in HIV immunity (79). Notably, in the Leishmania studies, cells making multiple cytokines simultaneously also produced higher levels of each individual cytokine (as indicated by higher mean fluorescence intensity, MFI), suggesting that it might be possible to distinguish polyfunctional cells from monofunctional cells based solely on the MFI measurements from one cytokine (78).

The measurement of multiple cytokines simultaneously can also reveal the functional maturation of a response (59). Specifically, there is emerging evidence that memory cells modulate not only surface markers like CD45RA and CCR7, but also cytokine secretion, over the course of an immune response. It is postulated that the least differentiated antigen-specific cells produce IL2 alone or TNFα alone, while central memory (or intermediate)

cells may be polyfunctional. Furthermore, as the response matures, these cells become transitional (downregulating IL2 expression) and effector cells (producing IFNγ alone). Although this model is supported by recent and ongoing studies ((78), unpublished data), it has – to date – not been applied to HIV studies to determine if functional maturation is impaired in HIV disease.

Why Make These Measurements?

Flow cytometry has been used to address a wide variety of questions critical to our understanding of HIV and AIDS. The most fundamental of these questions asks which cell subsets are affected by disease. Early studies defined the changes to major cell lineages that occurred after HIV infection (a decrease in CD4+ T-cells and a reciprocal increase in CD8+ T-cells (80)), and identified the distribution of cellular co-receptors for the virus (CCR5 is expressed by memory T-cells, CXCR4 is found on almost all CD4+ T-cells (81)). Subsequently, researchers recognized the high rate at which T-cells were lost in HIV+ individuals, by performing kinetic analyses of T-cell turnover (82), (83). These studies relied heavily on flow cytometry to enumerate CD4+ T-cells before and after therapy. Within just a few years, flow cytometric quantification and purification of naïve T-cells also showed that the production mechanisms needed to replace these lost cells failed as individuals progressed to AIDS (53). Finally, elevated levels of activated T-cells are well-described in HIV+ individuals (reviewed above).

Though these studies greatly advanced our understanding of HIV pathogenesis, a key question remained unresolved until about five years ago. Given the relatively low proportion of cells infected with HIV during the chronic phase of disease (0.05% of CD4+ T-cells), what accounted for the dramatically reduced level of CD4+ T-cells found in HIV+ people? Flow cytometric studies of acute infection, first in the animal (SIV) model (50) and subsequently in humans (84), shed light on this issue by revealing very high levels of infected cells, and a profound loss of over 60% of the body's CD4+ memory T-cells within weeks of infection. This depletion was particularly striking in the gut, since this tissue contains primarily memory CD4+ T-cells. Notably, subsequent studies revealed that damage to the gut had severe consequences, as bacterial products were found in the systemic circulation of individuals in the early stages of HIV disease (84). As a recent study showed, these bacterial products bind toll-like receptors on CD4+ and CD8+ T-cells (85), perhaps causing generalized immune activation.

Thus, with the help of flow cytometry, a better understanding of how the T-cell compartment is remodeled upon infection has emerged. Initially, HIV destroys large numbers of memory CD4+ T-cells, causing particular damage to the gut, which allows the translocation of bacterial products into systemic circulation. Bacterial ligands for toll-like receptors, bind CD4+ and CD8+ T-cells, activating them and amplifying the already significant loss of T-cells that accompanies early HIV disease. To compensate for this loss, homeostatic pathways for cell replacement are invoked. However, over time, continued infection of memory T-cells and the loss of cells to activation-induced cell death strains the homeostatic apparatus. A final blow is delivered before AIDS diagnosis, when CXCR4-tropic strains of virus emerge and deplete immature and naïve cells. Although this represents a broad account of the disease process, it is by no means complete. The myriad of markers described earlier in this review (and summarized in Table 1) has been examined across a wide variety of patient groups, ranging from those with acute/early infection to those dying of AIDS. For more detailed information, readers are encouraged to consult a variety of thoughtful reviews (86), (87), (8).

Flow cytometry studies have also provided important clinical information that helps predict disease outcome and guide treatment decisions. To this end, the strongest predictors of disease progression (and thus the need for therapeutic intervention) are CD4+ T-cell count (88) and viral load (89). Though these measures are the gold-standard for initiation of therapy, they do not fully capture an individual's risk for disease progression. For example, in a recent retrospective study of HIV+ people infected for less than 18 months, a substantial proportion of patients who had more than 350 CD4+ T-cells/uL blood (the current threshold for treatment initiation) were destined to progress to AIDS within only three years. This suggests that additional tools are needed to help identify, early in disease, more HIV+ individuals at risk for rapid disease progression. Despite substantial investigation, only a few such markers have been reported. For example, in the setting of early HIV disease, the ability of Ki-67 to predict disease progression independently of CD4+ T-cell count or viral load was recently recognized (15). Similarly, in the setting of chronic infection, CD38 expression is a CD4− and viral load-independent predictor of disease outcome (12).

The identification of these kinds of predictive measures is not only important for guiding treatment decisions, but also for the development of preventive and therapeutic HIV vaccines. Vaccine efficacy trials require large patient cohorts, followed for long periods of time, therefore great effort is devoted to finding any early evidence of an effective immune response. To this end, a number of flow cytometry-based studies have examined HIV-specific T-cells, identified by ICS or tetramers, in individuals with less pathogenic HIV disease. These individuals include LTNP, defined by their ability to maintain normal CD4+ T-cell counts and undetectable levels of virus, without treatment, for years after infection. (Some of these people can also be identified by their expression of "protective" HLA alleles B27 and B57 (90).) Early research described high levels of HIV-specific CD8+ T-cells in LTNP; however, the frequency of these cells alone could not explain reduced disease burden, since HIV-specific CD8+ T-cells were readily detectable in normal progressors as well (91), (92). Instead, it could have been the quality of these cells that mattered. For example, the breadth of the HIV-specific CD8+ T-cell response (as indicated by the number of epitopes of HIV-Gag protein targeted) correlated with low viral load and high CD4+ T-cell counts (93). Moreover, HIV-specific CD8+ T-cells from LTNP were more likely to: 1) be polyfunctional (76), 2) express the cytolytic enzymes perforin and granzyme B (94), 3) kill infected cells rapidly (95), (94), 4) and recognize lower levels of peptide (suggesting that they expressed high avidity T-cell receptors) (95). In theory, vaccines that generate similar T-cell responses may be effective.

Finally, flow cytometry has established itself as an important tool for the study of a number of other HIV-related questions. For example, with the advent of highly active anti-retroviral therapy (HAART), many studies were aimed at defining the immunologic benefits of therapy. Moreover, studies sought to understand the source, maturation, and function of the cells that reconstituted the T-cell compartment after therapy. As the prevalence of treatment grew, it also became apparent that some individuals would not respond to therapy (immunologic non-responders), and research was directed toward identifying these individuals and defining their T-cell characteristics. The body of literature addressing these issues is reviewed elsewhere (96), (97), (98).

Conclusion

Few diseases have produced the sheer volume of scientific literature that HIV has generated. Notably, the vast majority of these studies have relied on some form of flow cytometry technology, and therefore the history of these two fields is intimately linked. Clearly, flow cytometry is an important tool in the study of HIV pathogenesis, but given the multitude of immune measurements that can be made using this technique, researchers must think

carefully about which markers to examine, and ought to consider the subtleties involved in performing each of these measurements. Nevertheless, as demonstrated by the studies described in this review, carefully designed experiments can reveal important aspects of HIV pathogenesis, guide treatment decisions, and help evaluate the immunogenicity of vaccine candidates. Such efforts may stem the tide of the global HIV/AIDS epidemic.

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Table 1

Summary table describing markers commonly examined in studies of HIV disease.

Category	Marker(s)	Surface or Intracellular	Characteristics	Ref.
Activation	HLA-DR	Surface	Constitutive expression on APCs, upregulated on T-cells upon stimulation	19
	CD38	Surface	Constitutive expression on naïve cells, upregulated on memory with activation	22
	CD69	Surface	Early activation marker, diluted with cell proliferation	26
Senescence	CD28	Surface	Costimulatory molecule, expression reduced in HIV+ individuals	30
	CD57	Surface	Terminal cells, expressed by multiple subsets, marker for highly cytotoxic cells	37
	PD-1/CTLA-4	Surface	Inhibitory receptors that block activation, complex expression patterns	40
	CD95/FasL	Surface	Expressed by activated cells, involved in apoptotic pathways	49
Early Cells	CD127	Surface	IL7 receptor, expressed by naïve and central memory cells	15
	CD45RA, CD45RO, CD11a, CCR7, CD27, CD62L	Surface	Markers used to distinguish naïve, central memory, effector memory, and terminal cells.	54
Regulatory	CD25/FoxP3/CD127	Surface	Consensus that regulatory T-cells are CD25+ FoxP3+ CD127−; however, this is also phenotype of some activated cells.	66
	CD39	Surface	Produces adenosine, which has immunoinhibitory properties	67
	GITR	Surface	Costimulatory molecule for regulatory T-cells	68
	IL10/TGFβ	Intracellular	Immunosuppressive cytokines, expression very low even after *in vitro* stimulation	65
Cytotoxic Function	Granzyme A, Granzyme B, Perforin	Intracellular	Precise mechanisms of function unclear, released upon stimulation, kinetics of regeneration differ by molecule.	70
Proliferation	CFSE	Intracellular	Cell labeling dye, diluted over 5 day *in vitro* proliferation, not for effector cells	74
	Ki-67	Intracellular	Present during all active phases of cell cycle, but not during resting (G0) phase.	28
Cytokines	MIP1β, IL2, TNFα, IFNγ	Intracellular	Cytokines produced upon *in vitro* stimulation, markers of polyfunctional cells	76
Co-receptors	CCR5/CXCR4	Surface	Co-receptors for HIV	81