

Tracking the killers: how should we measure CD8 T cells in HIV infection?

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Most immunologists agree that CD8 T cells are important in controlling viruses, at least after the first week of infection – the time it takes for naive CD8 T cells to expand and differentiate into antiviral cytotoxic T lymphocytes (CTL). Nonetheless, CD8 T-cell measurements in HIV clinical studies show little relation to disease prognosis. There are two possible interpretations – either CD8 T cells are not important in established HIV infection or we are not measuring the right things.

What is the evidence that CD8 T cells protect against HIV and other viral infections? CD8 T cells suppress viral infection by direct cytotoxicity and secreting soluble anti-HIV factors. The evidence of CD8 cell-mediated immune protection in acute infection is strong. The most compelling evidence comes from the control of primary viremia coincident with the expansion of HIV-specific CTLs [1,2]. In simian immunodeficiency virus (SIV) infection, depleting CD8 T cells by injecting anti-CD8 antibodies increases viral burden and worsens prognosis [3,4]. The rapid emergence of CTL escape mutations in acute SIV infection also indirectly points to protective selection pressure from SIV-specific CD8 T cells [5]. Recent reports document increasing evidence for HIV escape from CD8 T-cell recognition [6–8]. CD8 T cells also secrete suppressor factors and interferons that contribute to protective

immunity via both antigen-dependent and independent mechanisms [9,10].

The evidence for viral control by CD8 T cells in chronic infection is weaker. Although a few human studies show correlation of antiviral CD8 T cells with disease progression, the data are much less striking, particularly given how many clinical studies examined this issue. Although high HIV-specific CD8 T-cell frequencies are maintained until late in disease, many antigen-specific T cells have restricted functional capacity [11,12]. Therefore counting the numbers of HIV-specific cells, for instance by tetramer staining, may not be that informative. Some T-cell functions, such as IL-2 production and cytotoxicity, are impaired more than others [i.e., interferon- γ (IFN γ) production] [13]. Freshly isolated HIV-specific CD8 T cells from chronically infected donors are rarely cytotoxic, and only a small minority highly express perforin, which is required for cytotoxicity [14–19]. Even in early-stage donors with well-preserved CD4 cell counts, only about half of the HIV-specific CD8 cells produce IFN γ in response to HIV-infected target cells; in more advanced patients (even with controlled viral load) only a minority do. Following acute SIV infection or vaccination, almost all SIV tetramer-staining cells produce IFN γ when activated, but within 6 months of chronic infection the proportion that produce IFN γ

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drops significantly [20]. This finding contrasts with observations during acute mouse virus infections where virtually every antiviral CD8 T cell produces IFN γ upon activation. However, if *in vitro* activation is strengthened by adding IL-2, CD40L or other co-stimulatory signals, function in cell culture can often be observed [14,21].

The first clear-cut evidence of distinct CD8 T-cell differences associated with favorable prognosis was the finding that HIV-specific CD8 T cells from rare long-term non-progressors (LTNP) with undetectable plasma viremia uniquely retain the ability to proliferate in response to HIV-infected cells. They also stain brightly for perforin, the rate-limiting molecule for cytotoxicity [22]. CD8 T cells from more conventionally defined LTNPs (with detectable plasma viral RNA) do not proliferate or stain brightly for perforin. Therefore maintaining HIV-specific cytotoxicity may be key to viral control. This response may be even more difficult to maintain than CD4 T-cell HIV lymphoproliferative activity. In fact, when antiviral T cells are transferred into chronically lymphocytic choriomeningitis virus-infected mice, CD8 T-cell cytotoxicity is lost within a few weeks, whereas CD4 T cells continue to function for at least 1 month [23]. However, although CD8 T-cell lymphoproliferation and perforin expression correlate with viral control, we must keep in mind that they may not be responsible.

Because most clinical studies have not revealed signifi-

cant correlations of prognosis or therapeutic response with CD8 T-cell measurements, we need to re-examine the assays used to follow CD8 immune function (Table 1). The assays we use may not warrant the time, expense and use of precious clinical samples. A popular assay involves counting HIV-specific CD8 T cells by tetramer-staining for common HLA-restricted epitopes. Another common approach is measuring, by ELISpot assay or intracellular cytokine staining (ICS), IFN γ production to cells incubated with HIV peptides or infected with recombinant vaccinia virus expressing HIV antigens. The antigens in these assays are derived from consensus or laboratory virus strains.

Why might these assays be misleading? Most HIV tetramer+ cells lack a functional phenotype and a large proportion are not functional when tested directly *ex vivo* [14–18]. Moreover, HLA expression does not predict HIV epitope recognition and the most common HLA alleles (for which tetramers are available) infrequently restrict the immunodominant CD8 T-cell responses [24,25]. Moreover, these epitopes are frequently mutated [7]. In addition, counting tetramer+ cells does not correlate with responses to therapy or prognosis, except possibly during acute infection [26]. Therefore counting the numbers of tetramer+ cells is unlikely to be useful.

What about ELISpot or ICS assays? The assays currently used augment the weak signal provided *in vivo* by HIV-infected cells. HIV-infected primary cells dis-

Table 1. Assays for measuring antigen-specific CD8 T cell number and function.

| What we measure | Disadvantages |
|---|--|
| Tetramer staining [41] | Contains no functional information Only measures common alleles which infrequently restrict the dominant response Corresponding epitopes may be subject of viral escape mutations |
| IFN- γ production by ELISpot or intracellular cytokine staining [38,42] | Does not correlate with cytotoxicity Stimulated production of other cytokines may be important for protection (i.e. IL-2, TNF- α) or inhibiting protection (i.e., TGF- β , IL-10, IL-13) Adding costimulatory antibodies gives false impression of <i>in vivo</i> function |
| Simulators: peptide-loaded or recombinant vaccinia virus-infected targets | Antigen provided in excess may overcome weak presentation on infected cell Vaccinia or peptide sequences from laboratory strain virus may have no relation to recognition of autologous virus |
| What we should measure | Advantages |
| IL-2, IFN- γ , TNF- α , TGF- β , IL-10 production by ELISpot or ICS [38,42] Stimulators: HIV-infected primary cells (CD4 T cells, macrophages) [33] – in substudies with autologous virus[29] with and without costimulatory cytokines, IL-2 or CD40L | Captures protective as well as potentially immunosuppressive responses Best mimic for natural <i>in vivo</i> targets Ratio of response with and without co-stimulation will measure the extent of CD8 dysfunction |
| LYSISpot assay (release of granzymes, perforin in response to HIV-infected primary cells with and without exogenous co-stimulatory molecules) [36] | Measures most critical CD8 T-cell function in response to natural targets Ratio of response with and without exogenous costimulation gives an indication of the extent of CD8 dysfunction |
| HIV production (by p24 enzyme-linked immunosorbent assay) in primary blood mononuclear cells directly or with varying numbers of CD8 and CD4 T cells [32] | Captures effective protection (secreted and cytotoxic) without bias Uniform population of infected cells can be used to quantify the ratio of CD8 T cells required to suppress infection by 50% or 90% |

play limiting amounts of viral antigen because of competition for binding with other host peptides and Nef-mediated down-modulation of cell surface HLA [27,28]. Infected T cells will not stimulate as well as professional antigen-presenting cells because they lack co-stimulatory molecules. Current assays get around this problem by providing a surfeit of antigen (peptides in vast excess, vaccinia which stops host gene synthesis) and by adding co-stimulatory antibodies. These 'improvements' compensate for limited antigen presentation by infected cells but are not good surrogates for *in vivo* function. Moreover, although IFN γ production has antiviral effects, it is less effective than killing infected cells, which is what we really want to measure.

Another potential harder-to-overcome problem is using consensus viral sequences to construct antigens. Mutation of a virus to escape from CTL recognition has been increasingly documented [6–8]. CD8 T cells that recognize earlier HIV variants persist, like memory cells for resolved infections. These memory cells are likely to have better functional properties than the T cells that recognize antigens in the currently replicating viruses. In fact, the IFN γ response to a laboratory HIV strain is often stronger than the response to the patient's own virus [29]. In that study, T-cell receptor usage did not overlap and there was no cross-reactive lysis of autologous and lab-strain virus, in all symptomatic patients and in one of three asymptomatic donors [29]. Therefore functional measurements using consensus epitopes may be irrelevant to the viruses infecting the patient. Although a few studies have attempted to address this question by generating recombinant vaccinia viruses or peptides based on individual patient sequences, this is a Herculean task, only possible for a few subjects [30,31]. This problem is confounded by the continuous *in vivo* evolution of autologous virus. Epitope mapping to study the breadth of responses to consensus epitopes probably has little relevance to maintaining functional CD8 cells. In fact as the infected individual develops CD8 T cells against non-dominant epitopes, these subdominant T cells probably have lower avidity interactions with infected cells and may be less functional.

It is time to think about alternative approaches. Assays that might be more difficult to perform can be done in smaller studies or as subset studies of larger trials. Choosing the right assays is especially important for studying interventions that seek to improve on drug therapy to establish effective immunosurveillance against HIV. An operational way to measure immune control over HIV is by designing drug interruption (analytical treatment interruption or ATI) into the study. Immune assays that predict viral containment after treatment interruption would be valuable. The closer an assay is to *in vivo* conditions, the more likely it will distinguish relevant immune differences. Co-culture assays that measure *in vitro* immune control of

virus make sense [32], but have not won wide acceptance. Perhaps they should be revisited in the context of ATI.

Ferrari and colleagues developed methods to use HIV-infected cells as stimulators or targets [33]. Taking advantage of CD4 down-modulation by Nef, a uniform population of HIV-infected target cells can be prepared by deleting CD4+ cells from infected cultures. These physiologically relevant target cells have been used to distinguish functional from dysfunctional CD8 T cells in ^{51}Cr release, ICS and ELISpot assays [16,22,34,35]. ELISpot assays are the easiest to perform reproducibly. It makes sense to mimic the *in vivo* situation as closely as possible by using HIV-infected primary cells as stimulators and omitting co-stimulatory antibodies. Moreover, ELISpot assays can also measure IL-2 production (linked to both lymphoproliferative capacity and perforin expression) and release of cytolytic proteins [36,37]. ELISpot cytotoxicity assays ('Lysispots') may need to be adapted to chronic infections where perforin expression is limiting. Instead of measuring granzyme B release, measuring perforin+ spots may be more useful since degranulation without perforin might not result in target cytolysis. The number of spots increases linearly with the numbers of specific killers so quantification is improved over ^{51}Cr release assays. Moreover, IFN γ secretion and cytotoxicity can be simultaneously detected by using two color indicators to measure the proportion of cells that produce IFN γ , but are not cytotoxic [36]. Some assays could also compare function with and without co-stimulatory antibodies or ligands and/or added IL-2. This approach would provide estimates of the proportion of functioning HIV-specific cells in the universe of potentially functioning cells. In a subset study, autologous virus could be isolated to infect CD4 T cells and/or monocyte-derived macrophages to measure the relevant response to the patient's own virus [29,35]. Flow-based assays of lymphoproliferation and cytotoxicity are also available, but may be more difficult to adapt to clinical settings [22,38,39]. However, they will be easier to standardize than ^3H -thymidine incorporation or ^{51}Cr release assays. It may also be possible to use antigen-presenting cells, such as monocyte-derived macrophages or dendritic cells, incubated with inactivated virus, rather than live virus, for some of these assays [40]. With inactivated virus, more laboratories, particularly at international sites, could do testing in real time to avoid inevitable artifacts from performing assays on stored cells. However, a comparison of live and inactivated virus needs to be done.

CD8 T-cell immunity is nonlinear in relation to antigen load (Fig. 1). The frequency of antigen-specific T cells declines with successful highly active antiretroviral therapy as viremia becomes undetectable. However, the function of each cell is preserved only when viral loads

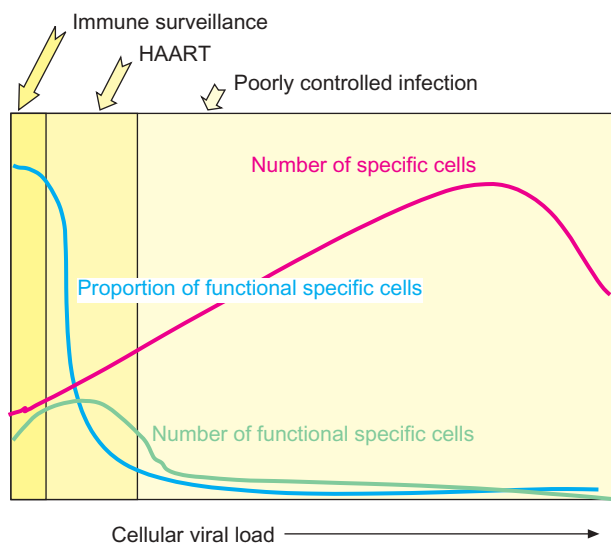


Fig. 1. Hypothetical model for CD8 T-cell function in chronic HIV infection. The number of HIV-specific cells (for instance as measured by tetramer staining, red) has little relation to the numbers of functional cells (green). With HAART, the viral load goes down and the numbers of functional cells may increase somewhat. However, it is not enough to provide effective immune surveillance without drugs. The proportion of functional (especially cytotoxic) HIV-specific cells (blue) may be the best predictor of immune control of HIV infection. I argue that this response is what should be measured. I predict that it will be high only in patients who have effective immune surveillance.

are lowest. If my analysis is correct, having a high proportion of HIV-specific CD8 T cells that are cytolytic (especially against autologous virus), rather than a high frequency of HIV-specific CD8 T cells or even a high frequency of functional HIV-specific cells, will best predict effective HIV immunosurveillance. That is why counting tetramer+ cells or counting the numbers of cells which can be artificially stimulated to function has not been useful in chronic infection. It is time to consider doing not what's easy, but what makes sense.

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