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Cytotoxic CD8+ T cells play a major role in the central immune response against viruses. However, the dynamics of CD8+ T cell responses during the course of a human infection are not well understood. Using tetrameric complexes in combination with a range of intracellular and extracellular markers, we present a detailed analysis of the changes in activation and differentiation undergone by Ag-specific CD8+ T cells, in relation to Ag-specific CD4+ T cell responses, in the context of a human infection: HIV-1. During primary HIV-1 infection, the initial population of HIV-specific CD8+ T cells is highly activated and prone to apoptosis. The Ag-specific cells differentiate rapidly from naive to effector cells at a perforin low intermediate stage of differentiation, later forming a stable pool of resting cells as viral load decreases during chronic infection. These observations have significant implications for our understanding of T cell responses in human viral infections in general and indicate that the definition of effector and memory subsets in humans may need revision.

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Materials and Methods

Study subjects and samples

Samples were taken from a well-characterized cohort of volunteers with acute HIV-1 infection in San Diego, CA. The local Institutional Review Boards and Ethical Committees approved this study. PBMCs were separated from heparinized blood and cryopreserved for subsequent studies. HLA typing was conducted by amplification refractory mutation system-PCR using sequence-specific primers, as previously described (21).
Fourteen patients were selected for the study of CD8+ T cells, on the basis of their having an HLA type for which we had relevant tetramers, and following the detection of HIV-specific CD8+ T cell populations using these tetramers. Patients were classified in two groups: 1) those individuals who were diagnosed before or at the time of HIV-1 seroconversion (referred to as preseroconversion acute HIV, n = 7), defined by symptomatic disease, recent high risk exposure, high plasma HIV-1 RNA (ranging from 3 × 10^5 to 3 × 10^6 copies/ml (mean 8.3 × 10^5 copies/ml)) and either a negative HIV-1 ELISA or a negative/indeterminate HIV-1 Western blot; and 2) those individuals diagnosed after HIV-1 seroconversion, but within 180 days of primary HIV-1 infection (postseroconversion primary HIV, n = 7) with viral load ranging from 1.2 × 10^4 to 6.4 × 10^4 copies/ml (mean 2 × 10^5 copies/ml). Upon diagnosis of acute HIV-1 infection, the majority of the subjects were treated with antiretroviral therapy (ART). Samples from group 1 were analyzed at multiple time points when possible, and the first time points were taken before the start of ART.

For comparison, another group of individuals with chronic HIV-1 infection was studied: individuals with low viral load (ranging from 3 × 10^3 to 1.6 × 10^5 copies/ml (mean 5.6 × 10^4 copies/ml)) for at least 3 years with or without treatment.

**Ags and Abs**

Peptides were synthesized by F-Moc chemistry and corresponded to previously defined and optimized CTL epitopes. Anti-CD8 (PerCP), anti-CD27 (FITC), and anti-CD28 (FITC) Abs were purchased from BD Biosciences (Mountain View, CA) or BD PharMingen (San Diego, CA).

**Preparation of HLA-peptide tetrameric complexes**

The HLA molecule H chain cDNAs were modified by substitution of the transmembrane and cytosolic regions with a sequence encoding the BirA biotinylation enzyme recognition site, as previously described (18). These peptides were synthesized by F-moc chemistry and corresponded to previously defined epitopes for HIV-1 (10^5 to 3 × 10^5 copies/ml) and either immune control or the prompt institution of ART (Fig. 1A). CD8 T cells were perforin high. The quadrants shown are not intended to separate, and therefore with the degree of activation of the cells (Fig. 2B). Ki67 is an intracellular marker of proliferation; expression is restricted to cells in cycle. As expected, its expression in HIV-specific CD8+ T cells was mainly detected during the expansion phase of acute infection, and subsequently decreased (Fig. 1B). In our group of acute seroconverter patients, the percentage of tetramer-staining cells in cycle ranged from 11 to 45% (data not shown).

Intracellular levels of Bcl-2 in HIV-specific CD8+ T cells were reduced during acute infection but increased thereafter to reach high levels during chronic infection (Fig. 2C). The level of Bcl-2 expression showed a close inverse correlation with CD38 expression, and therefore with the degree of activation of the cells (Fig. 1C). Changes in Fas expression also occurred. In contrast to naïve CD8+ T cells (CD27+CD28+CD45RO+), Ag-experienced CD8+ T cells demonstrate extracellular Fas expression, which is significantly increased when the cells are activated (Fig. 2D). Accordingly, HIV-specific CD8+ T cells expressed a high level of Fas CD8 magnetic beads (Dynal Biotech, Oslo, Norway) for 30 min on ice (5.1; beads:PBMC), followed by two washes with RPMI, 2% FCS (the depletion was efficient at 96–99% by FACS analysis). A total of 10^6 CD8+ PBMCs was then plated out in the presence of either staphylococcal enterotoxin B (2 μg/ml), CMV lytase (10 μg/ml; Advanced Bio-technology, Columbia, MA), HIV-1 p24 (10 μg/ml, rHIV-1 IIIB gag p24 produced in the baculovirus system), HIV-1 gp120 (10 μg/ml, rHIV-1 IIIB envelope expression gp120 produced in the baculovirus system), HIV-1 tat (10 μg/ml, rHIV-1 IIIB tat produced in the baculovirus system), or peptide pools of nef (10 μg/ml for each peptide; HIV-1 LAI, thirteen 15-mers with 5-aa overlap) or HIV-1 p17 (10 μg/ml for each peptide; HIV-1 SP2, twenty mers with 10-aa overlap). The plates were incubated overnight at 37°C, 5% CO2, and washed with PBS/0.05% Tween before addition of the second, biotinylated anti-IFN-γ mAb, 7-B6-1 biotin (Mabtech) at 1 μg/ml and incubated at room temperature for 3 h. After washing, streptavidin-conjugated alkaline phosphatase (Mabtech) was added at room temperature for 90 min. Individual cytokine-producing cells were detected as dark spots after a 20-min reaction with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium using an alkaline phosphatase-conjugate substrate (Bio-Rad, Richmond, CA). The number of specific T cells was calculated by subtracting the negative control values, and expressed as either the number of spot-forming units (SFU) per 10^6 PBMC (CD8+ responses) or SFU per 10^6 CD8+ “depleted” PBMC (CD4+ responses). To derive the total HIV-1 response, responses toward all the HIV-1 Ags used were added together.

**Results**

Ag-specific CD8+ T cells are highly activated and susceptible to apoptosis during acute HIV-1 infection

High levels of plasma viremia are generated during acute HIV-1 infection, which is immediately associated with the expansion of a range of HIV-specific CD8+ T cells. The numbers of virus-specific CD8+ T cells decline as viral load falls, whether this is due to either immune control or the prompt institution of ART (Fig. 1A). The magnitude of each CD8+ T cell expansion may vary according to the Ag, with strong responses often seen toward nef epitopes. Using HIV-specific tetramers, the phenotype of these expanded populations was monitored from primary infection onward. During acute infection, the great majority of the virus-specific cells were activated, expressing high levels of the activation marker CD38 (Fig. 2A). CD38 expression fell rapidly, closely following the viral load, and was expressed only at low levels during chronic infection. The activation state of the cells correlated with the levels of TCR expression, as measured by the brightness of tetramer staining: activated cells displayed slightly lower tetramer staining than when resting (Fig. 2B), as described recently (22, 23). Ki67 is an intracellular marker of proliferation; expression is restricted to cells in cycle. As expected, its expression in HIV-specific CD8+ T cells was mainly detected during the expansion phase of acute infection, and subsequently decreased (Fig. 1B). In our group of acute seroconverter patients, the percentage of tetramer-staining cells in cycle ranged from 11 to 45% (data not shown).

Intracellular levels of Bcl-2 in HIV-specific CD8+ T cells were reduced during acute infection but increased thereafter to reach high levels during chronic infection (Fig. 2C). The level of Bcl-2 expression showed a close inverse correlation with CD38 expression, and therefore with the degree of activation of the cells (Fig. 1C). Changes in Fas expression also occurred. In contrast to naïve CD8+ T cells (CD27+CD28+CD45RO+), Ag-experienced CD8+ T cells demonstrate extracellular Fas expression, which is significantly increased when the cells are activated (Fig. 2D). Accordingly, HIV-specific CD8+ T cells expressed a high level of Fas

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1 Abbreviations used in this paper: ART, antiretroviral therapy; SFU, spot-forming unit.
during acute infection, while this was much lower in resting cells, during the chronic phase (Fig. 1D). HIV-specific CD8+ T cells may thus be highly prone to apoptosis during primary infection, presumably through either of the main apoptotic pathways, and may be more protected from cell death and hence more stable during chronic infection. This susceptibility to apoptosis is closely related to the activation status of the cells, and therefore also to the Ag load present in the patient. When viral rebound occurs, HIV-specific CD8+ T cells again exhibit high CD38 levels (22) and also reduced Bcl-2 expression (data not shown). Our observations of the activation state of virus-specific cells were not directly influenced by the use of ART, but more closely followed the viral load, as a similar phenotype was seen in both treated and untreated patients (Fig. 2, A and C, and data not shown). We were able to use a panel of tetramers, including A2 p17, A2 pol, B8 nef, B8 p24, B27 p24, and A11 nef for studies in the acute seroconverters, and no significant differences were observed between CD8+ populations with different specificities.

Rapid, but incomplete differentiation of HIV-specific CD8+ T cells during the course of HIV infection

Ag stimulation drives the differentiation of naive CD8+ T cells into Ag-primed cells, which can be further distinguished, according to cell surface expression of the costimulatory receptors CD28 and CD27: CD28+CD27− cells are seen as precursors or early differentiated cells, and CD28−CD27+ are fully or late differentiated cells, which express higher levels of perforin (24–26). We followed the differentiation of the HIV-specific CD8+ T cell population from acute to chronic infection, measuring CD28, CD27, CD45RA, and perforin expression in these cells. Early during acute infection, high numbers of CD28+CD27− cells were observed, which differentiated rapidly (within 2–4 wk) into CD28−CD27+ intermediate differentiated cells (Figs. 1E and 2, E and F). The maturation process was very rapid. As soon as the cell activation diminished and the expansion phase ended, phenotypes resembled that observed during chronic infection, with the majority of the cells displaying CD27, but not CD28. Furthermore,
no clear separation between CD28+ and CD28− cells could be observed during acute infection, to suggest ongoing differentiation (Fig. 1E). Substantial, but low numbers of CD28− CD27− cells could be observed later, toward the end of the acute infection. As expected, the majority of cells were also CD45RA negative, even very early in acute infection (data not shown). IFN-γ ELISPOT assays conducted using specific CTL epitopes suggested that the capacity of the HIV-specific CD8+ T cells to produce IFN-γ during acute infection could vary, ranging from lower levels to ones similar to those observed during chronic infection (data not shown). In accordance with the phenotypes previously described, low levels of perforin were found in these cells at all time points (Fig. 2G).

The CD8+ T cell phenotype appears to be independent of the HIV-specific CD4+ T cell response

HIV-specific CD4+ T cells secreting IFN-γ in response to viral Ags are usually found in low numbers in HIV carriers (27, 28). They are thought to disappear early during primary infection (29), presumably because as activated cells homing to areas of viral replication, they are a prime target for viral infection and destruction. Loss of CD4+ T cell help may be a cause of impairment of CTL function, rendering CTL less able to control HIV-1 replication (30). In this study, Ag-specific CD4+ T cell responses were measured using IFN-γ ELISPOTs conducted on CD8+ T cell-depleted PBMCs. Fig. 3A shows a comparison between numbers detected in our cohort of patients undergoing primary HIV infection before treatment, and numbers detected in a cohort of patients undergoing chronic infection with and without treatment. In both groups, most responses were either nonsignificant (<50 SFU per 10^6 cells) or relatively low (between 50 and 500 SFU per 10^6 cells). Although there may be a trend toward higher CD4+ T cell responses during acute infection, of borderline statistical significance, the difference is minimal compared with the level of CMV-specific responses in the same individuals, which were generally much higher (>500 SFU 10^6 cells).

Two patients with high numbers of HIV-specific CD4+ T cells also displayed HIV-specific CD8+ T cells, which could be stained with relevant tetramers. No difference compared with other patients in the CD8+ T cell phenotype was found, despite the presence of strong HIV-specific CD4+ T cell responses. Neither of these two patients exhibited more CD8+ T cells with a CD8+ CD27− (more differentiated) phenotype (with high perforin levels and assumed to be more cytotoxic) (Fig. 3B). This suggests that the phenotype of the HIV-specific CD8+ T cells is independent of the virus-specific CD4+ T cell response detected in the periphery, at least as far as IFN-γ secretion is concerned.

The phenotype of the whole CD8+ T cell populations is altered in both acute and chronic HIV-1 infection

To assess the impact of HIV infection on the CD8+ T cell population, we compared the changes in activation and differentiation between an individual HIV-specific CD8+ T cell population and the remaining CD8+ T cells in that patient. Surprisingly, the CD8+ T cell activation status as a whole, as measured by the expression of CD38, Bcl-2, and Ki67, was very similar to what was observed in the HIV-specific population, in both acute and chronic infections (Fig. 4A). A majority (~80%) of CD8+ T cells expressed CD38 and low levels of Bcl-2, with about one-fifth of the CD8+ T cells staining positive for Ki67 during acute infection, similar to the proportions seen for the B8 nef-specific population. Thus, it appears that HIV infection has an impact on the great majority of the CD8+ T cell population.

In contrast to the uniformity of the activation phenotype, the pattern of CD8+ T cell differentiation, analyzed by staining for CD28, CD27, and perforin expression, was distinct for chronic infection (Fig. 4B). In healthy donors, CD8+ T cells are mainly found in the naive and presumably early differentiated Ag-primed cells, both CD28−CD27− (as exemplified in Fig. 4B). During
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acute HIV infection, similar to the pattern displayed by HIV-specific CD8+ T cells, the whole CD8+ T cell population also displayed a shift toward the CD28+CD27+ subsets (Fig. 4B). However, during chronic infection, the CD8+ T cell population as a whole displays a distribution distinct from that of the HIV-specific CD8+ T cells, with clear enrichment of CD28+CD27- cells (31). While the activation phenotype appears similar between the two populations, the differentiation phenotype differs. This implies that HIV is not the only cause of the differentiation phenotype changes undergone by the whole CD8+ T cell population.

Discussion

The schematic representation in Fig. 5 illustrates the most common model depicting the evolution of the HIV-specific cellular immune response: beginning with a peak of viremia, followed by the appearance of HIV-specific CD8+ T cells, characterizing the acute phase, leading to the asymptomatic chronic phase, in which CTL numbers are reduced and viral load has fallen because of either immunological control or antiretroviral treatment. Our observations parallel the pattern of CD8+ T cell responses described in murine models, in which both effector and memory phases have been characterized. During the initial (acute or effector) phase, the virus-specific CD8+ T cells are activated and expand to a large population that is poised between proliferation and apoptosis. Then as the antigenic challenge diminishes, the cells stop proliferating and their numbers are substantially reduced through apoptosis. As cells enter the resting phase, their susceptibility to apoptosis is reduced, accompanied by up-regulation of Bcl-2 and down-regulation of Fas. These characteristics show clear parallels with the dynamics of CTL responses to SIV in rhesus monkeys (32). During the second (chronic or memory) phase when viremia is low, the pool of memory CD8+ T cells remains apparently stable. These cells might be maintained by the ongoing transcription of particular HIV genes (notably nef) that is known to occur in treated chronic infection even when viral load remains undetectable (33–35), as we have recently mentioned (36). However, this may be unlikely, as the cells are mainly resting, in contrast with cells responding to viral rebound. Moreover, recent reports have demonstrated that naive CD8+ T cells undergo programmed differentiation, following a brief encounter with Ag, to generate ultimately a pool of memory T cells that can survive and maintain their rapid-response mode in the absence of Ag (23, 37, 38).

The study of the differentiation of Ag-primed cell from acute through to chronic phases enables the clarification of memory and effector subsets in humans. In 1997, Hamann et al. (24) had described the existence of several subsets of CD8+ T cells exhibiting distinct functional activity. However, it appears now that the allocation of terms such as memory (CD28+CD27+) and effector (CD28-CD27-) cells to these subsets is somewhat oversimplified and not appropriate. Indeed, in HIV-1 infection, the effector phase is marked by an increase in CD28-CD27+ CD8+ T cells, likely to be generated from a population of naive cells. These cells mature rapidly into cells at an intermediate stage of differentiation (CD28+CD27-). The memory phase shows an enrichment of CD28+CD27+CD8+ T cells mixed with much smaller numbers of CD28+CD27+ and CD28-CD27+ cells. In humans, the assignment of different functional subsets of Ag-primed cells into memory and effector subsets is not so simple. For instance, a recent report by Hislop et al. (39) showed that neither cytotoxicity nor cytokine production in EBV-specific CD8+ T cells is restricted to CD28+ or CD27- fractions.

In recent studies, we and others suggested that a paucity of IFN-γ-producing CD8+ T cells specific for HIV-1, in comparison with the levels of CMV-specific help, may be a cause for incomplete or impaired maturation of the CD8+ T cells (40). However, our present data show that HIV-specific CD8+ T cells do not appear to differentiate any further even in individuals exhibiting unusually high HIV-specific CD4+ T cell responses in primary infection. This suggests that CD8+ T cell maturation may be independent of the numbers of specific CD4+ T cells, in keeping with recent observations (23, 37). It is still open to debate whether HIV-specific CD8+ T cells are functionally deficient or whether the phenotype of the CD8+ T cells is an appropriate host response for this particular infection, despite the low perforin levels (41). In favor of the second hypothesis is the observation that during primary infection, when virological control appears to be achieved for a time, the cell phenotype does not differ significantly from that observed during chronic infection. The numbers of HIV-specific IFN-γ-producing CD4+ T cells are generally quite low during primary infection, and their numbers do not seem dependent on the stage of infection. This may conceivably be a normal response to HIV-1 infection, but it seems more likely that these cells are lost .

Ki67, CD27, CD28, and perforin is shown in CD8 T cells during viral infection is limited, and that the majority of activated T cells in the mouse have shown that bystander activation triggered – acute HIV-1 infection, it seems unlikely that 80% of them showing active proliferation, a similar proportion to that observed in the HIV-specific CD8+ T cell populations. Recent studies in the mouse have shown that bystander activation triggered during viral infection is limited, and that the majority of activated CD8+ T cells are virus specific (8, 9). However, in the case of acute HIV-1 infection, it seems unlikely that 80–90% of the CD8+ T cells are HIV specific (although we have not ruled this out). An important part of the general activation of CD8+ T cells may be a consequence of bystander activation (42), but it is also feasible that this could also be due to immune responses to other pathogens and viruses, either reactivated or appearing in the context of HIV-induced immunosuppression. The differences in the distribution of differentiation subsets between HIV-specific CD8+ T cells and the whole CD8+ T cell population are particularly striking for the CD28+CD27- subset. We know that the majority of CMV-specific CD8+ T cells occur in this subset. These are therefore one potential example that could account for the expanded CD28+CD27- subset in the general CD8+ T cell population, but other opportunistic infections could also be activated, particularly in the context of HIV-induced immunosuppression, and thus lead to changes in the phenotypic distribution of the whole CD8+ T cell population.

In this study, we have provided a detailed analysis of the dynamics and phenotype of the CD8+ T cell response during acute and chronic HIV-1 infection and its relationship to the number of specific CD4+ T cells. This is the first such analysis in the context of a human infection, and shows close parallels with the findings previously obtained in mouse models. Our observations have important implications for the understanding of effector and memory responses in human infections in general, and HIV-1 in particular.

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References


CORRECTIONS


The authors found in Figure 5 that wild-type EGFP-CIITA had been used inadvertently for immunofluorescence localization instead of the L469P mutant. They found the bona fide CIITA-L469P is localized predominantly in the cytoplasm; however, it also transits through the nucleus since it shows strong nuclear accumulation after leptomycin treatment. Residual nuclear localization of CIITA-L469P is also found by biochemical analysis of the patient fibroblasts subcellular fractions and is supported by the residual transactivation potential of CIITA-L469P. The main findings and conclusions of the paper remain unchanged.


Phenotype CD28−CD27− was not observed in the original experiment and should be changed to CD28−CD27− throughout the entire paper to match the reality and data initially presented.