CD8+ T Cells Specific for EBV, Cytomegalovirus, and Influenza Virus Are Activated during Primary HIV Infection

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Primary viral infections, including primary HIV infection, trigger intense activation of the immune system, with marked expansion of CD8⁺ CD8⁻ T cells. Whether this expansion involves only viral-specific cells or includes a degree of bystander activation remains a matter of debate. We therefore examined the activation status of EBV-, CMV-, and influenza virus (FLU)-specific CD8⁺ T cells during primary HIV infection, in comparison to HIV-specific CD8⁺ T cells. The activation markers CD38 and HLA-DR were strongly expressed on HIV-specific CD8⁺ T cells. Surprisingly, CD38 expression was also up-regulated on CD8⁺ T cells specific for other viruses, albeit to a lesser extent. Activation marker expression returned to normal or near-normal values after 1 year of highly active antiretroviral therapy. HIV viral load correlated with CD38 expression on HIV-specific CD8⁺ T cells but also on EBV-, CMV-, and FLU-specific CD8⁺ T cells. In primary HIV infection, EBV-specific CD8⁺ T cells also showed increased Ki67 expression and decreased Bcl-2 expression, compared with values observed in HIV-seronegative control subjects. These results show that bystander activation occurs during primary HIV infection, even though HIV-specific CD8⁺ T cells express the highest level of activation. The role of this bystander activation in lymphocyte homeostasis and HIV pathogenesis remains to be determined. The Journal of Immunology, 2004, 173: 2410–2418.

However, two points must be considered. First, even though most activated cells were virus specific, the latter do not account for all activated cells. Second, several groups, including our own, have reported that HIV-specific CD8⁺ T cells usually represent <10% of all CD8⁺ T cells, contrasting with much higher numbers of activated CD8⁺ T cells (8, 19–21). These observations point to a degree of bystander activation.

To determine the degree of bystander activation during primary HIV infection, we examined the expression level of the activation markers CD38 and HLA-DR on CD8⁺ T cells specific for EBV, CMV, and influenza virus (FLU). HIV-specific CD8⁺ T cells were analyzed in parallel, both during acute HIV infection and after 1 year of effective highly active antiretroviral therapy (HAART). To detect a possible impact of bystander activation on the differentiation, function, and proliferation of virus-specific CD8⁺ T cells, we also examined the expression of several markers. Maturation stage was assessed by measuring CCR7, CD28, CD27, and CD45R0 expression; apoptotic and proliferative status were examined in terms of Bcl-2 and Ki67 expression, respectively; and cytotoxic potential was assessed by measuring intracellular perforin content.

Materials and Methods

Study population

We studied 23 subjects with primary HIV-1 infection from the French multicenter PRIMO cohort (Agence Nationale de Recherche sur le Syndrôme d’Immuno-Déficience Acquise EP08). Primary infection was defined by HIV RNA positivity and a negative or emerging Ab response shortly after a recent high-risk HIV exposure event. After providing informed consent, subjects were included in the study (month 0) and were prescribed HAART. They comprised 21 men and 2 women, with a median age of 37 years. The 23 subjects were studied a median of 28 days after symptom onset, and 11 (45%) of the subjects were evaluated before seroconversion. The median baseline plasma HIV RNA level (measured with the Amplicor HIV Monitor assay, version 1.0; Roche Diagnostic Systems,
somerville NJ was 4.9 log copies/ml (interquartile range IQR), 4.5-5.7 log copies/ml). The median CD4+ T cell count was 585 cells/μl (IQR, 477–765 cells/μl). Twelve HIV-1-seronegative individuals were studied as controls.

Cells and peptides
PBMC were isolated by density gradient centrifugation (Ficoll-Paque; Pharmacia, Peapack, NJ). HLA genotyping was performed by ACTGene (Evry, France). A set of 88 peptides corresponding to described optimal HIV-CTL epitopes was used (National Institutes of Health HIV Molecular Immunology Database: http://www.hiv.lanl.gov/content/immunology/index.html). Peptides were synthesized by NeoSystem (Strasbourg, France). Lyophilized peptides were diluted to 1 mg/ml in H2O containing 10% DMSO, then aliquoted and stored at –20°C. Peptides were used at a final concentration of 1 μg/ml.

CD8+ ELISPOT assay
IFN-γ secretion by virus-specific CD8+ T cells was quantified with the ELISPOT assay as described elsewhere (22). Briefly, 96-well nitrocellulose plates were coated with 1 μg/ml mouse monoclonal anti-human IFN-γ capture Ab. PBMC were plated in duplicate at 10^5 cells/well. Appropriate stimuli were added, and the plates were incubated for 24 h at ±37°C with 5% CO2. Wells were then washed and filled with 100 μl of biotinylated monoclonal mouse anti-human IFN-γ and finally with alkaline phosphatase-labeled extravidin. Spots were developed by adding a chromogenic alkaline phosphatase-conjugated substrate. IFN-γ spot-forming cells (SFCs) were counted with a KS-ELISPOT system (Carl Zeiss Vision, y, 96-well nitrocellulose plate). IFN-γ levels were determined by ELISA (Maxisorp, Nunc, Roskilde, Denmark). IFN-γ ELISPOT assays were performed in duplicates.

Frequency of virus-specific CD8+ T cells
The frequency of virus-specific CD8+ T cells was estimated by tetramer staining and IFN-γ ELISPOT assay.

HIV-specific CD8+ T cells were detected in 21 of the 23 infected subjects (91%) by ELISPOT assay with a wide range of appropriate synthetic peptides derived from the HIV-1 Env, Gag, Pol, and Nef proteins. On average, HIV-specific CD8+ T cells represented 0.73 ± 0.96% of circulating CD8+ T cells (maximum, 4.47%). The number of recognized peptides ranged from 0 to 9 (median, 3).

Tetramer staining was performed using seven MHC-peptide complexes. HIV-specific tetramer-stained CD8+ T cells were found in 15 of the 23 infected subjects (mean, 1.56 ± 2.91%; maximum, 12.50%; Table I).

Thus, except in subject H10, HIV-specific CD8+ T cells represented <5% of all CD8+ T cells in all of the subjects tested.

Tetramer staining was also used to examine two EBV specificities, one CMV specificity and one FLU specificity. The mean frequencies of these heterologous virus-specific T cells were also low (Table II). Similar low proportions of EBV- and FLU-specific CD8+ T cells were present in the HIV-infected subjects. A moderate but nonsignificant increase in the frequency of CMV-specific CD8+ T cells was also found in the HIV-infected subjects (2.3% compared with 0.23% in controls).

Activation of CD8+ T cells specific for HIV and other viruses
The majority of HIV-specific CD8+ T cells were highly activated as shown in Fig. 1B, 83 ± 17% of these cells expressed CD38+ (range, 30–99%). This high level of activation was confirmed by the detection of HLA-DR expression on 65 ± 11% of HIV-specific CD8+ T cells (Table II).

We then examined CD38 expression on CD8+ T cells specific for EBV, CMV, and FLU. Surprisingly, CD38-expressing CD8+ T cells specific for each of the three heterologous viruses were more frequent in the subjects with primary HIV infection than in the HIV-seronegative controls (EBV: 45 ± 24% vs 12 ± 8%, respectively, p < 0.0001; CMV: 44 ± 33 vs 8% ± 2%, p = 0.0348; FLU: 11 ± 4% vs 3 ± 3%, p = 0.0007). Like HIV-specific CD8+ T cells, the increase in the number of these CD38-expressing cells was further amplified as a result of the expansion of the entire CD8+ T cell population (data not shown).

Analysis of HLA-DR expression confirmed the activated status of heterologous virus-specific CD8+ T cells (Table II). The percentage of HLA-DR on CMV-specific CD8+ T cells increased from 12 ± 3% in the HIV-seronegative controls to 26 ± 15% in the subjects with primary HIV infection (p = 0.04). A similar trend was observed for EBV-specific CD8+ T cells: 28 ± 18% and 45 ± 25% in controls and subjects with primary HIV infection, respectively (p = 0.07).

Results
Magnitude of CD8+ T cell activation
As well-documented in primary HIV infection, we observed marked CD8+ T cell expansion. The median CD8+ T cell count was 1197 cells/μl (IQR, 789-1759 cells/μl) compared with 615 cells/μl (IQR, 558–658 cells/μl) in HIV-seronegative controls. CD38+ cells represented 8 ± 3% of the CD8+ T cell compartment in controls and 53 ± 22% in the subjects with primary HIV infection (p < 0.0001; Fig. 1A). Thus, the expansion of the CD8 compartment was mostly due to an increase in cells expressing the CD38 activation marker. The number of CD38+CD8+ T cells increased from 48 ± 20/μl in control subjects to 715 ± 530 in subjects with primary HIV infection.

The activation status of CD8+ T cells was confirmed by an increase in the proportion of CD8+ T cells expressing HLA-DR (8 ± 4% and 41 ± 19% in controls and subjects with primary HIV infection, respectively; p < 0.0001).
These results show that heterologous virus-specific CD8+ T cells are also activated during primary HIV infection, albeit to a lesser extent than HIV-specific CD8+ T cells.

**Relationship between HIV viral load and virus-specific CD8+ T cell activation**

Since the percentage of CD38+CD8+ T cells has been linked to HIV viral load, we measured CD38 expression after 1 year of successful HAART. The percentage of CD38-expressing cells among total CD8+ T cells fell from 53 ± 22% before treatment to 17 ± 13% after 1 year of HAART, a value similar to that observed in controls (p < 0.0001; Fig. 1A).

A marked decrease in the proportion of CD38-expressing cells was also observed among virus-specific CD8+ T cells. The proportion of CD38+ HIV-specific CD8+ T cells fell markedly, from 83 ± 17% at baseline to 10 ± 8% after 1 year of successful HAART (p < 0.0001). Interestingly, the proportions of heterologous virus-specific CD8+ T cells showed similar changes: CD38+ EBV-specific CD8+ T cells fell to 13 ± 8% (p < 0.0001 vs baseline), CD38+ CMV-specific CD8+ T cells fell to 12 ± 5% (p = 0.02 vs baseline), and CD38+ FLU-specific CD8+ T cells fell to 3 ± 3% (p = 0.002 vs baseline). These values after 1 year of HAART were not significantly different from values in healthy controls (Fig. 1B).

The link between HIV viral load and CD38 expression on virus-specific CD8+ T cells was further assessed by examining the relationship between viral load and CD38 expression (Fig. 2). As described elsewhere, the percentage of CD38-expressing cells among total CD8+ T cells correlated strongly with viral load (r = 0.70, p < 0.0001; Fig. 2A) (10, 11). A strong correlation was also found between viral load and the frequency of CD38+ HIV-specific CD8+ T cells (r = 0.76, p < 0.0001; Fig. 2B). Interestingly, this was also the case of CD38+CD8+ EBV-specific T cells (r = 0.70, p = 0.0003; Fig. 2C), CD38+CD8+ CMV-specific T cells (r = 0.63, p = 0.008), and CD38+CD8+ FLU-specific T cells (r = 0.84, p = 0.04).

**Virus-specific differentiation and proliferation of CD8+ T cells**

To further determine whether activation of HIV- and heterologous virus-specific CD8+ T cells was associated with proliferation, maturation, and/or functional changes, we examined the expression of surface and intracellular markers (Table II).

Proliferation was estimated by measuring the expression of Ki67, a nuclear marker associated with cycling cells. Ki67 Ag was more frequently expressed on CD8+ T cells from subjects with primary HIV infection than in controls (27 ± 22% vs 2 ± 1%, respectively, p < 0.0001; Fig. 3A). As expected, HIV-specific CD8+ T cells showed high Ki67 expression (51 ± 27%; Fig. 3B). This percentage was lower than that of CD38+ T cells, suggesting that not all activated cells proliferated. However, a strong correlation was found between CD38 and Ki67 expression (R = 0.73, p = 0.0003; Fig. 3C), suggesting that a high activation level may drive proliferation. The increase in Ki67 expression was much more moderate on heterologous virus-specific CD8+ T cells. The percentage of Ki67+ cells among EBV-specific CD8+ T cells was significantly higher in the subjects with primary HIV infection (13 ± 12%) than in the controls (4 ± 2%, p = 0.047) but no differences were observed for CMV- and FLU-specific CD8+ T cells (Fig. 3B). However, a significant relationship was also observed between the levels of CD38 and Ki67 expression for these heterologous virus-specific CD8+ T cells (r = 0.56, p = 0.04).

We then focused on the differentiation markers CCR7, CD28, CD27, and CD45RO (Table II). HIV-specific CD8+ T cells were predominantly CD45RO+, CCR7+, and CD27+ in the subjects with primary HIV infection, as reported by Appay et al. (23, 24). Most EBV-specific CD8+ T cells also shared this phenotype. In...
Table 1. Baseline characteristics of HIV-infected subjects, CD38 expression percentage in their total CD8\(^+\) T cells, and their HIV-specific CD8\(^+\) T cells responses analysed by tetrameric staining and IFN-\(\gamma\) ELISPOT

<table>
<thead>
<tr>
<th>Subject(^a)</th>
<th>HIV Viral Load(^a)</th>
<th>CD4(^+) T Cell Count</th>
<th>CD8(^+) T Cell Count</th>
<th>CD38 % in Total CD8(^+) T Cells</th>
<th>Total HIV-specific SFC (% ELISPOT(^a))</th>
<th>Single HIV-specific CD8(^+) T Cells</th>
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\(^a\) Only HIV-infected subjects whose PBMC contained HIV tetramer-positive CD8\(^+\) T cells are shown (n = 15).
\(^b\) Log\(_{10}\) copies HIV-RNA/ml.
\(^c\) Sum of HIV peptide-specific IFN-\(\gamma\)-SFCs (expressed as percent and reported to CD8\(^+\) T cells).
\(^d\) Number of HIV peptide recognized in ELISPOT assay.
\(^e\) Expressed as percent tetramer-positive cells among CD3\(^+\)CD8\(^+\) T cells.

The subjects with primary HIV infection, EBV-specific CD8\(^+\) T cells expressed slightly, although not significant, lower levels of CCR7 and CD28 than the controls, suggesting a trend toward a later differentiation stage. However, measurement of CD27 expression showed that these cells did not reach the late Ag-experienced stage. Conversely, CMV-specific cells were more differentiated, a significant proportion of them expressing a CD27\^-CD45RA\^- phenotype, as illustrated in Fig. 4, a phenotype characteristic of terminally differentiated effector cells. By contrast, FLU-specific CD8\(^+\) T cells expressed high levels of CCR7 and CD28 markers, characteristic of an early differentiation stage. No difference in the differentiation stage of these cells was found between the controls and the subjects with primary HIV infection.

We also analyzed the cytotoxic potential of these specific T cells by measuring intracellular perforin expression (Fig. 4). Cells may express high or low levels of perforin, and we defined only perforin\(\text{high}\) cells as positive. As previously reported, a very small proportion of HIV-specific CD8\(^+\) T cells expressed high levels of perforin (23). This was also the case of EBV- and flu-specific CD8\(^+\) T cells in both the controls and the subjects with primary HIV infection. This was expected, as cells expressing CD27 do not usually express high levels of perforin. Accordingly, CMV-specific CD8\(^+\) T cells, which contain significant proportions of CD27\^- cells, expressed substantial levels of perforin.

Finally, we examined the expression level of the anti-apoptotic factor Bcl-2. Decreased expression was observed in the total CD8\(^+\) T cell population of subjects with primary HIV infection (mean fluorescence intensity (MFI), 7.1 \(\pm\) 3.6 vs 15.2 \(\pm\) 4.4 in controls, \(p = 0.0002\)). HIV-specific CD8\(^+\) T cells displayed very low Bcl-2 expression (MFI, 3.7 \(\pm\) 2.6), indicating a proapoptotic state associated with strong activation. Surprisingly, as illustrated in Fig. 5, EBV-specific CD8\(^+\) T cells from the subjects with primary HIV infection exhibited comparable low Bcl-2 expression (MFI, 5.7 \(\pm\) 4.0 vs 11.6 \(\pm\) 4.7 in controls, \(p = 0.03\)).

**Discussion**

Marked immune activation and CD8\(^+\) T cell expansion are classically observed during the acute phase of viral infections in both humans and animal models (1–7). These phenomena were initially attributed to nonspecific mechanisms and referred to as bystander activation (7, 14–16). More recent studies have challenged this view, favoring direct virus-specific activation (5, 6, 17, 18). This discrepancy resulted from differences in the quantitation of virus-specific cells (25). Despite significant technical improvement, the HIV-specific CD8\(^+\) T cell response remains hardly quantifiable with precision. In this study, we provide evidence that, during primary HIV infection, CD8\(^+\) T cells specific for the infecting virus accounted for the majority of activated cells, but not for all of them: between one-third and one-half of CD8\(^+\) T cells were marked, activation of heterologous virus-specific cells (25).
virus-specific CD8⁺ T cells observed here are consistent with the results of another study showing that cycling cells were about four times less frequent among activated bystander cells than among cells specific for the infecting virus (14).

Several mechanisms may be involved in this bystander activation. One involves multiple virus-specific activation. A variable degree of immune deficiency is frequently observed during acute viral infections (26) and this could favor replication of viruses such as EBV and CMV. However, EBV replication is not increased during acute HIV infection, in contrast to chronic HIV infection (27). Moreover, there was no evidence of acute CMV infection or reactivation in the patients studied (no IgM response and plasma CMV viral load <250 copies/ml by real-time quantitative RT-PCR).

Finally, the increase in CD38 expression on FLU-specific CD8⁺ T cells could clearly not be explained by influenza virus reactivation.

The second mechanism involves cross-reactivity (15, 16). This may require antigenic similarities between HIV and other viral epitopes. Cross-reactivity leading to activation of heterologous virus-specific CD8⁺ T cells has been documented in mice. This cross-reactivity could lead to marginal activation with or without proliferation depending on variable TCR-peptide affinities (5, 6, 28). Cross-reactivity between viral epitopes has been described in humans between FLU and other viruses such as HCV, EBV, rotavirus, and Dengue virus (29–31). In addition, cross-reactivity may be observed without amino acid sequence analogy, suggesting that T cells may be much more cross-reactive than usually thought (32). Indeed, a cross-reactivity between two immunodominant HLA-A*0201-restricted peptides (HIV-1 gag 77–85 and FLU mat 58–66) has been recently reported (33). Our results cannot thus exclude this hypothesis.

The third mechanism involves cytokines. Tough et al. (7) clearly demonstrated that, during viral infection, IFN-α induces marked proliferation and activation of CD44high memory CD8⁺ T cells, as assessed by BrdU integration and expression of Ly6-C, the murine counterpart of CD38. Subsequent in vivo studies demonstrated that IL-15 could also be responsible for bystander activation (34, 35). In vitro studies have also shown that IL-15 can induce the proliferation and enhance the cytotoxic activity of memory cells (36–38). IL-12 and IFN-γ have also been implicated in various models (39, 40). These cytokines may be produced in cascade events. Indeed, IL-12 can induce IFN-γ production, while IL-15 can be induced by IFN-α, inefficiently by IL-12, and not at all by IFN-γ (39, 40). This suggests that IL-12/IFN-γ and IFN-α/IL-15 could represent independent pathways. These cytokines are classically produced during primary viral infections and could act on heterologous virus-specific cells in the vicinity of cells directly activated by the infecting virus. However, several studies suggest that the cytokine burst might be smaller during primary HIV infection than in other acute infections (41–44). IL-12 and IFN-α have not been precisely quantified during primary HIV infection, but these cytokines are produced by myeloid and plasmacytoid dendritic cells, respectively, whose numbers and functions are altered during primary HIV infection (45–48). Data on IL-15 are contradictory, with reports of increased or decreased serum levels, increased or decreased cellular production, and increased levels of IL-15Ra mRNA (47–52).

Interestingly, we observed a relationship between HIV viral load and the percentage of CD38⁺ cells on CD8⁺ T cells (9, 10). This relationship had previously only been observed with total CD8⁺ T cells. It must be underlined, however, that although viral load and activation correlate with each other, they act independently on the course of HIV infection, suggesting a direct deleterious effect of activation (11, 12).

Ag-driven stimulation through the TCR usually leads to activation, proliferation, and differentiation of specific T cells. We found a significant number of cycling Ki67⁺ HIV-specific CD8⁺ T cells in subjects with primary HIV infection, as previously reported (24). Most activated T cells will then die by apoptosis. Indeed, most HIV-specific CD8⁺ T cells express very low levels of Bcl-2, suggesting they are prone to apoptosis, as previously shown in primary HIV infection (23, 24, 53). Finally, HIV-specific CD8⁺ T cells remained in an intermediate state of differentiation, most cells lacking CCR7 and CD28 but remaining CD27⁺ and expressing low levels of perforin (23). Whether this specific pattern implies a defect in maturation or is rather the appropriate response to this particular virus remains a matter of debate (23, 54).

The bystander activation of heterologous virus-specific CD8⁺ T cells was not limited to increased CD38 and HLA-DR expression. Indeed, we observed an increase in Ki67 expression on EBV-specific CD8⁺ T cells during primary HIV infection. The relationship between Ki67 and CD38 suggests that activation may directly drive some activated cells to proliferate. These cells will then contribute to the overall increase of CD8⁺ T cells observed in primary HIV infection, in addition to specific-Ag driven clonal expansions.
This is in line with a report that increased Ki67 expression during primary HIV infection involves all TCR-vβ families to a similar extent (55). The differences observed in the levels of CD38 and Ki67 expression among different viral-specific CD8+ T cells as well as the magnitude of changes between the controls and the subjects with primary HIV infection may be due to different thresholds required for activation, proliferation, and function (6, 15). IL-15 in particular has been shown to activate cells without inducing their proliferation (36).

No major change in differentiation pattern of viral-specific cells has been observed between the controls and the subjects with primary HIV infection except a trend toward the intermediate CD28+CD27+ phenotype for EBV-specific CD8+ T cells. Cytokines, and particularly IL-15, can stimulate naive and memory CD8+ T cells and induce a moderate shift in their differentiation status, without loss of CD27 (56). A sustained activation, as observed in untreated chronic HIV infection, may lead to a further step in differentiation (57).

Finally, the low Bcl-2 expression observed on EBV-specific CD8+ T cells suggests apoptosis of these cells and is in line with the higher level of activation and Ki67 expression observed on these cells compared with CMV- and flu-specific cells. It is noteworthy that bystander apoptosis has been reported in TCR-transgenic mice. Other cytokines might be involved, as IL-15 is known for up-regulating Bcl-2 and for its antiapoptotic properties (52, 58). This point requires further studies focusing on apoptosis of specific CD8+ T cells. The pathophysiological relevance of bystander activation is unclear, but is probably of no major biological consequence (18).
Bystander activation of T cells specific for EBV, CMV, and FLU probably has few, if any, implications. Indeed, no clinical manifestations related to these viruses are observed during the first years of HIV infection, and CMV infection and EBV-driven lymphomas are only observed in the advanced stages of HIV infection when CD4⁺ T cell depletion is severe. In addition, no reduction in the pool of EBV-specific memory cells has been observed after HIV infection, suggesting, once again, that apoptosis of these cells is marginal or that the capacity to regenerate such cells is not compromised (59).

Bystander activation may play a role in lymphocyte homeostasis by influencing the quantity and quality of the memory T cell pool. Indeed, it may contribute to maintaining the pool of memory cells in the absence of stimulation by the cognate Ag (7, 15, 16, 31). Conversely, bystander apoptosis may be necessary for the expansion of new memory cells (60, 61). In contrast, the general immune activation associated with HIV infection is clearly pathogenic. Even in the nonpathogenic model of sooty mangabey infection by SIVsmm, a negative correlation was recently reported between CD8⁺ T cell activation and the circulating CD4⁺ T cell count (13). Aberrant immune activation following HIV infection could thus contribute directly to pathogenesis.

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References


