CD8+ T Cells Are Activated in an Antigen-Independent Manner in HIV-Infected Individuals

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CD8+ T Cells Are Activated in an Antigen-Independent Manner in HIV-Infected Individuals

Sonia Bastidas,* Frederik Graw,†,1 Miranda Z. Smith,*2 Herbert Kuster,‡ Huldrych F. Günthard,‡ and Annette Oxenius*

Hyperactivation of T cells, particularly of CD8+ T cells, is a hallmark of chronic HIV 1 (HIV-1) infection. Little is known about the antigenic specificities and the mechanisms by which HIV-1 causes activation of CD8+ T cells during chronic infection. We report that CD8+ T cells were activated during in vivo HIV-1 replication irrespective of their Ag specificity. Cytokines present during untreated HIV-1 infection, most prominently IL-15, triggered proliferation and expression of activation markers in CD8+ T cells, but not CD4+ T cells, in the absence of TCR stimulation. Moreover, LPS or HIV-1–activated dendritic cells (DCs) stimulated CD8+ T cells in an IL-15–dependent but Ag-independent manner, and IL-15 expression was highly increased in DCs isolated from viremic HIV-1 patients, suggesting that CD8+ T cells are activated by inflammatory cytokines in untreated HIV-1 patients independent of Ag specificity. This finding contrasts with CD4+ T cells whose in vivo activation seems biased toward specificities for persistent Ags. These observations explain the higher abundance of activated CD8+ T cells compared with CD4+ T cells in untreated HIV-1 infection. The Journal of Immunology, 2014, 192: 1732–1744.

Continuous loss of CD4+ T cells and hyperactivation of T cells, particularly CD8+ T cells, are hallmarks of chronic HIV-1 infection (1). In contrast to many acute and resolved infections where activation of adaptive immune cells is transient and CD38 expression on Ag-specific CD8+ T cells decreases after resolution of infection, a continuous and even gradual increase of activation marker expression (including CD38 and HLA-DR) is observed on virus-specific and on nonspecific CD8+ T cells throughout the natural course of HIV-1 infection (1, 2). Furthermore, the degree of immune activation defined by the expression of these markers on CD8+ T cells has prognostic significance (1-3). Usually only 6–8% of all circulating CD8+ T cells are HIV-1 specific (4, 5), whereas the level of CD8+ T cells exhibiting activated phenotypes (CD38+ HLA-DR+) can be as high as 60% (6). The mechanisms underlying this increased activation of non–HIV-1–specific T cells are still poorly defined (7, 8).

It is believed that most of these cells are activated by nonspecific mechanisms, including cross-reactivity and cytokine-driven activation (7, 9), a phenomenon referred to as “bystander activation.” Furthermore, systemic translocation of gut microbial products including LPS, owing to increased permeability of the gut epithelium, is suggested to contribute to general immune activation (10). However, the mechanisms of how systemic exposure to bacterial pathogen-associated molecular patterns (PAMPs) translates into activation of CD8+ T cells remain undefined. Because there is currently little known about the antigenic specificities of bystander-activated CD8+ T cells and the mechanisms by which HIV-1 causes activation of CD8+ T cells during chronic infection, we investigated these questions in this study in a cohort of HIV-1–infected individuals undergoing cessation of antiretroviral therapy (ART) and hence experiencing HIV-1 recrudescence. In addition to a control cohort, which continued to received ART, we assessed whether viral rebound was associated with activation of non–HIV-1–specific CD8+ T cells and whether these exhibited particular Ag specificities.

Based on our data, we propose that CD8+ T cells are activated during HIV-1 replication irrespective of their Ag specificity. We observed that CD8+ T cells are more susceptible to cytokine stimulation, since CD8+ T cells, in contrast to CD4+ T cells, were strongly activated (assessed by coexpression of CD38 and HLA-DR and proliferation) by cytokines present during untreated HIV-1 infection, such as IL-15 and IL-12 (11). A different susceptibility toward cytokine activation was also apparent between different T cell subsets, with memory CD8+ T cells being activated more efficiently than naive CD8+ T cells by in vitro cytokine stimulation. We provide evidence that LPS- or HIV-1–activated monocyte-derived dendritic cells (MDDCs) can stimulate CD8+ T cells through IL-15 in an Ag-independent manner, and we demonstrate that myeloid dendritic cells (mDCs) isolated from HIV-1–infected patients with active HIV-1 replication produce higher amounts of IL-15 compared with dendritic cells (DCs).
isolated from the same patients when successfully treated with ART. We propose that these IL-15–expressing mDCs contribute directly to CD8+ T cell activation in vivo, as shown for IL-15–producing MDDCs in vitro.

These findings strongly suggest that, in the context of HIV-1 replication, memory CD8+ T cells are efficiently activated by inflammatory cytokines and independently of Ag specificity. This finding is in contrast to CD4+ T cells whose in vivo activation during HIV-1 replication seems biased toward specificities for persistent Ags (12, 13). These observations might help to explain the higher abundance of activated CD8+ T cells compared with CD4+ T cells in untreated HIV-1 infection.

**Materials and Methods**

**Ethics statement**

Approval was obtained from the University Hospital Zurich Ethics Committee, and written informed consent was received from all subjects.

**Study population**

We recruited a study cohort of 41 HIV-1–positive individuals who initiated ART during the acute phase of infection (Table I). These patients participated in the Zurich Primary HIV-1 Infection Study (ZPHI), a prospective long-term observational single study. In this protocol, patients are offered early ART. After 1 y of suppressed viremia (<<400 HIV-1 RNA copies/ml plasma), patients can choose to interrupt ART (14-18). Study details are listed under http://www.clinicaltrials.gov; ID NCT00537966. All individuals were screened for CMV and EBV seropositivity prior to enrolment. From the recruited patients, 25 patients interrupted ART (rebound cohort), and 16 patients continuously received ART (control cohort).

For the analysis of dendritic cells, 10 study individuals were recruited from a previously described study cohort of HIV-1–infected individuals also participating in the ZPHI study who initiated ART during the acute phase of infection and then chose to undergo treatment interruption (12). Details of the patients of this study are listed in Table IV. For analyses we used cryopreserved PBMC samples from one time point during ART and one time point off ART.

**Cell preparations**

For CD8+ T cell ELISPOT analysis, cryopreserved PBMCs were thawed and rested overnight and depleted of CD4+ cells by magnetic separation (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer’s protocol.

For the generation of MDDCs, fresh PBMCs were isolated from whole blood using Ficoll-Hypaque density gradient centrifugation; monocytes were isolated by magnetic separation using CD14-microbeads (Miltenyi Biotech) according to manufacturer’s protocol. Cells were adjusted to 5 × 107/ml in RF-10 (RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM t-glutamine [all GE Healthcare Europe, Glattbrugg, Switzerland]) and cultured for 4–6 d in the presence of IL-4 (1000 U/ml) and GM-CSF (1000 U/ml) (19).

**IFN-γ ELISPOT**

CD8+ T Cells were cocultured with 10% fresh autologous MDDCs. Cells were stimulated with PHA (5 μg/ml per peptide) (21), with overlapping peptides spanning the CMV pp65 and IE1 proteins (20). The number of spot-forming cells (SFCs) per well was calculated by subtracting the negative control values from the specific responses. Positive responses were defined as three SDCs above background. Spots were quantified using and automated spot counter (AID, Strassberg, Germany). Unless stated differently, Ag-specific CD8+ T cell responses are shown as SFCs per 1 × 10^6 CD4+ T cell–depleted PBMCs.

**In vitro stimulation assays**

Stimulations were performed using 2 × 10^6 purified T cells in 96-well U-bottom plates in a final volume of 200 μl CD4+ and CD8+ T cells from healthy donors were purified from freshly isolated or cryopreserved PBMCs by MACS (Miltenyi) and labeled with 5 μM CFSE (Invitrogen). For cytokine stimulation assays, T cells were stimulated with IL-15 (50 ng/ml; R&D Systems), IL-7 (50 ng/ml; R&D Systems), IL-12 (3 ng/ml; Insight Biotechnology), IL-21 (25 ng/ml; Life Technologies), and IL-2 (300 U/ml; a gift from the National Cancer Institute, Fisher BioServices) and cultured for 3–d following analysis by flow cytometry. For LPS activation, MDDCs were stimulated overnight with 0.5 μg/ml LPS (Sigma-Aldrich, Buchs, Switzerland). For IFN-γ activation, MDDCs were stimulated for 24 h with AT-2 inactivated HIV-1 particles (50 μg/ml p24) or matching concentrations of control microvesicles (23). For IL-15 blocking assays, LPS or IFN-γ–activated MDDCs were incubated for 3 h with anti–IL-15 mAb (5 μg/ml; R&D Systems) before the addition of purified CD4+ and CD8+ T cells. MDDCs (2 × 10^5 cells) were cultured in RPMI 1640 medium (GE Healthcare Europe) supplemented with 10% human serum with purified autologous CD4+ or CD8+ T cells for 3–d and analyzed by flow cytometry. For Ag stimulation assays, whole PBMCs were cultured for 6 h. For intracellular cytokine production, cells were cultured at 37°C, 5% CO2 for 2 h. Next, 10 μg/ml Brefeldin A was added, and cells were cultured for an additional 4 h at 37°C.

**Flow cytometry**

Cells were surface stained with anti-CD4 Am cyan (Becton Dickinson, Allschwil, Switzerland), anti-CD25 PE-Cy7 (eBioscience), anti-CD3 Pacific Blue, anti-CD8 PerCP, anti-CD38 PE, anti-HLA-DR APC, and anti–CD69 FITC (all from BioLegend). For intracellular staining, cells were permeabilized with a solution containing 1% BD FACS Lyse and 0.25% Tween 20. Cells were stained intracellularly with anti–IFN-γ PE-Cy7 (Becton Dickinson), anti–IL-2 FITC and anti–TNF-α (BioLegend). Doublets were excluded using side- and forward-scatter height and width parameters. Fluorescence minus one controls were used to set gates for HLA-DR and CD38. For MDDC analyses, cells were stained with anti–HLA-DR-APC, anti–CD11c Pe-Cy7 (all BioLegend), and anti–IL-15–PE (R&D Systems). Samples were acquired on a three-laser, nine-parameter LSII Flow cytometer (Becton Dickinson), and analysis was performed using FlowJo (Tree Star, Ashland, OR).

**Cell sorting**

For T cell subset sorting, MACS-purified CD8+ and CD4+ T cells were surface stained with anti–CD3 Pacific Blue, anti–CD4–Am cyan, anti–CD62L APC, anti–CD8–PerCP, and anti–CD45RA FITC (all from BioLegend). Cells were subsequently sorted into naive T cells (CD45RA-, CD62L+), effector memory T cells (CD45RA-, CD62L), and central memory T cells (CD45RA+, CD62L+) with a FACSAria cell sorter (Becton Dickinson). Directly after sorting the cells were used for in vitro stimulation assays or for RNA isolation.

For DC sorting, cryopreserved PBMCs from HIV–1–infected patients were thawed on ice and immediately surface stained with anti lin–1–FITC, anti–CD34–FITC, anti–HLA-DR–APC-H7, anti–CD123–PerCP-Cy5.5 (all from Becton Dickinson), and anti–CD11c–PE-Cy7 (eBioscience) as described previously (12). Cells were subsequently sorted into plasmacytoid dendritic cells (pDCs; lin–1–CD34–, HLA-DR+, CD11c+CD123+) and mDCs (lin–1–CD34–, CD11c+, CD123+) with a FACSAria cell sorter (Becton Dickinson). Directly after sorting the cells were used for in vitro stimulation assays or for RNA isolation.

**Real-time quantitative PCR**

Total RNA was isolated from MACS-purified mDCs, pDCs, and T cell subsets using Trizol reagent (Invitrogen, Basel, Switzerland) and cDNA was generated using M-MLV Reverse Transcriptase RNase, H Minus (Promega, Dübendorf, Switzerland). Real-time PCR was performed using a Rotorgene 3000 machine (Corbett Research, Eight Miles Plains, Australia) to measure SYBR green (Sensi Mix; R&D Systems) incorporation. The following primer sets were used: IL-12p35: 5‘-CTCTTGACACCTTCAG-TTG-3‘ and 5‘-GGTGAAGGACATGGGACATTT-3‘; IL-12p40: 5‘-GCAGGGCCTGGCCCTCACTCAAGAATA-3‘ and 5‘-CTCTCCAGCAGAGCTGGAAATC-3‘; IL-15Rβ: 5‘-CTCCTGAGAGATGGCACCCTGGT-3‘ and 5‘-CTCCTCAGCA-CTTCTAGTGG-3‘; IL-15: 5‘-CTCGTGTGATGGCCATTTACCACTTTGATCTTTTCGA-3‘ and 5‘-TCTCCAGTCTCTCCTCATTCTTCT-3‘; GADPH: 5‘-GGACGGG-
GAGCCAAAAGGG-3' and 5'-TGGCAAGCCCCAGCTCAAAG-3' (25). The amount of mRNA was normalized to GADPH RNA levels for each sample. The fold induction was calculated according to the relative expression in unstimulated controls.

**Statistical analysis**

Spearman correlation analysis with a two-tailed test of significance was performed using SPSS software (SPSS). Results were analyzed and graphed using Prism version 5.04 (GraphPad Software). Nonparametric Wilcoxon signed-rank tests for paired or unpaired data were used to calculate significance. The p values were adjusted for multiple comparisons using the Bonferroni-correction method. Dynamical correlation analysis was performed as described elsewhere (26). This method quantifies the covariation of components of multivariate longitudinal data. It estimates a correlation for the trend rather than the magnitude of the measured parameters. Standard R Software was used for analysis; p < 0.05 was considered significant.

**Results**

**HIV-1 rebound drives expansion of CD8+ T cells irrespective of their Ag specificity**

CD4+ T cell depletion and chronic immune activation are major characteristics of HIV-1 infection; however, their causal relation is poorly defined. In previous studies, we have observed that during HIV-1 rebound (after interruption of ART), the dynamics of HIV-1–specific CD4+ T cells is highly correlated with the dynamics of CD4+ T cells specific for persistent Ags derived from various members of the Herpes virus family, whereas CD4+ T cell responses toward nonpersistent Ags were unaffected by HIV-1 replication (12). However, aberrant immune activation is not restricted to CD4+ T cells, and in fact the extent of CD8+ T cell replication (12). However, aberrant immune activation is not restricted to CD4+ T cells, and in fact the extent of CD8+ T cell activation is far more pronounced compared with CD4+ T cells. In addition, the level of chronic immune activation in CD8+ T cells is a better prognostic indicator of disease progression than the extent of CD4+ T cell activation (1). To investigate the effect of HIV-1 replication on T cell activation, with particular interest in the specificities of CD8+ T cells, we analyzed the in vivo dynamics of HIV-1–specific and non–HIV-1–specific CD8+ T cells (specific for CMV, EBV, influenza [Flu], and adenovirus) in a cohort of 26 patients undergoing interruption ART and thus experiencing viral rebound (patient details are summarized in Table I). For each patient, multiple samples before and after therapy interruption were analyzed.

HIV-1 recrudescence induced a dynamic response in the HIV-1–specific CD8+ T cell population. However, HIV-1 rebound also affected the dynamics of CD8+ T cell responses specific for non–HIV-1 Ags. Interestingly, within the selection of Ag specificities assessed, we did not find any bias toward certain Ags—that is, responses specific for persistent herpes virus–derived Ags (CMV, EBV) were comparably modulated by HIV-1 recrudescence as CD8+ T cells responses with specificity for nonpersistent Ags (influenza and adenovirus). Thus, all tested non–HIV-1–specific CD8+ T cell responses were highly correlated with the HIV-1–specific CD8+ T cell response. Fig. 1A and 1B and Supplemental Fig. 1 show scatterplots of all the individual data points. Because baseline responses (on ART) and responses after ART interruption differed significantly in magnitude between individuals, we refrained from marking data points from before and after therapy interruption in these compiled data graphs. However, to account for the interdependency of different time points originating from the same individual, we also analyzed the data by dynamical correlation (Table II). Indeed, the significance of the correlation between HIV-specific and non–HIV-specific CD8+ T cell responses was confirmed by the two independent statistical analyses: Spearman’s correlation (Rₛ; Fig. 1A, 1B) and dynamical correlation analysis (Rₒ; Table II). The more stringent dynamical correlation analysis (Rₒ) accounts for the fact that multiple data points originate from the same individual and are thus interrelated (26).

Importantly, the observed correlation between the dynamics of HIV-1–specific CD8+ T cell responses and CD8+ T cell responses specific for non–HIV-1 Ags was dependent on HIV-1 rebound, as no correlation was found in a matching control cohort that continuously received ART (Fig. 1C). Thus, HIV-1 recrudescence and an increase in plasma virus load and consequently Ag availability boosted HIV-1–specific CD8+ T cell responses, but at the same time existing CD8+ T cell responses with specificities for persisting or nonpersisting non–HIV-1 Ags were activated and underwent dynamic changes in their frequencies that were comparable to the HIV-1–specific CD8+ T cell response. These observations suggest non–HIV-1–specific CD8+ T cell activation, in contrast to non–HIV-1–specific CD4+ T cell activation (Supplemental Table I) (12, 13), seems to be induced after HIV-1 rebound in an Ag-independent manner.

**HIV-1 rebound drives increased activation marker expression on CD8+ T cells specific for different Ags**

To study directly the activation level of Ag-specific CD8+ T cells, whole PBMCs from HIV-1 infected donors were stimulated in vitro with the same group of Ags as shown in Fig. 1 (CMV, EBV, adenovirus, influenza). Ag-induced IFN-γ and TNF-α production was analyzed, as well as the HLA-DR and CD38 coexpression within the Ag specific CD8+ T cells (Fig. 2).

First, Ag-specific CD8+ T cell activation was analyzed in a patient from the cohort studied previously (Table I), and responses to each Ag were compared in samples before and after stopping ART (Fig. 2A). All tested Ags induced the production of IFN-γ and TNF-α.

**Table I. Characteristics of patient cohort**

<table>
<thead>
<tr>
<th>Study Group (n)</th>
<th>Age (Years)</th>
<th>Gender</th>
<th>HIV-Positive</th>
<th>Days</th>
<th>Maximum HIV (Copies × 10³/ml)</th>
<th>CD4 Count (Cells/ml)</th>
<th>CD8 Count (Cells/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (Range)</td>
<td>% Male</td>
<td>Median (Range)</td>
<td>Median (Range)</td>
<td>Before ART Stop</td>
<td>Before ART-Stop</td>
<td>Before ART-Stop</td>
</tr>
<tr>
<td>Stop-ART</td>
<td>25</td>
<td>38 (25–57)</td>
<td>92</td>
<td>&lt;6</td>
<td>536 (357–777)</td>
<td>341 (41–1007)</td>
<td>528 (3–33,150)</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>39 (32–61)</td>
<td>81</td>
<td>&lt;6</td>
<td>256 (151–3027)</td>
<td>442 (63–1238)</td>
<td>255 (17–33,000)</td>
</tr>
</tbody>
</table>

(Ranges of HIV-1 positive days, Maximum HIV, CD4 Count, and CD8 Count are provided.)

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TNF-α within CD8+ T cells, and overall there was an increase in cytokine-producing CD8+ T cells after HIV-1 viral rebound, indicating that HIV-1 replication induced the expansion of non–HIV-1–specific CD8+ T cells. Next, the level of activation (HLA-DR+ CD38+) on the Ag-specific cells was analyzed, and the overall increase in cytokine-producing CD8+ T cells was reflected by an increase of activation marker expression within Ag-specific CD8+ T cells, as significantly higher HLA-DR and CD38 coexpression was observed in Ag-specific CD8+ T cells in off-ART samples. These results corroborate the notion that HIV-1 rebound drives the activation of CD8+ T cells independent of Ag specificity.

Because these results were obtained from samples of one patient with HIV-1 infection, we decided to extend this analysis using a cross-sectional readout. Seventeen patients with chronic untreated HIV-1 infection (cohort details in Ref. 13) and 10 healthy, age-matched controls (details in Ref. 13) were involved. Whole PBMCs from HIV-1–infected patients and healthy donors were stimulated in vitro with the same Ags as in Fig. 1 (CMV, EBV, adenovirus). Ag-induced IFN-γ production was analyzed, and so was HLA-DR and CD38 coexpression within Ag-responsive CD8+ T cells. In this cross-sectional analysis, an overall increase of total CD8+ T cell activation was observed within the HIV-1–positive donors compared with healthy donors (data not shown). In addition, Ag-specific responses (measured by IFN-γ production) were generally higher in HIV-1 donors (Fig. 2B). More importantly, the extent of CD38 and HLA-DR coexpression on IFN-γ–producing T cells was significantly increased in HIV+ individuals compared with healthy donors (Fig. 2B). These results further support the hypothesis that HIV-1 rebound drives the activation of CD8+ T cells independent of their Ag specificity.

**IL-15 selectively activates CD8+T cells**

We observed that CD8+ T cells were activated independent of their Ag specificities after in vivo HIV-1 rebound; therefore, we tested
percentages of IFN-α and off-ART samples were pooled for analysis. Points on (Flu). Samples were analyzed from multiple time points on (EBV, HIV, CMV, adenovirus, and Flu). Stimulation of purified CD4+ and CD8+ T cells from healthy donors of TLR ligands (owing to microbial translocation) (10), we first performed comparative analyses for both T cell subsets. Failed to observe any direct stimulation of purified CD4+ and CD8+ T cells with different TLR ligands (e.g., lipopeptide [TLR2/1 or TLR2/6 ligand], LPS [TLR4 ligand], flagellin [TLR5 ligand], and bacterial DNA-containing CpG motifs [TLR9 ligands]), but we consistently failed to observe any direct stimulation of purified CD4+ and CD8+ T cells (Supplemental Fig. 2).

Besides microbial translocation, increased levels of many proinflammatory cytokines, such as IL-2, IL-15, IL-7, IL-12, IL-21, and IFN-α, have been reported during HIV-1 infection (8, 11, 27). Therefore, we investigated how cytokines being upregulated during HIV-1 infection affect T cell activation by performing in vitro stimulation assays. For these assays, purified CD4+ and CD8+ T cells were stimulated with different members of the γ-chain (γc) cytokine family (IL-7, IL-2, IL-21, IL-15), IFN-α, and IL-12, and different activation markers were measured, including HLA-DR and CD38, which are found to be upregulated on non-HIV-1–specific T cells in untreated HIV-1 infection (2, 3) as well as proliferation by CFSE dilution. By far the most pronounced effect, with respect to upregulation of activation markers and proliferation, was observed for IL-15, which acted selectively on CD8+ T cells (Table III). IL-2 and IL-7 also induced activation and proliferation of CD4+ and CD8+ T cells; however, the extent of CD8+ T cell activation was much lower compared with IL-15. Finally, the combination of both IL-15 and IL-12 slightly increased CD8+ T cell activation compared with IL-15 treatment.

Table III. Cytokine stimulation of MACS purified CD4+ or CD8+ T cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CD4+ Activation</th>
<th>CD4+ Proliferation</th>
<th>CD8+ Activation</th>
<th>CD8+ Proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2+</td>
<td>4.9% ± 2.8%</td>
<td>2.3% ± 1.4%</td>
<td>15.3% ± 5.9%</td>
<td>11.2% ± 3.9%</td>
</tr>
<tr>
<td>IL-7+</td>
<td>5.3% ± 2.6%</td>
<td>4.4% ± 1.1%</td>
<td>15.7% ± 3.1%</td>
<td>18.9% ± 1.1%</td>
</tr>
<tr>
<td>IL-12+</td>
<td>1.9% ± 1.7%</td>
<td>2.8% ± 2.7%</td>
<td>5.1% ± 0.6%</td>
<td>5.1% ± 4.6%</td>
</tr>
<tr>
<td>IL-15+</td>
<td>3.1% ± 3.2%</td>
<td>1.2% ± 0.7%</td>
<td>35.7% ± 3.2%</td>
<td>27.4% ± 5.5%</td>
</tr>
<tr>
<td>IL-21+</td>
<td>1.3% ± 0.1%</td>
<td>0.1% ± 0.04%</td>
<td>2.9% ± 1.4%</td>
<td>0.5% ± 0.3%</td>
</tr>
<tr>
<td>IL-15 + IL-12</td>
<td>4.8% ± 0.9%</td>
<td>6.3% ± 3.1%</td>
<td>50.0% ± 3.1%</td>
<td>52.4% ± 10.1%</td>
</tr>
<tr>
<td>IL-15 + IL-7</td>
<td>7.1% ± 1.1%</td>
<td>6.7% ± 1.9%</td>
<td>34.2% ± 0.7%</td>
<td>37.3% ± 1.1%</td>
</tr>
<tr>
<td>IL-15 + IL-2</td>
<td>3.6% ± 1.6%</td>
<td>7.6% ± 2.7</td>
<td>33.8% ± 3.1%</td>
<td>28.7% ± 7.4%</td>
</tr>
</tbody>
</table>

*aAverage HLA-DR+CD38+ T cells on day 6 after cytokine stimulation.
*bAverage percentage of CFSElow T cells on day 6 after cytokine stimulation.
*cCytokine concentrations: IL-2 = 50 ng/ml; IL-7 = 50 ng/ml; IL-12 = 3 ng/ml; IL-15 = 50 ng/ml; IL-21 = 25 ng/ml.
alone, indicating a synergistic effect of these two cytokines on CD8+ T cell activation.

Because we observed a pronounced effect of IL-15 on TCR-independent CD8+ T cell activation, we decided to focus on this cytokine in the following analyses. When CD4+ and CD8+ T cells from healthy donors were stimulated for 3 or 6 d in the absence or presence of IL-15, CD8+ T cells but not CD4+ T cells selectively upregulated the activation markers CD25, CD69 (not shown), and HLA-DR and CD38, with CD25 and CD69 being transiently upregulated early during stimulation and CD38 being upregulated most prominently by day 6 after stimulation (Fig. 3). Specifically, although IL-15 induced HLA-DR CD38 coexpression in 35.7% ± 3.2% CD8+ T cells, it did so in only 3.1% ± 0.8% of CD4+ T cells. In addition, 27.4% ± 5.5% of CD8+ T cells were CFSElo after IL-15 stimulation, but this was the case for only 1.2% ± 0.7% of CD4+ T cells. Furthermore, the concomitant addition of IL-12 even increased the extent of activation and proliferation within CD8+ T cells (50.9% ± 3.1% HLA-DR+ CD38+ CD8+ T cells and 52.4% ± 10.1% CFSElo CD8+ T cells). These results demonstrate that CD8+ T cells are far more susceptible to IL-15 stimulation than CD4+ T cells, and that this Ag-independent stimulation induces proliferation and long-lasting upregulation (up to 12 d, data not shown) of HLA-DR and CD38 on CD8+ T cells.

Comparative IL-15 stimulation experiments were also done in samples from nine HIV-1+ donors who received ART for at least 1 y with undetectable HIV-1 viral load. CD4+ and CD8+ T cells from HIV-1 donors were stimulated for 6 d in the presence or absence of IL-15 and CD8+ T cells, but not CD4+ T cells, showed selective upregulation of the activation markers HLA-DR and CD38 (Supplemental Fig. 3), thereby confirming the results obtained with healthy donor samples.

**FIGURE 3.** IL-15 stimulates proliferation and activation of CD8+ but not of CD4+ T cells. (A) Representative staining of one donor. Dot plots represent HLA-DR and CD38 expression on T cells. Numbers indicate percentage of HLA-DR+ CD38+ CD4+ or CD8+ T cells. Histograms show proliferation of T cells; the negative control is shown in gray. (B) Percentage of HLA-DR+ CD38+ (top) and CFSElo (bottom) T cells after stimulation with IL-15 ± IL-12. Error bars indicate the SD within a group. Each analysis was performed at least twice, and one representative result is shown. Data show the median of n = 8 healthy donors. *p < 0.05, **p < 0.025, adjusted for multiple comparisons using the Bonferroni method.
IL-15 activates memory CD8+ T cells more efficiently than naive CD8+ T cells

Next, we investigated whether IL-15 stimulation exhibits different potency in stimulating naive versus memory CD8+ T cells. To this end, different memory T cell subsets were FACS sorted from healthy donors based on the expression of CD45RA and CD62L: naive (TN, CD45RA+CD62L+), central memory (TCM, CD45RA−CD62L+) and effector memory (TEM, CD62L−, CD45RA+). Afterwards, the cells were cultivated for 6 d in the presence or absence of IL-15 with or without IL-12. Interestingly, the more differentiated the CD8+ T cells were, the more susceptible they were to IL-15–mediated activation (Fig. 4A). Whereas only 12.2% ± 7.2% of the TN cells expressed HLA-DR and CD38 6 d after IL-15 stimulation, 25.1% ± 5.9% of TCM and 48.8% ± 18.4% of TEM were HLA-DR+CD38+. Similar differences were observed for proliferation. Again, the addition of IL-12 increased this effect with 18.2% ± 4.9% TN, 44.7% ± 5.2% TCM and 72.3% ± 10.3% TEM being HLA-DR+CD38+. Thus, memory CD8+ T cells are more susceptible to IL-15 stimulation than TN CD8+ T cells are, and CD8+ T cells are overall more sensitive to IL-15 stimulation than CD4+ T cells are. To address the question why TN, TCM, and TEM CD8+ T cells exhibited different responsiveness toward IL-15 stimulation, we assessed expression levels of IL-15Rβ transcripts. Therefore, TN, TCM, and TEM CD8+ T cell subsets were isolated by FACS sorting, and mRNA levels of IL-15Rβ were quantified with quantitative PCR. The expression level of IL-15Rβ was higher in CD8+ T cells than in CD4+ T cells (Fig. 4B), and the expression level within CD8+ T cells was highest in TEM, followed by TCM and TN cells, consistent with the IL-15 sensitivity profile of these cell subsets.

**FIGURE 4.** Memory CD8+ T cells are more efficiently activated by IL-15 stimulation than naive CD8+ T cells. (A) Flow cytometric gating strategy for naive T cells (CD45RA+, CD62L+), effector memory T cells (CD62L−, CD45RA−, and CD45RA+) and central memory T cells (CD45RA+, CD62L−). (B) Percentage of HLA-DR+CD38+ (top), CFSElo (bottom) T cells after stimulation with IL-15 ± IL-12. (C) Relative IL-15Rβ mRNA expression (relative to GADPH) was determined with quantitative PCR. Error bars indicate the SD within a group. Each analysis was performed at least twice, and one representative result is shown. Data show the median of n = 8 healthy donors. Effector memory cells (TEM) shown in black, central memory cells (TCM) in white, and naive cells (TN) in gray. *p < 0.05, **p < 0.025, adjusted for multiple comparisons using the Bonferroni method.
**LPS- or HIV-1–activated dendritic cells (DCs) stimulate CD8+ T cells through IL-15 in an Ag-independent manner**

Based on our observation that IL-15 seems to be able to selectively activate CD8+ T cells and in combination with the knowledge that IL-15 is normally trans-presented by APCs (28–30), we tested the hypothesis that activated DCs (as observed during HIV-1 infection) (12) express IL-15 and that IL-15 expression in DCs leads to TCR-independent CD8+ T cell activation. We decided to activate DCs by LPS because systemically increased LPS levels have been found in HIV-1 patients and SIV-infected rhesus macaques as a result of microbial translocation (10, 31). Therefore, MDDCs were activated by LPS, and IL-15 expression was analyzed by RT-PCR and by flow cytometry at several time points following stimulation. The expression level of IL-15 peaked at day 3 (Fig. 5A), and this was confirmed with flow cytometry (Fig. 5B). In addition, IL-12 (subunits p35 and P40) followed expression kinetics similar to IL-15 after LPS stimulation (Fig. 5C).

We then used these IL-15–expressing MDDCs in coculture experiments with purified CD4+ or CD8+ T cells and measured their activation 6 d later by means of HLA-DR and CD38 expression and by proliferation (CFSE dilution; Fig. 6). Consistent with the experiments conducted by adding soluble IL-15, LPS-treated DCs selectively activated CD8+ T cells: CD8+ T cells: 25.8% ± 10.3% HLA-DR+CD38+ and 37.8% ± 11.1% CFSElo; compared with CD4+ T cells: 2.3% ± 1.6% HLA-DR+CD38+ and 1.7% ± 1.5% CFSElo.

To confirm that this activation was mediated by DC-derived IL-15, we added an IL-15 blocking Ab (αIL-15) to the LPS-activated MDDCs. Indeed, αIL-15 completely abrogated the activation of CD8+ T cells, both with respect to HLA-DR/CD38 expression and proliferation (Fig. 6A). We conclude that LPS-activated DCs activate CD8+ T cells in a TCR-independent and an IL-15–dependent manner, which might provide a link between microbial translocation and how bacterial products such as LPS are contributing to the activation of CD8+ T cells in HIV-1–infected individuals.

Besides the secondary effect of microbial translocation, HIV-1 replication itself is known to be a key driver in systemic immune activation. We therefore addressed the question of whether HIV-1 can also mediate activation of CD8+ T cells via IL-15 induction in DCs. MDDCs were exposed to AT2-inactivated HIV-1 (iHIV) (23); iHIV-treated MDDCs were cultured with autologous purified CD4+ or CD8+ T cells ± αIL-15 for 6 d when HLA-DR and CD38 expression and proliferation (CFSE dilution) were analyzed. As for LPS-activated MDPCs, iHIV-1 activated MDDCs strongly activated CD8+ T cells (17.1% ± 2.8% HLA-DR+CD38+) and promoted proliferation (16.1% ± 1.8%), and this effect was significantly reduced by blocking IL-15 (HLA-DR+CD38+ 5.9% ± 3.9, and proliferation 3.7% ± 3.4%; Fig. 7). As for LPS-treated MDDCs, this activation effect was exclusively seen for CD8+ T cells and not for CD4+ T cells.

**mDCs from HIV-1–infected patients show increased IL-15 production**

Next, we analyzed whether mDCs or pDCs isolated from HIV-1–infected patients also showed evidence for increased IL-15 production. To address the effect of HIV-1 replication on DC activation and IL-15 expression on an individual basis, we isolated mDCs and pDCs from 10 patients at one time point on and off ART and compared within one individual DC activation and IL-15 expression simultaneously. mDCs and pDCs were isolated from the PBMCs to high purity (Fig. 8A) stained for CD40 expression to assess their activation phenotype and subjected the purified cells to quantitative RT-PCR analysis for IL-15 expression. Detailed patient characteristics are listed in Table IV.

mDCs and pDCs isolated during the off-ART time point showed increased activation judged by CD40 expression compared with the on-ART time point (Fig. 8B). Importantly, IL-15 mRNA expression was markedly upregulated in mDCs, but not pDCs, isolated from the off-ART time point.

**Discussion**

HIV-1 infection is characterized by sustained high levels of "general" immune activation (32–35), which is reflected by increased expression of activation markers on T lymphocytes (1), increased cell cycling, and increased propensity of apoptosis (7, 36–38). The mechanisms underlying hyperactivation of non–HIV-1 specific T cells during chronic HIV-1 infection are still poorly understood. Because it is of prime importance to understand the mechanisms underlying such “general” immune activation, particularly as the extent of CD8+ T lymphocyte activation correlates with disease progression (1, 33, 34, 39, 40), we addressed this question on two levels by analyzing (1) whether the activated and

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**FIGURE 5.** IL-15 expression in MDDCs after LPS stimulation. (A) IL-15 quantitative PCR and (B) surface expression in LPS stimulated MDDCs. (C) IL-12p35 (left) and IL-12p40 (right). mRNA values indicate the fold increase of mRNA levels relative to unstimulated controls; each sample was normalized to GADPH expression. Each analysis was performed at least twice, and one representative result is shown. Data show the median and SD of n = 8 healthy donors. *p < 0.05, **p < 0.025, adjusted for multiple comparisons using the Bonferroni method.
hence expanded T lymphocytes exhibit a bias toward certain specificities (including persistent herpes or nonpersistent viral Ags), and 2) whether there is a contribution of inflammatory mediators (e.g., proinflammatory cytokines and TLR agonists) leading to CD4+ or CD8+ T cell activation independent of TCR triggering, which is referred to as “bystander activation” (7, 9).

In this study, we focused on CD8+ T cells because their immune activation status is one of the best predictors of disease progression (1). We show in a longitudinal cohort of HIV-1–infected patients who have stopped ART, that HIV-1 replication leads to activation of CD8+ T cells irrespective of their Ag specificity, including those with specificity for nonpersistent Ags (influenza or adenovirus). This result is in contrast to CD4+ T cells whose in vivo activation during HIV-1 replication seems biased toward specificities for persistent Ags (12). The correlation observed between the dynamics of HIV-1–specific CD8+ T cell responses and CD8+ T cell responses specific for non–HIV-1 Ags was dependent on HIV-1 rebound. This conclusion is supported by the fact that successful ART treatment, and consequent control of HIV-1 replication, results in a drop in proliferating T cells, despite slow recovery of CD4+ T cells, indicating that increased CD8+ T cell proliferation is driven by HIV-1 replication (41–43). This conclusion is further supported by the fact that the increased turnover of T cells observed during chronic HIV-1 infection is

**FIGURE 6.** Ag-independent activation of CD8+ T cells is dependent on IL-15 (LPS-activated DCs). (A) Representative staining of one donor. Dot plots represent HLA-DR and CD38 expression on T cells. Numbers indicate the percentage of HLA-DR+ CD38+ CD4+ or CD8+ T cells. Histograms show proliferation of T cells; the negative control is shown in gray. (B) Percentage of HLA-DR+ CD38+ (top), CFSElo (bottom) T cells after stimulation with autologous LPS-activated DCs. Data after IL-15 blockade are shown in white. Error bars indicate the SD within a group. Each analysis was performed at least twice, and one representative result is shown. Data show the median of n = 9 healthy donors. *p < 0.05, **p < 0.025, adjusted for multiple comparisons using the Bonferroni method.
driven by HIV-1–associated immune activation (41, 44, 45), and this increased T cell turnover is observed in blood and lymph nodes of HIV-1 patients (44, 46–49). Furthermore, CD8+ T cells show evidence of increased proliferation during HIV-1 infection (50). Together these data indicate that HIV-1 represents a dominant source of cellular activation and proliferation for both CD4+ and CD8+ T cells.

The observation that HIV-1 replication impinges on influenza-specific CD8+ T cell responses is supported by other studies reporting an activated phenotype on influenza-specific T cells during primary HIV-1 infection, correlating with CMV- and EBV-specific responses (3). These data indicate that bystander activated CD8+ T cells appear early in infection, but they also support our finding that CD8+ T cells are activated regardless of whether they are specific for persistent or nonpersistent Ags. It is possible that influenza-specific T cells are activated in the context of HIV-1 infection because of cross-reactivity for HIV-1 peptides (51). Although such cross-reactivity might contribute, it would not readily explain the observation that adenovirus-specific CD8+ T cell responses are similarly affected and that overall levels of CD8+ T cell activation and proliferation correlate with HIV-1 viral load (42, 43). This less restrictive manner of bystander activation of CD8+ T cells might explain why greater frequencies of CD8+ T cells exhibit an activated phenotype in HIV-1–infected individuals compared with CD4+ T cells. Because it also has been shown that in lymphopenic conditions—that is, the CD4+ T cell
loss in HIV-1 infection, sepsis (52), or after chemotherapy (53)—homeostatic proliferation stimulates the reconstitution of CD8+ T cells much faster than that of CD4+ T cells.

So far the relevance of TCR-independent T cell activation, involving cytokine stimulation or engagement of pattern recognition receptors, in chronic HIV-1 infection is not known, but it is possible that the inflammatory environment present during untreated HIV-1 infection has a significant effect on the induction of T cell hyperactivation and consequently contributes to disease progression (8, 11, 27). The apparent difference in the activation requirements of CD4+ and CD8+ T cells during HIV-1 replication, namely persistent Ag specificity of the former but not of the latter, prompted us to analyze whether PAMPs or cytokines have different abilities to activate CD4+ and CD8+ T cells. We found that IL-15 selectively activates CD8+ T cells and not CD4+ T cells, both with respect to upregulation of the activation markers CD38 and HLA-DR, which are commonly used to characterize activated T cells in HIV-1–infected patients and with respect to proliferation. Notably, IL-15 activated memory CD8+ T cells more efficiently than naive CD8+ T cells, which is consistent with the notion that activated and memory CD8+ T cells show increased turnover rates in HIV-1–infected patients compared with naive CD8+ T cells (54). These results support the idea that Ag-independent cytokine-mediated activation contributes to the aberrant CD8+ T cell activation observed during HIV-1 infection (7) and is in line with a report showing that activated T cells in HIV-1–infected individuals rarely show signs of recent TCR stimulation (55). Furthermore, it has also been reported in mice that IL-15 regulates the homeostatic proliferation of memory CD8+ but not CD4+ T cells (56).

Systemic translocation of gut microbial products including LPS, because of increased permeability of the gut epithelium, is suggested to contribute to the general immune activation (10). However, the mechanisms for how systemic exposure to bacterial PAMPs translates into activation of CD8+ T cells remain undefined. It is possible that TLR stimulation of CD8+ T cells in combination with cytokines directly results in their activation, supported by the observation that T cells respond directly to TLR2, TLR5, and TLR7/8 stimulation in the absence of TCR engagement but require the presence of proinflammatory cytokines like IL-15 or IL-2 (57–59). Furthermore, LPS stimulated the proliferation of memory CD8+ T cells in mice, and the authors suggest that this stimulation is mediated by an indirect pathway that involves the release of type I IFN (60). Finally, stimulation of PBMCs with TLR3, 4, 5, and 9 ligands induced the expression of CD69 on CD8+ T cells and cell cycle entry of CD4+ T cells (61). However, most studies have assessed the effects of TLR and cytokine receptor stimulation in whole PBMCs, making it difficult to draw conclusions about direct activation of T cells as other cells present in the PBMCs that might respond to the TLR ligands or cytokines, such as DCs or monocytes, which subsequently activate T cells via production of cytokines such as IL-15, IFN-α, or IL-12 (62).

In chronically HIV-1–infected individuals, partial activation of DCs (characterized by the upregulation of CD40/CD86 and by spontaneous production of proinflammatory cytokines/chemokines) (63-65) is observed, likely because of the presence of proinflammatory cytokines and TLR ligands, which are abundant because of microbial translocation (10, 66, 67). This partial activation of DCs likely contributes to increased activation of persistent herpes virus–specific CD4+ T cells during chronic HIV-1 infection.

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Table IV . Characteristics of patient cohort for mDC and pDC analysis

<table>
<thead>
<tr>
<th></th>
<th>On ART</th>
<th>Off ART</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days on ART before stopping</td>
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<td>28-863</td>
</tr>
<tr>
<td>Day of sample taken for analysis</td>
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<td>180.5</td>
</tr>
<tr>
<td>CD4 count</td>
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<td>HIV (RNA copies/ml)</td>
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<td>2e3-5e5</td>
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<td>% pDC</td>
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<tr>
<td>CD40 pDC (MFI)</td>
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<tr>
<td>% mDC</td>
<td>0.36</td>
<td>0.1-1.34</td>
</tr>
<tr>
<td>CD40 mDC (MFI)</td>
<td>294</td>
<td>98-1379</td>
</tr>
</tbody>
</table>

MFI, Mean fluorescence intensity.
(12, 13). In this study, we provide strong evidence that LPS- or HIV-1–activated DCs can effectively stimulate CD8+ T cells, but not CD4+ T cells, through IL-15 in an Ag-independent manner. We propose that LPS, being increased in the plasma of HIV-1–infected patients (10) and in tissues of SIV-infected macaques (31), in combination with HIV-1 particles, directly stimulate DCs to produce cytokines, such as IL-15, that activate CD8+ T cells, thereby contributing to their increased activation. In line with this assumption, it has been shown that IL-15 is increased in lymph nodes of HIV-1 patients (11), and monocytoid were identified as a possible source of IL-15 (68). In this study, we extend these observations by showing that mDCs from HIV-1–infected patients also show strikingly increased IL-15 production during active viral replication when comparing mDCs longitudinally within individuals on and off ART. Such an increase in IL-15 production is not seen in pDCs, which are known to produce mainly type I IFNs (69, 70). As it was shown that maturation of mDCs can also be induced by soluble factors secreted by HIV-1–activated pDCs (71), this bystander activation might also induce IL-15 production and thereby contribute to the activation of CD8+ T cells. These results are compatible with a scenario in which HIV-1–, PAMP- or cytokine-driven mDC activation and consequently IL-15 production in viremic HIV-1–infected individuals would represent a source for TCR-independent activation of CD8+ T cells and thus contribute to the increased activation status of these cells during HIV-1 infection.

In conclusion, we demonstrate that in vivo HIV-1 recrudescence is associated with concomitant activation of CD8+ T cells bearing specificities for persistent and nonpersistent Ags. This result is in contrast to non–HIV-1–specific CD4+ T cells whose activation depends on the presence of cognate Ag. This apparent discrepancy might be explained by the differential responsiveness of CD4+ and CD8+ T cells toward inflammatory cytokines, in particular IL-15, that activate CD8+ T cells. These results are compatible with a scenario in which HIV-1–, PAMP- or cytokine-driven mDC activation and consequently IL-15 production in viremic HIV-1–infected individuals would represent a source for TCR-independent activation of CD8+ T cells and thus contribute to the increased activation status of these cells during HIV-1 infection.

Acknowledgments

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HIV–ASSOCIATED IMMUNE ACTIVATION


