The Efficacy of T Cell-Mediated Immune Responses Is Reduced by the Envelope Protein of the Chimeric HIV-1/SIV-KB9 Virus In Vivo

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The Efficacy of T Cell-Mediated Immune Responses Is Reduced by the Envelope Protein of the Chimeric HIV-1/SIV-KB9 Virus In Vivo

Liljana Stevceva, Victor Yoon, Angela Carville, Beatriz Pacheco, Michael Santosoosso, Birgit Koroith-Schmitz, Keith Mansfield, and Mark C. Poznansky

Gp120 is a critical component of the envelope of HIV-1. Its role in viral entry is well described. In view of its position on the viral envelope, gp120 is a part of the retrovirus that immune cells encounter first and has the potential to influence antiretroviral immune responses. We propose that high levels of gp120 are present in tissues and may contribute to the failure of the immune system to fully control and ultimately clear the virus. Herein, we show for the first time that lymphoid tissues from acutely HIV-1/SIV (SHIV)-KB9-infected macaques contain deposits of gp120 at concentrations that are high enough to induce suppressive effects on T cells, thus negatively regulating the antiviral CTL response and contributing to virus survival and persistence. We also demonstrate that SHIV-KB9 gp120 influences functional T cell responses during SHIV infection in a manner that suppresses degranulation and cytokine secretion by CTLs. Finally, we show that regulatory T cells accumulate in lymphoid tissues during acute infection and that they respond to gp120 by producing TGF-β, a known suppressant of cytotoxic T cell activity. These findings have significant implications for our understanding of the contribution of non-entry-related functions of HIV-1 gp120 to the pathogenesis of HIV/AIDS. The Journal of Immunology, 2008, 181: 5510 –5521.

HIV-1-specific CD8⁺ CTL can be detected in the peripheral blood (PB) during the first 4–6 wk following acute retroviral infection and before a neutralizing Ab response is demonstrable. The appearance of these cells in blood coincides with an initial decrease in plasma viremia during acute HIV infection (1, 2) as well as in animal models of simian immunodeficiency virus (SIV) and feline immunodeficiency virus infection (3–5). In this setting, CTL numbers have been shown to negatively correlate with the viral load (6). In the SIV model of infection, CD8⁺-depleted macaques lose control of SIV replication that is subsequently restored with the reappearance of CD8⁺ cells (7). However, although CTL responses control viremia, they fail to eradicate the virus.

It is not fully understood why the body fails to eradicate or control the virus, but several phenomena have been described that support the thesis that the retrovirus is capable of evading the host cell-mediated immune response. HIV-1 utilizes various mechanisms to evade the immune response, such as mutation of its envelope protein, maintenance of a latent state, and active infection and killing of immune cells, including CD4⁺ T cells (8–10). CTLs may also be ineffective because of viral escape and the emergence of resistant strains (1, 11–18).

HIV-specific T cell clones that are present at the time of acute infection have also been shown to rapidly disappear (19). This phenomenon is not thought to be the result of mutations in viral epitopes recognized by these clones (19). It has been proposed that this reflects a deletion process caused by high levels of viral Ag. Clonal exhaustion/deletion is a well-documented phenomenon where an excess of Ag on APCs in the lymphoid organs induces all Ag-specific responsive T cells, resulting in the death of all matured effector T cells within a few days and the deletion of this specific response from the T cell repertoire (20). Both X4 and R5 HIV-1 gp120 itself has been shown in vitro to dysregulate T cell function, including the induction of T cell apoptosis, inhibition of functional T cell responses to recall Ags, and interference with the expression of costimulatory molecules and TCR desensitization (21–25, 26). Additionally, it has been demonstrated that both recombinant and oligomeric forms of gp120 directly influence T cell migration and that high concentrations of the retroviral protein (200 ng/ml) cause active movement of Ag-specific T cells away from the protein via a chemokine (C-X-C motif) receptor 4 (CXCR4)-mediated mechanism, termed fugetaxis or chemorepulsion (22). Consequently, it has been suggested that HIV-specific immune effector cells may fail to migrate to those areas in which HIV proliferation and gp120 expression are high, as in the lymph node (LN) (27). The question therefore arises as to whether high enough concentrations of gp120 exist in vivo to dysregulate T cell migration and suppress their function.

In this study, we used a model of acute chimeric HIV (SHIV) infection to examine the distribution and concentration of gp120 deposits and T cell subpopulations in infected tissues and the effects of the envelope protein on the functional status of T cell populations (28, 29). We chose this time point during the retroviral infection because...
it occurs before the development of Ab responses that might mask the presence of gp120 by binding it into immune complexes (30). Herein, we demonstrate that during acute infection, a significant amount of the gp120 protein is deposited in the LN where priming and proliferation of lymphocytes occur. Furthermore, we show that T cells from these areas lose their ability to respond to gp120. This defect is Ag-specific, as LN T cells are able to respond to unspecific or SIV

Materials and Methods

SHIV, animals, and LN and PBMC sampling

The SHIV chimeric virus used in this study was created by using SIV<sub>mac239</sub> and the env gene of a cytopathic primary patient isolate of HIV-1<sub>89.6</sub>, which causes an AIDS-like disease in rhesus macaques (28, 29). SHIV-specific CD<sub>8</sub> CTL are known to be present during the early weeks following initial infection and are able to respond to unspecific or SIV<sub>mac239</sub> Gag upon stimulation with SHIV gp120. These findings broaden our understanding of the non-entry functions that HIV-1 gp120 may contribute to the pathogenesis of HIV/AIDS.

<table>
<thead>
<tr>
<th>Macaques</th>
<th>Route of Viral Entry</th>
<th>Virus</th>
<th>Time of Sacrifice</th>
<th>Viral Load at Time of Sacrifice</th>
<th>% of CD&lt;sub&gt;4&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt; T Cells at Time of Sacrifice</th>
<th>% of CD&lt;sub&gt;8&lt;/sub&gt;&lt;sup&gt;+&lt;/sup&gt; T Cells at Time of Sacrifice</th>
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<tbody>
<tr>
<td>179-93</td>
<td>i.v.</td>
<td>SHIV-KB9</td>
<td>Day 42</td>
<td>55,398</td>
<td>8.34 (PB), 11.4 (LN)</td>
<td>88.8 (PB), 78.2 (LN)</td>
</tr>
<tr>
<td>212-92</td>
<td>i.v.</td>
<td>SHIV-KB9</td>
<td>Day 42</td>
<td>151,841</td>
<td>17.1 (PB), 6.77 (LN)</td>
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<td>249-92</td>
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<td>159,406</td>
<td>19 (PB), 12.3 (LN)</td>
<td>71.1 (PB), 78.6 (LN)</td>
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<td>24.9 (PB), 22.3 (LN)</td>
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<td>Day 42</td>
<td>134</td>
<td>46.2 (PB), 55.1 (LN)</td>
<td>68 (PB), 44.2 (LN)</td>
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<td>84 (PB), 89.3 (LN)</td>
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<td>15.2 (PB), 11.7 (LN)</td>
<td>72.3 (PB), 78.1 (LN)</td>
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<td>529-99</td>
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<td>8,125</td>
<td>13.5 (PB), 13.5 (LN)</td>
<td>80.7 (PB), 79.6 (LN)</td>
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<td>N/A</td>
<td>57.8 (PB)</td>
<td>41.6 (PB)</td>
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<td>N/A</td>
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<td>N/A</td>
<td>N/A</td>
<td>52.6 (PB)</td>
<td>46.2 (PB)</td>
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<td>475-99</td>
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<td>Control</td>
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<td>N/A</td>
<td>76.6 (LN)</td>
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<td>N/A</td>
<td>Control</td>
<td>N/A</td>
<td>N/A</td>
<td>56.1 (LN)</td>
<td>39.1 (LN)</td>
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<tr>
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<td>Control</td>
<td>N/A</td>
<td>N/A</td>
<td>58.0 (LN)</td>
<td>37.3 (LN)</td>
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<tr>
<td>211-93</td>
<td>N/A</td>
<td>Control</td>
<td>N/A</td>
<td>N/A</td>
<td>56.2 (LN)</td>
<td>37.7 (LN)</td>
</tr>
<tr>
<td>165-91</td>
<td>N/A</td>
<td>Control</td>
<td>N/A</td>
<td>N/A</td>
<td>70.3 (LN)</td>
<td>28.4 (LN)</td>
</tr>
</tbody>
</table>

* Eight acutely SHIV-KB-9-infected animals and nine uninfected animals were used in this study. PB and LNs were collected at the time of sacrifice. Plasma SHIV-KB9 RNA levels were measured by an ultrasensitive branched DNA-amplification assay with a detection limit of 125 RNA copies/ml (Bayer Diagnostics). The plasma SHIV-KB9 RNA levels and percentage CD4<sup>+</sup> and CD8<sup>+</sup> T cells were determined at time of sacrifice.

Quantifying the amount of gp120 in infected LNs and plasma

To confirm results obtained by immunofluorescent staining and flow cytometry for surface gp120 expression, we quantitated the amount of gp120 in LNs and plasma from four acutely SHIV-infected and four uninfected monkeys by using an ELISA. LNs were fragmented mechanically, and the subsequent mononuclear cell suspension was prepared by Ficoll gradient centrifugation. Cells were counted, resuspended in 1 ml of RPMI 1640, and sonicated on ice. The gp120-producing cell line CHO-NL-4-3 was processed in the same way and used as a positive control, while the parent, a gp120-negative CHO cell line, was used as a negative control. Cell lysates were separated from extracellular debris by centrifugation and the supernatant was tested for presence of gp120 by using an ELISA kit (Immuno-Diagnostics). Plasma samples from infected and uninfected animals were concentrated (10-fold) using Microcon tubes (Millipore). HIV-1 gp120 levels were quantitated as ng/ml of tissue so that levels could be compared between tissue specimens and plasma samples and to make a preliminary determination about the magnitude of the gradient that existed between extracellular plasma and intracellular tissues by using this method was determined by spiking normal, uninfected tissues with known amounts of recombinant envelope protein. Cell lysates were performed as previously described, and gp120 was measured after extraction.
Phenotypic and functional characterization of isolated PB and LN lymphocytes

Lymphocytes were isolated from PB and LNs of acutely SHIV-KB-9-infected macaques. Phenotypic characterization was done by multiparameter staining for CD3, CD4, CD8, and CD25 (BD Biosciences) with DIVA software in conjunction with an LSR2 FACS analyzer (BD Biosciences). The source and details of Abs used throughout this study are shown in Table II (under Panel 1). Any significant spillover from one channel to another is indicated by the DIVA software. The negative population for each Ag was established by staining with all of the other Abs except the one of interest and subsequent FlowJo analysis. The same settings were then used for the staining of samples and the quadrant settings established in the negative population were used to analyze the samples.

The functional capacity of the lymphocytes was assessed following stimulation with the complete set of peptides spanning the entire SHIV 89.6 Env (overlapping by 11 aa) from the core of SHIV (NARRRP) and then by performing multiparameter surface and intracellular staining for CD3, CD4, CD107a (degranulation marker), perforin, IFN-γ, TFN-α, and IL-10 (BD Biosciences). Stimulation and staining were performed as previously published (31, 32, 43–45). SHIV 89.6 is the parent strain of SHIV KB9, and the sequence of the SHIV 89.6 Env is similar and binds equivalently. The core protein of the SHIV (KB9) virus is equivalent to the SIVmac239 core protein. Nonspecific stimulation with PMA and ionomycin was used as a control and to demonstrate that the cells are functionally capable of degranulating and producing the cytokines.

Isolated and previously frozen PB and LN lymphocytes were thawed and washed. Viability was assessed by trypan blue dye exclusion, and viable cells were counted. Cells were incubated for 1 h at 37°C in 5% CO₂ at a concentration of 10⁶/ml in the presence of 1 µl of anti-CD28 (BD Biosciences), 1 µl of anti-CD49d (BD Biosciences), and 1 µg/ml of the complete set of pooled SHIV 89.6 Env peptides. Cells were also stimulated with the complete set of pooled SIVmac239 Gag peptides or 25 ng of PMA (Sigma-Aldrich) and 1 µg of ionomycin (Sigma-Aldrich). PB and LN lymphocytes from any individual monkey were tested in this way at the same time and in parallel with the same amount of peptide. Cells were incubated for an additional 5 h with added GolgiPlug and GolgiStop (BD Pharmingen). Cells were then stained first with surface Abs and then for intracellular IL-10, IFN-γ, or TNF-α (BD Pharmingen) (Table II, Panel 2) by using a Cytofix/Cytoperm Plus kit (BD Pharmingen) according to the manufacturer’s instructions. At least 5 x 10⁵ cells were acquired per sample. Cells were gated on CD4⁺CD5⁺ or CD8⁺CD3⁺. The percentage of CD107a, IFN-γ, IL-10, or TNF-α cells that were induced by a specific stimulant (Env peptides, Gag peptides, or PMA and ionomycin) is equal to the percentage of positive cells in the stimulated minus the percentage of positive cells in the unstimulated sample (background). An increased response was therefore determined as the percentage increase in responding cells above the unstimulated background. A positive response to stimulation was defined as being greater than or equal to a 100% increase above background in unstimulated conditions.

Assessment of Treg cell responses to Env was done by stimulating with the complete set of pooled SHIV 89.6 Env peptides, as described above, and multiparameter staining for CD3, CD4, CD25, CD25, FoxP3, IL-10, and TGFβ (Table II, Panel 3). Stimulation with anti-CD3 and anti-CD28 was used as a positive control for TGFβ expression. All of the stained cells or at least 1 x 10⁵ cells were acquired. Cells were gated on CD4⁺CD3⁻ and then CD25, and FoxP3 markers.

Table II. Specificity, manufacturer, clone, and fluorochrome for the panels of mAbs used in this study

<table>
<thead>
<tr>
<th>Panel 1 (phenotypic characterization)</th>
<th>Ab</th>
<th>Manufacturer</th>
<th>Clone</th>
<th>Fluorochrome</th>
</tr>
</thead>
<tbody>
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<td>Anti-CD3</td>
<td>BD Biosciences</td>
<td>L200</td>
<td>PerCP-Cy5.5</td>
<td></td>
</tr>
<tr>
<td>Anti-CD4</td>
<td>BD Biosciences</td>
<td>L200</td>
<td>PerCP-Cy5.5</td>
<td></td>
</tr>
<tr>
<td>Anti-CD8</td>
<td>BD Biosciences</td>
<td>L200</td>
<td>PerCP-Cy5.5</td>
<td></td>
</tr>
<tr>
<td>Anti-CD9</td>
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<td>L200</td>
<td>PerCP-Cy5.5</td>
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</tr>
<tr>
<td>Anti-CD10</td>
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<td>Anti-CD11</td>
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<tr>
<td>Anti-CD12</td>
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<td>L200</td>
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<td></td>
</tr>
<tr>
<td>Anti-CD14</td>
<td>BD Biosciences</td>
<td>L200</td>
<td>PerCP-Cy5.5</td>
<td></td>
</tr>
</tbody>
</table>

In vitro exposure of PB lymphocytes to SHIV-KB9 gp120

SHIV-KB9 gp120 glycoprotein was cloned, between the KpnI and MfeI sites, into the plasmid pE7-HXBC2(IIHexE7pA-KnpI)’ (46) in which a stop codon had been introduced at the beginning of the gp41 region, to produce the plasmid pE7-KB9 gp120. The expression of HIV-1 gp120 glycoprotein was achieved in HEK 293T cells cotransfected with the pE7-KB9 gp120 plasmid and a pTat plasmid that express HIV-1 Tat protein. About 14 h after transfection, cells were washed once with PBS and cultured for 72 h in serum-free medium Pro293s-CDM (Cambrex) supplemented with 12 mM glutamine. Sodium butyrate was added to a 3 mM concentration 31 h before harvesting. The medium containing KB9 gp120 glycoprotein was harvested and incubated with 100 µl of IgG1-b12-Sepharose beads for ~16 h at 4°C. After washing the beads three times with washing buffer (Tris 0.1 M (pH 8.0), NaCl 0.5 M), the KB9 gp120 glycoprotein was eluted with Gly 0.1 M (pH 3.0) and neutralized immediately with 1/10 volume of Tris 1 M (pH 8.0). The purity and concentration of KB9 gp120 protein were determined by silver staining of an SDS-PAGE gel and spectrophometry, respectively (see Fig. 5a).

To assess the effect of HIV-1 gp120 on the functionality of T cells previously frozen, PB lymphocytes from acutely SHIV-KB9-infected macaques were thawed and washed in the absence or presence of 200, 400, and 800 ng/ml of the SHIV-KB9 gp120 glycoprotein. Viability of the cells was assessed by trypan blue exclusion and was >75% after treatment with gp120. Cells were then stimulated with the complete pooled SHIV-996 Env peptide mix or SIVmac239 Gag and stained for CD3, CD4, CD8, CD107a, IFN-γ, TNF-α, and IL-10 and analyzed by multiparameter flow cytometry and FlowJo as previously described.

Statistical analysis

All statistical analyses were performed using a Mann-Whitney U test. A value of p < 0.05 was considered significant. p-values were determined with and without single outlier values where present.
Results

LN tissues of acutely SHIV-KB9-infected macaques contain gp120 deposits at levels previously shown to induce T cell dysfunction

We demonstrated by immunofluorescent staining with V3 447-52D Ab directed to the V3 loop of gp120 a dense and widespread distribution of the envelope protein in LNs derived from acutely SHIV-infected macaques (Fig. 1, a and b). Staining for the SIV Gag protein (green) revealed less SIV Gag-positive cells that were differently distributed (c, original magnification ×20) from gp120. SIV Gag protein was detected at the plasma membrane in most of the positive cells (d, original magnification ×40). The white scale bar represents 100 μm in a and 20 μm in b.

Figure 1. Immunofluorescent staining for HIV gp120 and SIV Gag protein was performed on serial sections of LN tissues of macaques in primary SHIV infection. Gp120 expression (red) in the acutely infected LNs did not appear to be related to the CD3+ T cell zones (green) (original magnification ×10 (a) and ×40 (b)). Staining for the SIV Gag protein (green) revealed less SIV Gag-positive cells that were differently distributed (c, original magnification ×20) from gp120. SIV Gag protein was detected at the plasma membrane in most of the positive cells (d, original magnification ×40). The white scale bar represents 100 μm in a and 20 μm in b.

Significant effects of gp120 on T cell function have been previously described (21–26, 49). These effects were only seen at high concentrations of gp120 ≥20 ng/ml or 200 pM, but the precise relevance of these effects in vivo in HIV-1 infection is not clear. In the first instance we set out to see if a clinically relevant amount of gp120 is present in the LN tissues. We quantitated the amount of gp120 in SHIV-infected LNs and plasma from four acutely SHIV-infected and four uninfected monkeys using an ELISA-based system. Assessment was done on sonicated LN mononuclear cells as described in Materials and Methods. Concentrations of SHIV-KB9 gp120 in LN mononuclear cell lysates varied between 183 and 562 ng/ml (mean of 218 ng/ml) of LN tissue from acutely infected animals, whereas plasma levels varied between 0.33 and 3.73 ng/ml (mean of 1.55 ng/ml). These data indicate that a significant difference exists between the amount of gp120 present in the LN mononuclear cells and plasma. HIV-1 gp120 was detectable at a level of 123 ng/ml from lysed control CHO-NL-4-3 cells engineered to express the envelope protein and was otherwise undetectable in plasma and lysed LN mononuclear cells from uninfected monkeys or the parent CHO cell line (data not shown). Additionally, we measured percentage retrieval of gp120 from normal uninfected tissues spiked with a known amount of the recombinant protein and found that on average 66% (range 50–75%) of gp120 was retrieved by our described method. This measurement does not take into account gp120 bound to extracellular matrix in the LNs, which was detected by immunostaining. Consequently, we think that our measurements of gp120 in infected tissues may be an underestimate of total gp120 in the LN itself. These results demonstrate that gp120 is present during acute SHIV-KB9 infection in the LNs at high levels equivalent to those shown to dysregulate T cell function and migration in vitro.

LN T cells fail to degranulate and secrete less IFN-γ, TNF-α, and IL-10 when stimulated with gp120 peptides compared with PB T cells

The functional fitness of PB and LN T cells from SHIV-infected animals was assessed by determining the secretion of cytokines relevant to T cell function such as IFN-γ and IL-10 and by examining the capacity of T cells to perform cytotoxic function. CTL-mediated lysis of infected targets is initiated by the recognition of a MHC class I exogenous peptide complex on the surface of target cells, and can be achieved by granule-independent triggering of apoptotic processes in the virus-infected cells via TNF-α and
CD95 (reviewed in Refs. 50–52) or via a granule-dependent pathway. During the granule-dependent pathway of CTL-mediated lysis, perforin molecules insert themselves into the plasma membrane of target cells, thus enabling granzymes to enter the cell and to activate the precursors of caspases initiating self-destruction of the cell by apoptosis (reviewed in Refs. 50–52). The markers CD107a and CD107b are expressed on the cell surface during degranulation and were used in these studies in conjunction with perforin to assess the capacity of the T cells to degranulate in response to an Ag (53).
We made the robust finding that while stimulation with the SHIV Env peptide pool induced degranulation as measured by CD107a expression in CD4\(^+\) and CD8\(^+\) T cells (Figs. 2a and 3a), PB-derived T cells and CD4\(^+\) LN-derived T cells degranulated to a much lesser extent in response to this stimulant (\(p = 0.00031\)) (Figs. 2b and 3a). A similar pattern of CD107a expression was seen for CD8\(^+\) T cells derived from peripheral blood and LNs (\(p = 0.04\)) (Figs. 2c, 3d, and 3b). Note that CD107a measurements are prone to fixation artifacts and may be unduly sensitive to excess amounts of peptides. This was internally controlled in this study by comparing responses of PB T cells to LN-derived T cells to identical amounts of peptides and fixation methods. Similarly, while gp120 stimulation induced IFN-\(\gamma\) secretion in both CD4\(^+\) and CD8\(^+\) PB T cells (Figs. 2a and 3a, and 3c and d), LN CD4\(^+\) and CD8\(^+\) T cells demonstrated significantly reduced levels of these responses (Figs. 2b, 3d, and 3c and d) (\(p = 0.002\) and \(p = 0.011\), respectively). Secretion of IL-10 and TNF-\(\alpha\) responses of LN CD3\(^+\)CD4\(^+\) T cells were reduced in comparison to PB-derived CD8\(^+\) T cell responses, but this difference did not reach statistical significance. The statistical significance of differences between the responses of PBMC- and LN-derived T cells was measured using a two-tailed Mann-Whitney \(U\) test. Each symbol (●, PB-derived T cells; ▼, LN-derived T cells) represents data from an individual monkey.

**FIGURE 3.** Cumulative flow cytometric data from the stimulation of PB-derived and LN-derived CD3\(^+\)CD4\(^+\) (a, c, e, and g) and CD3\(^+\)CD8\(^+\) T cells (b, d, f, and h) from acutely SHIV-KB9-infected monkeys in response to the complete set of SHIV 89.6P Env peptides (gp120). Percentage of Env-specific responder T cells in the context of measurements of CD107a, IFN-\(\gamma\), IL-10, and TNF-\(\alpha\) are shown for both nonstimulated (NS) and gp120 peptide-stimulated cells. When compared with PB-derived CD3\(^+\)CD4\(^+\) T cells, LN CD4\(^+\) T cells (a) showed consistently less degranulation as measured by CD107a expression on their surface (\(p = 0.00031\) or \(p = 0.00078\) without the single outlier value). Secretion of IFN-\(\gamma\), IL-10, and TNF-\(\alpha\) in response to Env peptide stimulation was also significantly lower in the LN CD3\(^+\)CD4\(^+\) (c, e, and g, respectively) T cells as compared with those originating from the PB. These statistically significant differences were maintained when single outlier values where present in the dataset were excluded (bracketed \(p\)-value). A similar pattern of reduced Env-induced CD107a and IFN-\(\gamma\)-expression in LN-derived CD8\(^+\) T cells as compared with PBMC-derived CD8\(^+\) T cells was seen (b and d) (CD107a: \(p = 0.04\) and IFN-\(\gamma\): \(p = 0.011\), respectively). Env-specific IL-10 and TNF-\(\alpha\)-responses of LN CD3\(^+\) CD8\(^+\) T cells were reduced in comparison to PB-derived CD8\(^+\) T cell responses, but this difference did not reach statistical significance. The statistical significance of differences between the responses of PBMC- and LN-derived T cells was measured using a two-tailed Mann-Whitney \(U\) test. Each symbol (●, PB-derived T cells; ▼, LN-derived T cells) represents data from an individual monkey.

We made the robust finding that while stimulation with the SHIV Env peptide pool induced degranulation as measured by CD107a expression in CD4\(^+\) (Figs. 2a and 3a) and CD8\(^+\) (Figs. 2c and 3b), PB-derived T cells and CD4\(^+\) LN-derived T cells degranulated to a much lesser extent in response to this stimulant (\(p = 0.00031\)) (Figs. 2b and 3a). A similar pattern of CD107a expression was seen for CD8\(^+\) T cells derived from peripheral blood and LNs (\(p = 0.04\)) (Figs. 2c, 3d, and 3b). Note that CD107a measurements are prone to fixation artifacts and may be unduly sensitive to excess amounts of peptides. This was internally controlled in this study by comparing responses of PB T cells to LN-derived T cells to identical amounts of peptides and fixation methods. Similarly, while
demonstrated similar levels of IL-10 responses (Figs. 2, c and d, and 3h) \( (p = 0.17) \). In separate experiments nonspecific stimulation of CD3^+CD4^+ or CD3^+CD8^+ T cells with PMA and ionomycin showed robust responses as measured by CD107a and intracellular cytokine expression (Fig. 2, e and f).

We then set out to examine whether the suppressive effects of gp120 were Ag specific or also occurred in response to other viral Ags including Gag. Stimulation with SIVmac239 Gag peptide pool led to suppression of Env peptide pool-secreted IFN-\( \gamma \) in both PB and LN CD4^+ (Fig. 4c) and CD8^+ (Fig. 4b) T cells. Stimulation of lymphocytes from SIVmac239-infected macaques and stimulation with PMA and ionomycin were used as positive controls. Non-specific stimulation with PMA and ionomycin induced high IFN-\( \gamma \) (Fig. 4, c and d) and TNF-\( \alpha \) (data not shown) secretion from both blood and LN CD8^+ T cells. However, when compared with PBMCs, LN CD4^+ T cells responded poorly to PMA and ionomycin stimulation, and the percentage of LN CD4^+ T cells that were induced to produce both IFN-\( \gamma \) (Fig. 4c) or TNF-\( \alpha \) was significantly lower than those in PB \( (p = 0.049 \) and \( p = 0.01) \) (data not shown).

**Exposure of PB-derived lymphocytes to SHIV-KB9 gp120 suppresses CD4^+, and CD8^+ T cell function in a dose-dependent manner**

Having demonstrated that PB lymphocytes from SHIV-KB9-infected macaques responded well to stimulation with the SHIV-KB9 Env peptide pool by degranulating and increasing their secretion of IFN-\( \gamma \), TNF-\( \alpha \), and IL-10, we set out to examine whether this response could be abolished by the presence of gp120. Isolated PB lymphocytes were incubated overnight with SHIV-KB9 gp120 at concentrations that we had shown above to be present in LNs from acutely SHIV-infected monkeys (200, 400, and 800 ng/ml). The SHIV-KB9 gp120 was generated and purified as previously described (Fig. 5a). Lymphocytes were then stimulated with SHIV-KB9 envelope peptide pool and stained for CD3, CD4, CD8, CD107a, IFN-\( \gamma \), TNF-\( \alpha \), and IL-10. In these experiments, SHIV-KB9 gp120 at a concentration of 400 ng/ml suppressed CD4^+ T cell responses to a mean of 32 ± 21% of those of the nonexposed cells for TNF-\( \alpha \), 45 ± 22% for IL-10, and 35 ± 15% of the responses of nonexposed cells for TNF-\( \alpha \) (Fig. 5b).

Simultaneously, preincubation of CD8^+ T cells with recombinant KB-9 gp120 led to suppression of Env peptide pool-induced responses to 32 ± 17% for CD107a, 48 ± 20% for IFN-\( \gamma \), 34 ± 33% for IL-10, and 32 ± 21% for TNF-\( \alpha \) (Fig. 5c). In rare instances where suppression of one function of CD4^+ or CD8^+ cells did not occur, other functions were suppressed. In summary, exposure of the cells to concentrations of 400 and 800 ng/ml of SHIV-KB9 gp120 suppressed all functions in both CD4^+ and CD8^+ T cells and completely abolished gp120-induced degranulation or cytokine secretion in most of the six samples examined (Table III). This experiment confirmed that, when present at high concentrations, gp120 is able to suppress CTL responses. It is unclear whether other viral proteins are present at high levels in infected tissues and whether they may have a similar effect. We were unable to detect a high amount of the core protein SIVmac239 in acutely infected tissues by immunostaining (see Fig. 1). In control experiments to examine the relative effect of Gag protein, we exposed PB T cells to 0,
Table III. Quantitation of the suppressive effect of recombinant SHIV-KB9 gp120 on T cell responses to the complete pool of Env peptides by recombinant SHIV-KB9 gp120

<table>
<thead>
<tr>
<th>CD107a</th>
<th>IFN-γ</th>
<th>IL-10</th>
<th>TNF-α</th>
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<tbody>
<tr>
<td>a) Amount of gp120 (CD4+)⁹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 ng/ml</td>
<td>4/4</td>
<td>1/4</td>
<td>2/4</td>
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<td>400 ng/ml</td>
<td>4/4</td>
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<td>800 ng/ml</td>
<td>4/4</td>
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<td>4/4</td>
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<tr>
<td>b) Amount of gp120 (CD8+)⁹</td>
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<tr>
<td>200 ng/ml</td>
<td>4/4</td>
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<td>800 ng/ml</td>
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⁹ No. of inhibited CD4⁺ T cell responses/total no. of responses measured.

LNs but not PB of SHIV-KB9 acutely infected macaques contain a significantly higher percentage of regulatory T cells than do those of uninfected macaques

While significant depletion of the CD3⁺CD4⁺ T cells was seen in both compartments (Fig. 6a), the percentage of regulatory T cells as determined by high-level expression of CD25 on CD3⁺CD4⁺ cells was significantly up-regulated in LN lymphocytes (Fig. 6b) but not in PBMCs (Fig. 6c) of SHIV-infected macaques compared with the naive controls. CD4⁺CD25⁺ Treg cells normally constitute a small fraction of circulating CD4⁺ T cells in humans that belong to the memory T cell pool (54–57). They are commonly identified by the expression of the IL-2Ra (CD25) on their surface and by the expression of the transcription factor scurfin that is encoded by the FoxP3 gene. Upon further phenotypic analysis of regulatory T cells from SHIV-infected macaques, we observed that most CD3⁺CD4⁺ cells were CD25low from PB and LNs that expressed high levels of CD25 were CD127low (PB mean of 86.5%; LN mean of 84.0%) (Fig. 6d). We also observed a smaller majority of CD3⁺CD4⁺ T cells was significantly up-regulated in LN lymphocytes (Fig. 6d). We also observed a smaller majority of CD3⁺CD4⁺ T cells was significantly up-regulated in LN lymphocytes (Fig. 6d). However, no significant difference in the proportion of CD3⁺CD4⁺ T cells between PB and LNs was observed in this study. Accumulation of Treg cells has been previously documented and

FIGURE 5. PB lymphocytes were exposed overnight to SHIV-KB9 glycoprotein gp120. SHIV-KB9 gp120 was generated by expressing it into the plasmid pE7-HXBc2(IIIxE7pA-KpnII), transfecting the permissible cells, collecting the supernatants, and purifying the KB9 gp120. The purity of gp120 was determined by silver staining of an SDS-PAGE gel (a). (Lanes, left to right, products of flow-through (lane 1), three column washes (lanes 2–4), three lanes of eluted KB9-gp120 protein (lanes 5–7) and a molecular mass marker (lane 8). Pretreatment of CD3⁺CD4⁺ T cells (b) or CD3⁺CD8⁺ T cells (c) with recombinant SHIV-KB9 gp120 (gp120) at concentrations of 400 ng/ml and/or 800 ng/ml significantly reduced the percentage of responding cells from the PB of acutely SHIV-KB9-infected monkeys to a complete set of SHIV-KB9 Env peptides. Graphs show the percentage of CD4⁺ or CD8⁺ Env-induced responder cells (% of Env induced T cell responses/total no. of responses measured). Most T cell samples exposed to concentrations of 400 and 800 ng/ml of recombinant SHIV-KB9 gp120 reported reductions in CD107a and cytokine expression in comparison to T cells, which were not pretreated with recombinant gp120.
was attributed to the selective promotion of their survival by the HIV virus via a CD4-gp120 interaction (60).

Regulatory T cells that accumulate in the LNs of acutely SHIV-infected macaques respond to stimulation with SHIV Env peptide pool by secreting TGFβ and H9252.

To determine whether the Treg cells that accumulate in the LNs of acutely SHIV-infected macaques respond to gp120 and how gp120 affects their behavior, isolated LN lymphocytes were stimulated with the SHIV Env peptide pool and then stained for CD3, CD4, CD25, FoxP3, IL-10, and TGFβ and H9252. Adaptive Treg cells can be induced in the presence of IL-10, and in that case they exert their suppressive activity via the production of IL-10 or by TGFβ (61). Although the first mechanism described above and other potential factors cannot be excluded, in the samples that we tested, the Treg cells population secreted TGFβ but not IL-10 (unstimulated, blue; stimulated, red) (e). Data from three different SHIV-infected monkeys are shown.

TGFβ has been shown to be essential in mediating suppression of CTLs by impairing degranulation (62).

Discussion

Most HIV-infected persons only transiently control viral replication and ultimately progress to AIDS, despite the presence of robust CTL responses (63–65). This could be due in part to the fact that CTL responses documented in vitro do not always correlate with effective effector responses in vivo (12, 66). Additionally, although high HIV-specific CD8 T cell frequencies are maintained until late in disease, many HIV-specific T cells have a restricted ability to function (67, 68). We hypothesized in this study that the presence of high levels of gp120 in the LN contributes to the dysregulation of T cell function and assists the virus in evading the immune response. In this study we describe for the first time that high levels of gp120 are present in the LNs of SHIV-infected macaques. Furthermore, CD8+ and CD4+ T cells originating from those LNs respond poorly to stimulation with the gp120 peptide mix. The same result was not obtained in PBMCs from the same animals that were exposed to significantly lower levels of gp120 in the blood. Our data showed that the CD8+ effector cells were only

![FIGURE 6. CD4+ Treg cells were quantitated in LNs and PB of acutely SHIV-infected monkeys. Percentages are shown of CD3+ T cells expressing CD4 and CD8 markers in PBMCs (a, upper) and LNs (a, lower) of a representative naive (a, left) and SHIV-infected (a, right) macaque. The differences between PB CD3+CD4+CD25high T cells from infected and uninfected animals were not significant (p = 0.2) (b). In contrast, the percentages of CD4+CD25high T cells in SHIV-infected LN lymphocytes were significantly higher than in those LN lymphocytes derived from naive macaques (p = 0.0031; p = 0.005 without outlier) (p-values were calculated using a two-tailed Mann-Whitney U test) (c). Flow cytometric analysis of CD3+CD4+ T cells from SHIV-KB9-infected monkeys was also performed to examine the expression of CD25 and CD127 (d). Most (>80%) of the CD3+CD4+CD25high T cells were CD127low from both PB and LNs. When LN lymphocytes from SHIV-infected monkeys were stimulated with SHIV env peptide mix, the Treg cell population (gated on CD3+CD4+CD25+FoxP3+ cells) was shown to secrete TGFβ but not IL-10 (unstimulated, blue; stimulated, red) (e). Data from three different SHIV-infected monkeys are shown.]
unresponsive to stimulation with the SHIV Env peptide pool but not to nonspecific stimulation with PMA and ionomycin nor to stimulation with the core protein-derived SIV$_{mac239}$ Gag peptide mix. The defect seen in CD4$^+$ T cells was demonstrable not only in response to gp120 but also when nonspecific stimulation was used.

While there are extensive published studies on the effects of gp120 on Ab responses, surprisingly little is known about the effects of gp120 on T cells in vivo (30). In vitro studies have shown that gp120 induces movement of T cells toward (chemotaxis) or away from (fugueaxis) the protein in a concentration-dependent and CXCRI4 receptor-mediated manner (22). Others have shown that aberrant activation in infected and uninfected CD4$^+$ T lymphocytes by soluble or membrane-bound HIV envelope protein (24, 25) causes apoptosis (21, 23–26, 69). A recent study showed that HIV-1 gp120 activates immature dendritic cells in a manner that abrogates their normal function in host immune responses, and consequently disturbs the homeostatic balance of the immune response of the hosts to the infection (70). These authors suggested that HIV-1 gp120 may support sustained productive infection and transinfection of activated T cells that cluster with gp120-activated dendritic cells (70). In another study, when two L(d)-restricted epitopes derived from HIV-1 envelope gp160 (Env) and from CMV pp89 phosphoprotein were coexpressed, HIV-1$_{imm}$ Env, but not HIV-1$_{MN}$ Env variant, impaired recognition by a specific CTL of CMV pp89 epitope (71). It was also recently shown that natural mutations in an immunodominant Th epitope recognized by human CD4 clones specific for the envelope glycoprotein gp120 (from sequences of different HIV strains) escape CD4 T cell recognition. Furthermore, several natural analog peptides derived from gp120 exert an antagonistic function by inhibiting proliferative response of T cells specific to the envelope protein. If similar events occur in vivo, they may represent an additional escape mechanism for HIV (72).

Our findings support previous reports that gp120 suppresses the function of CD4$^+$ T cells (73–75). In our system, CD4$^+$ T cell suppression was observed in acutely SHIV-infected macaques, contrary to previously published reports that exposure to recombinant gp120 inhibits proliferative responses of CD4$^+$ helper T cells to various stimuli (76) in human, but not in chimpanzee, lymphocytes (77). Additionally, we demonstrated both CD4$^+$ and CD8$^+$ T cells had a decreased ability to release cytokine granules in response to gp120 as evidenced by CD107a staining. These aberrant responses to the viral envelope might be an important contributing factor to the failure of the cytotoxic lymphocytic response to clear the virus during acute infection. However, caution must be exerted when interpreting the results of this study. These experiments were done in a macaque model that utilizes SHIV-KB9, a chimeric virus that expresses the HIV-1 envelope glycoprotein on a SIV$_{mac239}$ core (28). It is unclear at this point to what extent the observed accumulation of Treg cells in the LNs of acutely SHIV-infected animals that we observed contributes to the unresponsiveness of the T cells. Previous studies have shown that removal of Treg cells from PBMCs leads to significant HIV-specific CD4$^+$ and CD8$^+$ T cells secretion of IFN-γ (78) and IL-2 (79). In PB of HIV-infected patients, the proliferative capacity of CD4 T cells to tuberculin, CMV, and p24 significantly increased following depletion of CD4$^+$ CD25$^+$ T cells. Furthermore, addition of increasing numbers of CD4$^+$CD25$^+$ T cells resulted in a dose-dependent inhibition of CD4$^+$CD25$^+$ T cell proliferation to tuberculin and p24 (80). Our results demonstrate that the accumulated Treg cells secrete TGFβ in response to stimulation with gp120. Treg cells that are induced by Ag feeding and are TGFβ dependent for their immunosuppressive activity were described for the first time as oral tolerance-mediating Th3 cells (81). These cells secrete TGFβ following Ag triggering, consequently suppressing immune responses and inducing tolerance to orally acquired Ags. TGFβ, on the other hand, can induce FoxP3 gene expression in CD4$^+$CD25$^+$ cells mediating their transition toward a Treg cell phenotype with potent immunosuppressive potential (82). Treg cell function beyond cytokine release was not assessed in our study due to limitations of cell numbers. The precise role that the accumulated Treg cells might play in the suppression of anti-HIV responses and the role that gp120 plays in this during acute infection are clearly worthy of further exploration.

The HIV-1 envelope used in this study binds both CCR5 and CXCRI4 for viral entry as opposed to mostly CCR5-tropic viruses that are usually detected in early HIV infection. Nevertheless, while most of the viruses isolated from early, asymptomatic HIV infection are CCR5-tropic, emergence of the CXCRI4-tropic strains of the virus coincides with CD4 cell depletion and progression to AIDS (83). Additionally, early infection with dual tropic viruses has been associated with CD4 depletion and progression to AIDS (84, 85). The effects of gp120 from purely CCR5-tropic HIV viruses are currently being investigated in our laboratory.

Our data support the thesis that significant amounts of HIV-1 gp120 present in lymphoid organs of acutely infected individuals dysregulate immune cell localization and function and thereby contribute a new mechanism by which the virus evades the immune system. These data also add to a growing body of evidence that supports the view that suggests that Env may have limited utility as a CD8 T cell immunogen (49, 86, 87). Additionally, given the apparent role that high levels of gp120 play in suppressing immune responses in vivo, the data may also contribute to the design of more efficacious vaccines and immunotherapies for HIV/AIDS.

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Disclosures

The authors have no financial conflicts of interest.

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