Simian immunodeficiency virus-specific cytotoxic T lymphocytes and cell-associated viral RNA levels in distinct lymphoid compartments of SIVmac-infected rhesus monkeys

Marcelo J. Kuroda, Jörn E. Schmitz, Aruna Seth, Ronald S. Veazey, Christine E. Nickerson, Michelle A. Lifton, Peter J. Dailey, Meryl A. Forman, Paul Racz, Klara Tenner-Racz, and Norman L. Letvin

Introduction

Although it is well established that levels of human immunodeficiency virus (HIV)-1 replication can differ significantly in different anatomic compartments in infected patients, we know little about the associated quantitative local differences in HIV-1–specific immune effector cells. It has long been appreciated that lymph nodes represent one of the main reservoirs for HIV-1 and that substantially more HIV-1 replication takes place in lymph nodes than in peripheral blood lymphocytes (PBL) in infected patients. Moreover, the recent demonstration in animal model studies of a bias in lentivirus infection-induced CD4+ T-lymphocyte loss in gut-associated lymphatic tissue suggests that regional differences in HIV-1 replication are likely to occur in secondary lymphoid tissue.

CD8+ cytotoxic T lymphocytes (CTL) play a major role in containing HIV-1 replication in chronically infected patients. Low virus load and stable clinical status are correlated with potent systemic CTL responses. In studies in nonhuman primates, the transient elimination of total body CD8+ lymphocytes through monoclonal anti-CD8 antibody infusions are associated with periods of high acquired immunodeficiency syndrome (AIDS) virus replication. Yet, although we appreciate the importance of CTL in containing HIV-1 spread, we know little about the regional trafficking of CTL to anatomic areas of high viral replication and about how the distribution of CTL might reflect local differences in viral replication.

To assess regional anatomic differences in HIV-1–specific CTL distribution, reproducible and quantitative assays are needed to enumerate these cells. Until recently, the detection of virus-specific CTL required the use of cumbersome, nonquantitative functional assays that measured the ability of a cell population to lyse target cells expressing viral antigen. However, it has recently been demonstrated that virus epitope-specific CD8+ CTL can be detected by flow cytometry, measuring the binding of these cells to fluorescent-labeled tetrameric major histocompatibility complex (MHC) class I–peptide complexes. This technology provides a powerful quantitative tool for detecting virus-specific CD8+ T lymphocytes in diverse anatomic compartments of an infected person.

The simian immunodeficiency virus of macaques (SIVmac)-infected rhesus monkey develops a disease similarly to HIV-1–induced disease in humans. This nonhuman primate infection provides an important animal model for the study of the immunopathogenesis of AIDS. We have made use of a dominant CTL response specific for the SIVmac Gag epitope p11C, C-M in rhesus...

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monkeys expressing the MHC class I molecule Mamu-A*01 to explore the role of CTL in containing SIVmac replication.6,13,15-17 In the current study, CTL specific for SIVmac have been characterized in various lymphoid compartments of infected, Mamu-A*01+ rhesus monkeys using both Gag peptide-specific functional CTL assays and tetrameric MHC class I–peptide complex staining techniques. Moreover, the association of these CTL with localized SIVmac replication has been assessed.

Materials and methods

Animals and viruses

EDTA-anticoagulated blood samples and lymphocytes from different lymphoid organs were obtained from euthanized rhesus monkeys (Macaca mulatta) infected with uncloned SIVmac strain 251 for more than 12 months. All rhesus monkeys used in this study were Mamu-A*01+ as determined both by polyclone chain reaction-based MHC class I typing15,16 and by functional CTL assays, as previously described.14 These animals were maintained in accordance with the guidelines of the Committee on Animals for the Harvard Medical School and with the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academic Press, Washington, DC, 1996).

Staining and phenotypic analysis of p11C, C-M–specific CD8+ T lymphocytes

Soluble tetrameric Mamu-A*01/p11C, C-M complex was made as previously described.14 The tetramer was produced by mixing biotinylated Mamu-A*01/p11C, C-M complex with phycoerythrin (PE)-labeled ExtrAvidin (Sigma Chemical, St. Louis, MO) or Alexa 488–labeled NeutrexAvidin (Molecular Probes, Eugene, OR) at a 4:1 molar ratio. The monoclonal antibodies (mAbs) used for this study were directly coupled to fluorescein (Molecular Probes, Eugene, OR) at a 4:1 molar ratio. The monoclonal antibodies (mAbs) used for this study were directly coupled to fluorescein (Molecular Probes, Eugene, OR) at a 4:1 molar ratio. The monoclonal antibodies (mAbs) used for this study were directly coupled to fluorescein (Molecular Probes, Eugene, OR) at a 4:1 molar ratio. The monoclonal antibodies (mAbs) used for this study were directly coupled to fluorescein (Molecular Probes, Eugene, OR) at a 4:1 molar ratio. The monoclonal antibodies (mAbs) used for this study were directly coupled to fluorescein (Molecular Probes, Eugene, OR) at a 4:1 molar ratio.

Cytotoxicity assay

Autologous B-LCL were used as target cells and were incubated with 5 μg/mL p11C, C-M (CTPYDINQMe), or the negative control peptide p11B (ALSEGCTPYDIN) for 90 minutes during chromium 51Cr labeling. For effector cells, peripheral blood mononuclear cells or single cells isolated from different lymphoid organs of monkeys chronically infected with SIVmac were cultured for 3 days at 4 × 10^6 cells/mL in the presence of 1 μM peptide p11C, C-M, and then were maintained for another 7 to 11 days in medium supplemented with recombinant human IL-2 (20 U/mL) (provided by Hoffman-La Roche, Nutley, NJ). Lymphocytes cultured according to this protocol were then centrifuged over Ficoll–Hypaque (Ficopaque; Pharmacia Chemical, Piscataway, NJ) and assessed as effector cells in a standard 51Cr release assay using U-bottom microtiter plates containing 10^4 target cells with effector cells at different effector:target ratios. All wells were established and assayed in duplicate. Plates were incubated in a humidified incubator at 37°C for 4 hours. Specific release was calculated as [(Experimental Release – Spontaneous Release)/ (Maximum Release – Spontaneous Release)] × 100. Spontaneous release was less than 20% of maximal release with detergent (1% Triton X-100; Sigma Chemical) in all assays.

Branched DNA quantitation of SIV RNA

SIV RNA was quantitated by a branched DNA (bDNA) signal amplification assay.18 Target probes were designed to hybridize with the pol region of the SIVmac group of virus strains, including SIVmac251, SIVmacc239, and SIVmne. SIV RNA was quantified per 10^6 CD4+ cells by comparison with a standard curve produced by purified, quantified, in vitro transcribed SIVmac239 pol RNA. The lower quantitation limit of this assay was 3,000 SIV RNA equivalents per sample.

In situ hybridization

A 35S-labeled, single-stranded, antisense RNA probe (Lofstrand Laboratories, Gaithersburg, MD) was used. Hybridization was performed on frozen

**Table 1. Binding of the tetrameric Mamu-A*01/p11C, C-M complex to freshly isolated lymph node CD8+ T cells simultaneously sampled from different anatomic compartments of SIVmac-infected, Mamu-A*01+ rhesus monkeys**

<table>
<thead>
<tr>
<th>Lymph nodes</th>
<th>Monkeys</th>
<th>PBL</th>
<th>Mandibular</th>
<th>Axillary</th>
<th>Inguinal</th>
<th>Mesenteric</th>
<th>Iliac</th>
</tr>
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<tbody>
<tr>
<td>575</td>
<td>5.3*</td>
<td>15.4</td>
<td>4.3</td>
<td>3.0</td>
<td>2.7</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>3KI</td>
<td>1.3</td>
<td>1.3</td>
<td>2.3</td>
<td>1.1</td>
<td>2.8</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>IPI</td>
<td>6.0</td>
<td>5.6</td>
<td>4.5</td>
<td>6.4</td>
<td>7.7</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>297</td>
<td>6.2</td>
<td>6.3</td>
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<td>8.6</td>
<td>6.2</td>
<td>4.9</td>
<td></td>
</tr>
</tbody>
</table>

*Percent staining by tetrameric Mamu-A*01/p11C, C-M complex on CD8+ T cells. CD8+ T cells are defined by gating on CD8+CD3- cells. p11C, C-M represents the 9-amino acid fragment optimal epitope of the SIV Gag 12-amino acid peptide p11C.
cells demonstrated similar percentages of CD8+ T lymphocytes binding the tetramer in the anatomically disparate lymph nodes of each monkey. Moreover, as we previously demonstrated in studies of single lymph nodes,19 the average percentage of tetramer-binding CD8+ T cells from the various lymph nodes sampled was remarkably similar to the percentage detected in PBL of the same monkey (Figure 1). The tonsils of these monkeys had variable levels of tetramer-binding CD8+ T lymphocytes. In 2 animals they were comparable to those observed in PBL and lymph node, whereas in the other 2 the levels were lower (Figure 1).

Distribution of tetramer-binding CD8+ T cells in secondary lymphoid compartments of SIVmac-infected rhesus monkeys

Gag epitope-specific CD8+ T cells were also quantitated in the spleens of these infected monkeys by the tetramer-binding assay. The percentage tetramer-binding CD8+ T cells was consistently greater in the spleen than in lymph nodes and PBL in all 4 monkeys studied (Figure 2). For example, the percentage of tetramer-binding CD8+ T cells in the spleen of monkey 297 was 11.7%, whereas these cells constituted 6.2% of CD8+ peripheral blood T cells (Figure 2).

Staining of CD8+ T cells from bone marrow and thymus with tetrameric Mamu-A*01/p11C, C-M complex was also assessed (Figure 2). In 3 of the 4 animals studied, the percentage tetramer-binding CD8+ T cells in bone marrow lymphocytes was even greater than that seen in splenic lymphocytes (Figure 2). In fact, 15.3% of CD8+ bone marrow T cells in monkey 297 bound the tetramer, whereas only 6.2% of CD8+ peripheral blood T lymphocytes were tetramer positive (Figure 2). The percentage tetramer-binding CD8+ T cells in the thymus was low in the 2 monkeys evaluated (Figure 2).

The functional p11C, C-M–specific lytic activity in peptide-stimulated lymphocytes was generally comparable to the percentage of tetramer-binding CD8+ T cells detected in these peptide-stimulated lymphocyte populations (Table 2). However, the ability of the freshly isolated tetramer-binding CD8+ T lymphocyte populations to expand in vitro after epitope peptide stimulation was variable. For example, tetramer-binding lymphocytes in PBL of monkey 3KI expanded from 1.3% to 61.3% CD8+ T cells, whereas lymphocytes in PBL of monkey 575 only expanded from 5.3% to 18.3% CD8+ T cells (Table 2). Because the assay used to assess functional CTL activity depends on in vitro expansion of effector T-lymphocyte populations, it was not possible to determine the correlation between functional CTL activity and numbers of freshly

### Table 2. Functional CTL activity of in vitro expanded cells correlates with tetrameric Mamu-A*01/p11C, C-M complex binding to CD8+ T cells in different secondary lymphoid compartments of SIVmac-infected, Mamu-A*01+ rhesus monkeys

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Fresh</th>
<th>p11C, C-M* expanded</th>
<th>PBL</th>
<th>Fresh</th>
<th>p11C, C-M expanded</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% tetramer</td>
<td>% lysis (5/3/1)</td>
<td>% tetramer</td>
<td>% tetramer</td>
<td>% lysis (5/3/1)</td>
</tr>
<tr>
<td></td>
<td>% tetramer</td>
<td>% lysis (5/3/1)</td>
<td>% tetramer</td>
<td>% tetramer</td>
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<td>% tetramer</td>
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</tr>
<tr>
<td>575</td>
<td>8.7%</td>
<td>29/21/15%</td>
<td>2.7</td>
<td>28.8</td>
<td>34/25/15%</td>
</tr>
<tr>
<td>3KI</td>
<td>3.0%</td>
<td>55/50/40%</td>
<td>2.8</td>
<td>43.9</td>
<td>57/51/40%</td>
</tr>
<tr>
<td>IPI</td>
<td>9.4%</td>
<td>55/54/36%</td>
<td>7.7</td>
<td>69.5</td>
<td>58/52/46%</td>
</tr>
<tr>
<td>297</td>
<td>11.7%</td>
<td>55/42/31%</td>
<td>6.2</td>
<td>69.5</td>
<td>55/53/45%</td>
</tr>
</tbody>
</table>

*p11C, C-M represents the 9-amino acid fragment optimal epitope of the SIV Gag 12-amino acid peptide p11C.
†E:T ratios.
‡Percent staining of tetrameric Mamu-A*01/p11C, C-M complex on CD8+ T cells. CD8+ T cells are defined by gating on CD8β+ CD3+ cells.
§Percent p11C, C-M-specific lysis was calculated as percent specific release by p11C, C-M-pulsed Mamu-A*01+ target cells minus the percent specific release by control peptide p11B-pulsed Mamu-A*01+ target cells.

### Results

#### Consistent distribution of tetramer-binding CD8+ T cells in lymph nodes and tonsils of SIVmac-infected rhesus monkeys

To explore the heterogeneity of distribution of SIV-specific CTL in various lymph node compartments, lymph nodes were sampled simultaneously from different anatomic locations (mandibular, axillary, inguinal, mesenteric, and iliac) in 4 chronically SIVmac-infected rhesus monkeys. Lymphocytes obtained from these nodes were evaluated for virus-specific CTL using tetramer-binding assays. Because the monkeys selected for these studies all shared the MHC class I allele Mamu-A*01, assays were performed to quantitate CD8+ T cells in the spleen of monkey 297 was 11.7%, whereas these cells constituted 6.2% of CD8+ peripheral blood T cells (Figure 2).

Consistent distribution of tetramer-binding CD8+ T lymphocytes in secondary lymphoid compartments of SIVmac-infected rhesus monkeys

Sections, as previously described.19 Sections were examined with a microscope equipped with epiluminescent illumination (Axio-phot; Carl Zeiss, Jena, Germany). Cells were considered positive for viral gene expression if the grain count was more than 6 times higher than the background count.

![Figure 2. Tetrameric Mamu-A*01/p11C, C-M complex binds to a larger percentage of spleen and bone marrow and a smaller percentage of thymic CD8+ T cells than CD8+ T cells from peripheral blood in SIVmac-infected, Mamu-A*01+ rhesus monkeys.](Image)

A whole blood specimen (PBL) and a single-cell suspension of spleen, bone marrow, and thymus from 4 SIVmac-infected, Mamu-A*01+ rhesus monkeys (575, 3KI, IPI, 297) were stained with PE-coupled tetrameric Mamu-A*01/p11C, C-M complex and analyzed by flow cytometry with gating on CD8β+ CD3+ T cells. ND, not done because of inadequate cell specimens.

![Table 2. Functional CTL activity of in vitro expanded cells correlates with tetrameric Mamu-A*01/p11C, C-M complex binding to CD8+ T cells in different secondary lymphoid compartments of SIVmac-infected, Mamu-A*01+ rhesus monkeys](Image)
isolated tetramer-binding CD8⁺ T lymphocytes in a particular cell population.

**Phenotypic characterization of tetrameric Mamu-A*01/p11C, C-M-binding CD8⁺ T cells from different lymphoid organs**

We have previously shown that the phenotypic profiles of tetrameric Mamu-A*01/p11C, C-M complex positive T lymphocytes obtained from lymph nodes and PBL are similar. Extending this phenotypic analysis to lymphocytes obtained from other lymphoid organs. Monoclonal antibody staining of both CD8⁺ T cells and CD8⁺ tetramer-binding T cells in mesenteric lymph node, spleen, bone marrow, tonsil, and thymus of the 4 SIVmac-infected rhesus monkeys was analyzed using 4-color flow cytometry. Data generated in the analyses of CD8⁺ T cells and tetramer-binding CD8⁺ T cells from these 5 anatomic compartments of all 4 monkeys are shown in Tables 3 and 4.

Phenotypic profiles of the tetramer-binding CD8⁺ T lymphocytes were similar to those of the unselected CD8⁺ T lymphocytes (Tables 3, 4). Moreover, the phenotypic appearance of the tetramer-binding CD8⁺ T lymphocytes was similar in each sampled lymphoid compartment. Very high and homogeneous expressions of CD11a, CD49d, and CD95 were seen in the expression of CD28. A high level of MHC class II-DR expression was observed on these lymphocytes from 3 of 4 animals studied. Only monkey 297 had MHC class II-DR expression on less than 50% of its lymphocytes. The MHC class II-DR expression by tetramer-binding CD8⁺ T cells in the 4 monkeys was higher than its expression on unselected CD8⁺ T cells (Tables 3, 4).

**Correlation between the percentage tetramer Mamu-A*01/p11C, C-M complex-binding CD8⁺ T cells and T-cell–associated viral RNA in lymphocytes from different anatomic locations of infected rhesus monkeys**

In view of the importance of virus-specific CTL for containing SIV replication, we sought to determine the association between the percentage of tetramer Mamu-A*01/p11C, C-M complex-binding CD8⁺ T cells and the quantity of SIV RNA in the lymphocytes sampled from these different anatomic locations in the infected monkeys. Single-cell suspensions prepared from the sampled tissues were analyzed for SIV viral RNA by bDNA assay and for SIV Gag-specific CTL by tetramer staining. Lymphocytes from 2 animals (3KI, IPI) had detectable SIV RNA copies (range, 0.017 × 10⁶-16 × 10⁶ copies/10⁴CD4⁺ cells); those from the other 2 animals (575, 297) had, for the most part, undetectable SIV RNA (less than 3 × 10⁴copies/10⁴CD4⁺ cells) (Figure 3). In concordance with these bDNA results, SIV RNA was also detected by in situ hybridization only from animals 3KI and IPI (Figure 4). Moreover, these results were consistent with the viral loads measured in the plasma of the monkeys. The 2 animals with
Surprisingly, there was no apparent correlation between the percentage of tetrameric Mamu-A*01/p11C, C-M complex-binding CD8+ T cells in a lymphoid compartment and the magnitude of the viral load in that compartment of an individual monkey (Figure 3). Furthermore, levels of detectable viral RNA in a monkey were not even correlated with our ability to detect tetramer-binding CD8+ T cells in that same animal.

**Discussion**

Data from previous studies have indicated that the extent of lentiviral replication in diverse lymphoid compartments is heterogeneous. More viral replication has been reported in lymph node lymphocytes than in PBL.7 Moreover, greater viral replication has been seen in sites in which lymphocytes are activated, such as gut-associated lymphocytes, than in relatively quiescent lymphocytes in the periphery of infected individuals.8 In fact, insufficient data were generated in the current study to confirm or refute these predictions because SIV was detected by RNA assays in lymphoid cells of only 2 of the evaluated monkeys. Nevertheless, consistent with earlier studies, substantially more viral RNA was detected in lymph nodes than in peripheral blood in those 2 animals. Contrary to predictions, however, these 2 monkeys had similar patterns of viral replication in the various lymph nodes that were evaluated. One monkey had high levels and the other had low levels of viral replication in the gut-associated lymph nodes. Overall, however, there was considerable homogeneity in the amount of viral replication detected in the various sampled lymph nodes.

With homogeneity seen in local viral replication in distinct lymph node compartments of the monkeys, an associated homogeneity was also seen in the representation of tetramer-positive CD8+CD3+ T cells in these tissues. The representation of tetramer-positive CD8+CD3+ lymphocytes in anatomically disparate lymph nodes was remarkably consistent in each individual monkey. This finding suggests that a dynamic process of CTL trafficking may obscure the tendency of CTL to localize to particular regional lymph nodes.

There were, however, consistent differences seen in the representation of tetramer-binding CD8+ T lymphocytes in various secondary lymphoid organs. Larger numbers were seen in spleen than in PBL, and even larger numbers were detected in the bone marrow (Figure 2). These differences did not reflect local variations in SIVmac replication. Rather, these findings suggested that CTL may be preferentially trapped in certain lymphoid compartments or that some lymphoid organs may provide milieus that are particularly conducive to CTL expansion.

We have previously shown that the MHC class I–SIVmac Gag peptide tetramer binding CD8+ T lymphocytes in PBL, lymph nodes, and even in semen of chronically infected rhesus monkeys are phenotypically similar.15,20 These lymphocyte populations all express activation- and memory-associated molecules, suggesting that they are active CTL. The current study indicates that the tetramer-binding CD8+ T lymphocytes in the spleen and bone marrow are also activated memory cells.

There are certainly caveats associated with the current study. The number of evaluated monkeys was small. Moreover, only a single viral epitope-specific CD8+ T lymphocyte population was evaluated. Nevertheless, this study clearly indicates that SIVmac-specific CD8+ T lymphocytes are present in similar frequencies in disparate lymph nodes but that they are present in higher frequencies in spleen and bone marrow. This study demonstrates the
usefulness of the tetramer technology to quantitate and compare antigen-specific CD8+ T lymphocytes from different lymphoid organs, and it suggests that there is no correlation between the percentage of tetramer-binding CD8+ T lymphocytes and the magnitude of the cell-associated SIV RNA level in particular lymphoid compartments of individual monkeys.

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References


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