Viral Suppression and Immune Restoration in the Gastrointestinal Mucosa of Human Immunodeficiency Virus Type 1-Infected Patients Initiating Therapy during Primary or Chronic Infection

Moraima Guadalupe,1 Sumathi Sankaran,1 Michael D. George,1 Elizabeth Reay,1 David Verhoeven,1 Barbara L. Shacklett,1 Jason Flamm,4 Jacob Wegelin,3 Thomas Prindiville,2 and Satya Dandekar1*

* Corresponding author. Mailing address: Dept. of Medical Microbiology and Immunology, GBSF, Room 5511, University of California, Davis, CA 95616. Phone: (530) 752-3409. Fax: (530) 752-7301. E-mail: sdandekar@ucdavis.edu.

Department of Medical Microbiology and Immunology,1 Department of Internal Medicine,2 and Department of Public Health Sciences,3 School of Medicine, University of California, Davis, California, and Kaiser Permanente Medical Group,4 Sacramento, California

Received 17 January 2006/Accepted 16 May 2006

Although the gut-associated lymphoid tissue (GALT) is an important early site for human immunodeficiency virus (HIV) replication and severe CD4+ T-cell depletion, our understanding is limited about the restoration of the gut mucosal immune system during highly active antiretroviral therapy (HAART). We evaluated the kinetics of viral suppression, CD4+ T-cell restoration, gene expression, and HIV-specific CD8+ T-cell responses in longitudinal gastrointestinal biopsy and peripheral blood samples from patients initiating HAART during primary HIV infection (PHI) or chronic HIV infection (CHI) using flow cytometry, real-time PCR, and DNA microarray analysis. Viral suppression was more effective in GALT of PHI patients than CHI patients during HAART. Mucosal CD4+ T-cell restoration was delayed compared to peripheral blood and independent of the time of HAART initiation. Immune phenotypic analysis showed that repopulating mucosal CD4+ T cells were predominantly of a memory phenotype and expressed CD11c, α4β7, CCR5, and CXCR4. Incomplete suppression of viral replication in GALT during HAART correlated with increased HIV-specific CD8+ T-cell responses. DNA microarray analysis revealed that genes involved in inflammation and cell activation were upregulated in patients who did not replenish mucosal CD4+ T cells efficiently, while expression of genes involved in growth and repair was increased in patients with efficient mucosal CD4+ T-cell restoration. Our findings suggest that the discordance in CD4+ T-cell restoration between GALT and peripheral blood during therapy can be attributed to the incomplete viral suppression and increased immune activation and inflammation that may prevent restoration of CD4+ T cells and the gut microenvironment.

The success of highly active antiretroviral therapy (HAART) is determined by the suppression of viral replication and increase in CD4+ T-cell numbers in peripheral blood (7, 46). However, the majority of the lymphocytes are harbored in the gut-associated lymphoid tissue (GALT) (28), which is an important early target of human immunodeficiency virus (HIV) replication and a site for severe CD4+ T-cell depletion (13, 35). Due to concerns about the potential of long-term toxicities, metabolic abnormalities, high cost, and emergence of drug-resistant variants, current guidelines recommend initiation of HAART in patients with CD4+ T-cell numbers below 350 cells/mm3 (2, 6, 19, 46). However, recent studies have shown rapid restoration of CD4+ T cells and preservation of both HIV-specific CD4+ and CD8+ T-cell responses in peripheral blood of patients initiating HAART during early HIV infection (1, 15, 21, 29, 32, 33, 42). Although the kinetics of viral suppression and CD4+ T-cell restoration in peripheral blood have been extensively investigated in HIV-infected patients undergoing HAART (8), our understanding of the impact of HAART on the restoration of the gastrointestinal mucosal immune system and function is limited.

Our previous studies have demonstrated that CD4+ T-cell depletion in GALT occurs during primary HIV infection (PHI), and a slow and incomplete gut mucosal CD4+ T-cell restoration was observed in patients starting HAART during chronic HIV infection (CHI) (13). However, the impact of initiating HAART during the primary or chronic stage of HIV infection on immune restoration and function in the gut mucosal lymphoid compartments remains largely underinvestigated. Other studies have demonstrated ongoing viral replication in GALT of patients receiving HAART, despite undetectable viral loads in the peripheral blood (3). Thus, the lack of complete viral suppression may contribute to the continuous loss of CD4+ T cells from mucosal tissues during HAART, resulting in discordant CD4+ T-cell restoration between GALT and the peripheral blood compartment. These studies highlight the importance of monitoring GALT for the assessment of antiretroviral therapies and HIV disease progression and underscore the need for investigation of gut mucosal immune restoration in HIV-infected patients initiating HAART during the early or late stage of infection.

Studies using the simian immunodeficiency virus (SIV)-infected rhesus macaque model of AIDS have demonstrated that high levels of viral replication, severe depletion of mucosal CD4+ T cells, and intestinal dysfunction in nutrient digestion and absorption occurred early during primary SIV infection (14, 24, 41, 43, 45). Functional impairment in mucosal CD4+ T cells has also been demonstrated in SIV-infected animals with incomplete suppression of viral replication in GALT during...
therapy (23). Furthermore, the initiation of antiviral therapy during primary SIV infection resulted in efficient restoration of CD4<sup>+</sup> T cells in GALT of infected rhesus macaques and correlated with the level of local viral suppression and increased gene expression of growth factors and mediators of intestinal mucosal repair and regeneration (11).

Our previous studies demonstrated that the sustained viral replication and severe CD4<sup>+</sup> T-cell depletion in GALT of chronically HIV-infected patients were coincident with increased expression of genes associated with inflammation, immune activation, and apoptosis. In addition, decreased expression of genes regulating epithelial barrier and digestive functions, mucosal repair, and regeneration was detected, suggesting disruption of the gut microenvironment (13, 34). In contrast, long-term HIV-infected nonprogressors displayed suppression of viral replication, normal levels of CD4<sup>+</sup> T cells in GALT, and a distinct gene expression profile consisting of increased expression of genes involved in mucosal repair and regeneration and decreased expression of genes associated with inflammation, cell activation, and apoptosis (34). Thus, protection against HIV disease in long-term HIV-infected nonprogressors may correlate with the level of integrity of the gut mucosal immune system and function.

To gain insights into the restoration of the gut mucosal immune system in HIV-infected patients during therapy, we determined the suppression of HIV replication, restoration of CD4<sup>+</sup> T cells, HIV-specific CD8<sup>+</sup> T-cell responses, and gene expression regulating pathological processes and antiviral immune responses in longitudinal peripheral blood and gut biopsy samples of patients initiating HAART during primary or chronic HIV infection. Our findings demonstrated that the kinetics of restoration of the gut mucosal CD4<sup>+</sup> T-cell restoration during therapy was slower than that observed in the peripheral blood compartment and independent of the time of initiation of HAART. This delay was attributed to the incomplete suppression of viral replication, persistent inflammatory and immune activation, and down modulation of growth and proliferation factors in GALT.

**Materials and Methods**

**Human subjects and sample collection.** HIV-infected patients (n = 10) participating in the study had initiated HAART during either the primary (group PHI, n = 3) or the chronic (group CHI, n = 7) stage of HIV infection. Longitudinal jejunal biopsy samples and peripheral blood samples were obtained prior to and following the initiation of HAART over a period of 36 months and assessed for the restoration of T-cell subsets and their function, viral loads, HIV-specific CD8<sup>+</sup> T-cell responses, and gene expression profiles (Table 1). Four HIV seronegative healthy individuals were enrolled in the study to provide baseline values as negative controls. Jejunal biopsy samples were collected by upper endoscopy under sedation as previously described (13, 34). Studies were performed under informed consent and an Institutional Review Board (IRB #2003110887)-approved protocol.

**Measurement of HIV loads.** HIV copy numbers in plasma samples were measured by branched DNA assay (Bayer Diagnostics, Emeryville, California) and in jejunal biopsy samples by real-time PCR as previously described (34).

**Isolation of gut lymphocytes.** Mononuclear cells were obtained from longitudinal jejunal biopsy samples according to previously published protocols (13, 24, 41). Briefly, jejunal biopsy samples were placed in lymphocyte isolation media containing RPMI 1640 (Invitrogen/Gibco BRL, Grand Island, New York), 5% fetal calf serum (Gibco BRL), 100 U/ml of penicillin (Gibco BRL), 100 U/ml of streptomycin (Gibco BRL), and 100 U/100 ml of collagenase type II (Sigma Chemical Co., St. Louis, Missouri) and subjected to shaking, at 37°C with 5% CO₂, for three periods of 30 min. Cells were then washed with RPMI media and left to rest overnight at 37°C with 5% CO₂. Cell viability (>95%) was determined by trypan blue exclusion assay.

**Immunophenotyping.** Surface analysis of immunophenotypic markers on lymphocytes from peripheral blood and GALT was performed as previously described (13, 24, 41). Briefly, peripheral blood mononuclear cells and isolated lymphocytes from jejunal biopsy samples were incubated with fluorescent mouse anti-human monoclonal antibodies for 30 min at 4°C. Cells were washed, fixed with 1% paraformaldehyde, and analyzed by four-color flow cytometry (FACS-calibur; Becton Dickinson). A minimum of 100,000 events were collected for each sample, and data were analyzed using FlowJo (Tree Star Inc., San Carlos, California).

**Monoclonal antibodies.** Antibodies used for the analysis of T cells in this study included CD3, CD4, CD28 pure, CD69, gamma interferon (IFN-γ) (Becton Dickinson, Mountain View, California); CD8 and CD11a (Caltag Laboratories, South San Francisco, California); and CD4, CXCR4, and Ki67 (Coulter Immunotech, Miami, Florida).

**HIV antigen-specific CD8<sup>+</sup> T-cell responses.** Isolated T lymphocytes from GALT and peripheral blood were placed in 96-well V-bottom plates (Costar/Corning Incorporated, Corning, New York) and stimulated with either HIV Gag peptides (BD), cytomegalovirus peptides (BD), staphylococcal enterotoxin B (SEB; Sigma), or media alone at 37°C with 5% CO₂, for three periods of 30 min. Cells were then washed, fixed with 1% paraformaldehyde, and analyzed by four-color flow cytometry (FACS-calibur; Becton Dickinson). A minimum of 100,000 events were collected for each sample whenever possible and analyzed using FlowJo (Tree Star Inc., San Carlos, California).

**Immunohistochemistry.** Immunohistochemical analysis was performed for the detection and localization of CD4<sup>+</sup> lymphocytes in jejunal biopsy samples. Tissue samples were embedded in tissue freezing medium (Triangle Biomedical Sciences, Durham, North Carolina) at the time of sample collection and flash frozen. Tissue sections were fixed in STRECK (Streck laboratories, La Vista, Nebraska) and incubated with AR10 (Biogenex, San Ramon, California) for 5 min. Nonspecific binding was blocked by reacting the slides with 10% goat serum (Jackson ImmunoResearch, West Grove, Pennsylvania) and 5% bovine serum albumin for 1 h. Tissue sections were incubated with anti-CD4 (BD clone L200; Becton Dickinson, Mountain View, California) and polyclonal anti-CD3 (Dako, Carpinteria, California) antibodies for 12 h at 4°C, followed by an incubation

**TABLE 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Group</th>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Duration of HAART (yr)</th>
<th>HAART Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>116</td>
<td>38</td>
<td>2.5</td>
<td>ABC, 3TC, NFV</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>37</td>
<td>1.2</td>
<td>ABC+3TC+ZDV, LPV+RTV</td>
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<tr>
<td></td>
<td>140</td>
<td>35</td>
<td>8 mo</td>
<td>ABC+3TC+ZDV, LPV+RTV</td>
</tr>
<tr>
<td>CHI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>120</td>
<td>39</td>
<td>2.0</td>
<td>ABC+3TC+ZDV, RTV, FPV</td>
</tr>
<tr>
<td></td>
<td>123</td>
<td>38</td>
<td>2.0</td>
<td>ABC+3TC+ZDV, RTV, APV</td>
</tr>
<tr>
<td></td>
<td>127</td>
<td>42</td>
<td>1.0</td>
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<td></td>
<td>136</td>
<td>25</td>
<td>1.0</td>
<td>EFV, 3TC, TDF</td>
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<td></td>
<td>144</td>
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<td>6.0</td>
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<tr>
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<td>145</td>
<td>54</td>
<td>5.0</td>
<td>3TC, FPV, RTV, TDF</td>
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<tr>
<td></td>
<td>132</td>
<td>53</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup> PHI, HIV seronegative patients starting HAART between 4 to 6 weeks postexposure.

* CHI, HIV seropositive patients starting HAART after 1 year post-HIV diagnosis.

**abc** Abacavir; 3TC, lamivudine; NFV, nelfinavir; ZDV, zidovudine; LPV, lopinavir; RTV, ritonavir; FPV, fosamprenavir; APV, amprenavir; EFV, etravirine; TDF, tenofovir; NVP, nevirapine; NA, not applicable; Zagaen, ABC; Epirav, 3TC; Viracept, NFV; Trizivir, ABC, 3TC, and ZDV; Kaletra, LPV and RTV; Norvir, RTV; Lexiva, FPV; Agenerase, APV; Sustiva, EFV; Viread, TDF; Viramune, NVP; Combivir, 3TC and ZDV.
with anti-mouse Cy3 (Jackson ImmunoResearch) and anti-rabbit Alexa 488 (Invitrogen, Carlsbad, California) for 1 h. The sections were mounted using Slow Fade with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were captured by confocal laser microscopy using an LSM 5 and PASCAL software (Zeiss, New York), and cells positive for both CD3 and CD4 markers were localized in the tissue samples.

**Gene expression analysis.** Intestinal mucosal gene expression was investigated by oligonucleotide microarray analysis as previously described (11, 34). Briefly, total RNA from jejunal biopsy samples was extracted using an RNAeasy kit (Qiagen, Valencia, California), and cDNA was synthesized (Superscript Choice System; Gibco Life Technologies, Rockville, Maryland) utilizing an oligo(dT) primer. Biotinylated cRNA was synthesized using a Bioarray high-yield RNA transcription labeling kit (ENZO Diagnostics, Farmingdale, New York) and purified through RNAeasy nucleic acid columns. The quality of the cRNA was evaluated by hybridization to Test3 GeneChips (Affymetrix, Santa Clara, California), and only samples whose 3′/5′ ratios were less than 3 were utilized for subsequent hybridization to HuGene U95-AV2 GeneChips (Affymetrix). The GeneChips were scanned using a laser scanner (Agilent Technologies, Palo Alto, California), and fluorescence data were processed initially through GeneChip Operating System, version 1.1 (Affymetrix). Background correction, normalization, generation of expression values, and statistical analysis of differential gene expression were performed using dChip analysis software (DNA-Chip Analyzer [dChip], version 1.3; Harvard University). Genes differentially expressed (|P| > 0.01) in GALT were hierarchically clustered to identify patterns of up (red) and down (green) regulation in response to HIV infection and HAART. The prevalence of enriched biological functions within up and down regulated genes was analyzed (EASE, version 1.0) to determine which processes were statistically overrepresented in the microarray data set.

**Statistical analysis.** Longitudinal patterns of change in CD4+ T-cell levels in HIV-infected patients following HAART were assessed using linear mixed-effects models. These models are useful in the analysis of longitudinal data in a small number of sample groups. In each model, the outcome variable represents percentage points of increase, determined as absolute CD4+ T-cell counts or the CD4+ T-cell percentage of total CD3+ T cells, with time as the predictor variable, measured by months from the initiation of HAART, and group PHI or CHI (early versus late initiation of HAART) as the covariate. These models provide, for each patient and for each group, an overall level and a rate of change in CD4+ T-cell numbers in PHI and CHI patients during HAART, which correlated with the suppression of viral load. Our results demonstrated a significantly lower (P = 0.00082) rate of CD4+ T-cell restoration in PHI patients during HAART. These results were expected, since PHI patients did not display substantial CD4+ T-cell depletion in peripheral blood prior to therapy.

**RESULTS**

**Clinical characteristics of HIV-infected patients.** HAART regimens and clinical data from all patients in the study are presented in Table 1. Patients 116, 134, and 140 were identified during primary HIV infection within 4 to 6 weeks following HIV exposure. Patients were diagnosed for HIV infection based on the viral RNA burden in plasma. All three patients (PHI) chose to initiate HAART shortly after diagnosis. At the time of enrollment, all three patients had viral loads of >500,000 copies/ml and >500 cells/mm3 absolute CD4+ T-cell numbers in the peripheral blood (Fig. 1A).

Patients 120, 123, 127, 133, and 136 were chronically HIV-infected individuals (CHI) who had viral loads of >50,000 copies/ml of plasma and <300 CD4+ cells/mm3 in peripheral blood prior to the initiation of HAART and were known to be HIV positive for >1 year (Fig. 1B). Patients 144 and 145 (CHI) were enrolled in the study after receiving therapy for a period of 5 to 6 years and served as long-term HAART controls. Therefore, longitudinal samples prior to and immediately after HAART were not available from these patients for analysis. These patients had undetectable viral loads, >500 cells/mm3 CD4+ T cells in peripheral blood (data not shown) and normal levels of CD4+ T cells in jejunal mucosal biopsy samples (43% and 65%, respectively).

**Rapid suppression of HIV replication and maintenance of CD4+ T cells in peripheral blood of patients initiating HAART during primary infection.** To determine whether the timing of the initiation of HAART influenced the level of viral suppression and CD4+ T-cell restoration in peripheral blood, we compared viral loads and CD4+ T-cell numbers in PHI and CHI patients. Prior to the initiation of HAART, both PHI and CHI patients displayed high plasma viral loads of >50,000 RNA copies/ml (Fig. 1A). PHI patients 116 and 140 had undetectable viral loads following 4 months of therapy, while patient 134 had <400 RNA copies/ml. In contrast, four of the five CHI patients still had detectable viral loads (347 to 6,682 RNA copies/ml) at 3 to 4 months post-HAART (Fig. 1B). Thus, the initiation of HAART during primary HIV infection resulted in a more rapid and sustained suppression of viral burden in the peripheral blood than the initiation of therapy during chronic HIV infection.

PHI patients did not experience marked CD4+ T-cell depletion in peripheral blood prior to initiation of HAART and maintained relatively high CD4+ T-cell numbers (510 to 1,146 cells/mm3) during HAART (Fig. 1A). In contrast, a gradual but steady increase in CD4+ T-cell numbers was seen in most CHI patients during HAART, which correlated with the suppression of viral loads. Patient 127 experienced a rebound in viral loads after 6 months of therapy and did not show progressive increase in the peripheral CD4+ T-cell numbers that correlated with lack of effective suppression of plasma viral burden (Fig. 1B). To determine differences in the rate of CD4+ T-cell restoration in peripheral blood of PHI and CHI patients, we evaluated the longitudinal patterns of change in CD4+ T-cell levels, measured as an average change in percentage points per month (Fig. 1C). Our results demonstrated a significantly lower (P = 0.00082) rate of CD4+ T-cell restoration in PHI patients during HAART. These results were expected, since PHI patients did not display substantial CD4+ T-cell depletion in peripheral blood prior to therapy.

**Incomplete viral suppression and delayed CD4+ T-cell restoration in GALT of HIV-infected patients during HAART.** To determine the effects of initiating HAART during primary or chronic HIV infection on viral suppression and restoration of CD4+ T cells in GALT, we evaluated HIV burden and distribution and function of CD4+ T-cell subsets in longitudinal jejunal biopsy samples from PHI and CHI patients. While both PHI and CHI patients had detectable HIV RNA levels in GALT prior to the initiation of therapy, the level of viral suppression was different between PHI and CHI patients during HAART (Fig. 2A). Viral loads were undetectable in GALT of PHI patients at 6 months post-HAART and remained undetectable thereafter. In contrast, suppression of viral replication was incomplete in GALT of CHI patients, suggesting that initiation of HAART during primary HIV infection may result in a more rapid and sustained suppression of viral replication in GALT than initiation during chronic HIV infection.

Both CHI and PHI patients displayed severe depletion of CD4+ T cells in GALT prior to HAART that was accompanied by an increase in the percentage of mucosal CD8+ T cells (Fig. 2B and C). During the first year of HAART, restoration of gut mucosal CD4+ T cells was slow in both groups of
patients and CD8+ T-cell percentages remained elevated in most patients, demonstrating that T-cell homeostasis in GALT was not normalized. Despite a modest level of mucosal CD4+ T-cell restoration during the initial 8 months of HAART, patient 116 (PHI group) experienced a strong and steady increase in gut mucosal CD4+ T-cell percentages (from 16% to 55%) during the course of therapy (Fig. 2B). PHI patients 134 and 140 were monitored for 8 and 14 months post-HAART, respectively. Both of these patients opted to discontinue HAART thereafter. CD4+ T-cell depletion in GALT of these patients (28% and 23%, respectively) was not as severe as that observed in patient 116 (7%) prior to therapy, and neither patient displayed a significant change in CD4+ T-cell levels in GALT during therapy (<14 months). We hypothesize that viral replication was not fully suppressed in these patients and may have contributed to the lack of CD4+ T-cell restoration during the initial 1 year of therapy.

None of the patients in the CHI group were able to restore mucosal CD4+ T cells to normal levels during HAART (Fig. 2C). After 24 months of HAART, patients 120 and 123 experienced a minimal increase in CD4+ T-cell levels. Patient 133 exhibited modest restoration of mucosal CD4+ T cells initially but then experienced a substantial loss of CD4+ T cells after 12 months of HAART (Fig. 2C). Patient 127 experienced fluctuations in the levels of mucosal CD4+ T cells, possibly due to a documented lack of adherence to the HAART regimen. Patient 136 maintained mucosal CD4+ T cells but showed a decline in the CD4+ T-cell levels at 12 months post-HAART. HIV RNA was detected in jejunal biopsy samples from these patients at various time points during therapy,
suggesting that the incomplete suppression of viral replication in GALT might have contributed to poor restoration of gut mucosal CD4⁺ T cells.

**Repopulating CD4⁺ T cells were localized to mucosal effector sites.** We performed fluorescence immunohistochemistry on jejunal biopsy samples to detect and localize the CD4⁺ T cells repopulating GALT during HAART (Fig. 3). While the CD3⁺ CD4⁺ T cells (yellow) were uniformly scattered throughout the villi and in the crypts of uninfected individuals, severe depletion of CD4⁺ T cells throughout the villi and crypts was observed in HIV-infected patients, with only a few CD4⁺ T cells in a nonspecific pattern near the base of villus. Major aggregates of lymphocytes, likely to be CD8⁺ T cells (CD3⁺ CD8⁺) (green), were detected near the epithelial layer surrounding the villus. At 6 months post-HAART, most of the repopulating CD4⁺ T cells were detected in the crypt region at the base of the villus, with a few cells in the villus. Although patients on long-term therapy (>2 years) showed uniformly scattered CD4⁺ cells in the crypts and villi, the numbers of cells were still lower than those detected in uninfected healthy controls. These data demonstrated that CD4⁺ T cells were depleted from the villus effector sites during HIV infection and that these sites were not adequately replenished even during long-term therapy.

**Immunophenotypic characteristics of CD4⁺ T cells repopulating GALT during HAART.** Immunophenotypic and functional characteristics of CD4⁺ T cells repopulating GALT during HAART were evaluated by determining the expression of
CD45RA and CD45RO (naive/memory markers), CCR5 and CXCR4 (HIV coreceptors), LFA-1 (CD11a), and αEβ7 (trafficking markers) in comparison to healthy HIV-negative controls. The majority of CD4+ T cells in GALT of uninfected healthy individuals were of the memory phenotype expressing CD45RO+ (>90%) (data not shown). While the naive phenotype (CD45RA+) predominated in the CD4+ and CD8+ T-cell subsets (>45% and >70%, respectively) in peripheral blood prior to and following HAART, the majority of the CD4+ T cells in GALT (>90%) during HAART were of a memory phenotype (Fig. 4A).

The expression of CCR5 and CXCR4 was detected on the CD4+ T cells repopulating GALT during HAART in HIV-infected patients (Fig. 4B). However, in both CHI and PHI patients, the percentages of CCR5+ (16% and 8%, respectively) and CXCR4+ CD4+ T cells (10% and 10%, respectively) were significantly lower than in healthy uninfected controls (38% and 51.0%, respectively). The expression of HIV coreceptors on repopulating CD4+ T cells in GALT highlights their susceptibility to infection by both R5- and X4-tropic viruses and provides further evidence that ongoing viral replication may impair the efficacy of the restoration.

The expression of the lymphocyte trafficking molecules, LFA-1 and integrin αEβ7, was also analyzed in CD4+ T cells repopulating GALT during therapy (Fig. 4B). Although levels of αEβ7 expressing CD4+ T cells were similar in PHI and CHI patients, increased levels of LFA-1-expressing mucosal CD4+ T cells were observed in PHI (56%) compared to CHI patients (27%). No significant change was observed in the levels of CD4+ T cells expressing Ki67, a cell proliferation marker (<3%) (data not shown). These findings suggest that increased mucosal T-cell trafficking and not local proliferation may be the major mechanism of CD4+ T-cell repopulation in GALT during early stages of HAART.

HIV-specific CD8+ T-cell responses in GALT and peripheral blood of HIV-infected patients during HAART. To examine the correlation between the HIV-specific antiviral cellular responses in GALT and viral suppression during therapy, HIV
Gag-specific CD8\(^+\) T-cell responses were measured from both PHI and CHI patients at various time points post-HAART. Gag-specific CD8\(^+\) T-cell responses were detected in GALT of both PHI and CHI patients with detectable viral loads in GALT at the time of the sample collection (Fig. 5). One patient (123) in particular displayed strong HIV-specific responses and had significantly higher viral loads in GALT at the time of sample collection. These data suggested that incomplete suppression of viral replication in GALT led to increased levels of HIV Gag-specific CD8\(^+\) T-cell responses.

**Mucosal gene expression profiles in GALT may be predictive of response to HAART.** To determine the molecular mechanisms involved in the restoration of the gut mucosal immune system during HAART, we compared changes in the mucosal gene expression profiles of PHI and CHI patients. We hypothesized that changes in gene expression patterns in the gut microenvironment may precede detectable changes at the cellular and tissue levels. Therefore, we compared the gut mucosal gene expression profiles prior to HAART and at 3 months posttherapy in two patients who showed efficient mucosal CD4\(^+\) T-cell restoration (116 and 133) with two patients who displayed poor mucosal CD4\(^+\) T-cell restoration following HAART (120 and 123). Hierarchical clustering of differentially expressed genes in these patients revealed a distinct pattern of upregulated gene expression in those patients with poor CD4\(^+\) T-cell reconstitution (120 and 123) after 3 months of HAART. This included increased expression of genes involved in innate and acquired immune responses, apoptosis, and cell injury (Fig. 6). Genes associated with stress and inflammation were also upregulated in patients with poor CD4\(^+\) T-cell repopulation, including Toll-like receptors 1 and 5, myxovirus resistance proteins, and numerous interferon-induced factors (Fig. 7A). In contrast, patients 116 and 133 showed a marked decrease in the level of immune and inflammatory response-related genes at 3 months posttherapy. Thus, molecular processes leading to immune activation and tissue damage dominated the gut microenvironment in patients who failed to restore mucosal CD4\(^+\) T cells.

In agreement with recent reports (34), we found decreased expression of genes involved in digestive and absorptive functions, lipid metabolism, and xenobiotic metabolism in all HIV-infected patients (Fig. 7B). Prior to therapy, the degree of suppression appeared more severe in patients 120 and 123. This functional category included genes involved in lipid, carbohydrate, and steroid metabolism as well as large- and small-molecule transporter proteins and catabolic factors. Importantly, several cytochrome P450 family members involved in metabolism of HAART compounds were substantially downregulated, indicating a potential to impact the efficacy of therapy.

Viral loads were readily detectable in GALT of patient 123 after 6 months of therapy (Fig. 8), coinciding with increased expression of genes associated with inflammatory response and immune activation in GALT (RANTES, NF-κB, multiple interferon response factors, and Mx-2). In contrast, patient 116 maintained a relatively normal to minimally decreased expression of these genes in GALT at 6 months post-HAART. Patient 116 also displayed increased expression of genes involved in mucosal trafficking (alpha integrins 4 and E, ICAM, and VCAM) and growth (Ki67, platelet and transforming growth factors, and trefoil factor 3). In summary, mucosal gene expression profiles prior to therapy differentiated patients who progressed to restore gut mucosal CD4\(^+\) T cells poorly or efficiently during the first few months of therapy. These data suggest that the extent of inflammation, cell activation, and apoptosis may be indicative of the degree of disruption in the mucosal microenvironment prior to therapy and may directly impact the efficacy of viral suppression and CD4\(^+\) T-cell restoration during therapy.

**DISCUSSION**

In the present study, we investigated the effects of HAART on the restoration of the gut mucosal immune system compared to the peripheral blood in HIV-1-infected patients. A longitudinal analysis of virologic, immunologic, and molecular characteristics of patients initiating HAART during primary or chronic HIV infection indicated that the degree of mucosal CD4\(^+\) T-cell restoration was associated with the level of viral suppression and the severity of disruption of the gut microenvironment, independent of the time of initiation of therapy. Our findings suggest that initiation of HAART prior to the manifestation of severe enteropathy may support effective restoration of the mucosal immune system. Thus, monitoring of gut lymphoid tissue will be important for an accurate assessment of disease progression and the efficacy of antiretroviral therapy. Although the numbers of patients in our patient cohorts were relatively small, the strength of this investigation lies in the longitudinal evaluation of the patients in the study. Future studies involving larger numbers of patients in the study groups will increase the statistical significance of our observa-

FIG. 5. HIV-specific CD8\(^+\) T-cell responses in GALT and peripheral blood of HIV-infected patients during HAART. HIV-specific CD8\(^+\) T-cell responses were detected in both GALT and peripheral blood of HIV-infected patients during HAART using intracellular cytokine flow cytometry.
tions and determine the variation inherent within different patient groups.

Our previous studies have shown that the severity of CD4⁺ T-cell depletion and viral burden in GALT are not adequately reflected in the peripheral blood of HIV-infected patients (13). In this study, we also showed that the course of CD4⁺ T-cell restoration in GALT during HAART was slow with biphasic kinetics compared to the peripheral blood compartment of both PHI and CHI patients (Fig. 2A and B). Since impaired thymic functions and disruption of lymph node architecture have been well documented in patients with chronic HIV infection (18, 39, 44), it was not surprising to observe the delay in CD4⁺ T-cell restoration in GALT of CHI patients. On the other hand, the delay in CD4⁺ T restoration was observed in GALT of patients beginning HAART during primary HIV infection, despite having normal CD4⁺ T-cell numbers and undetectable viral loads in peripheral blood. A possible explanation for this delay could be attributed to the severe pathological changes occurring in GALT very early during primary HIV infection. Gene expression studies indicated that patients displaying modest CD4⁺ T-cell restoration during HAART also displayed increased expression of genes involved in inflammation, innate and cell-mediated immune responses, and response to wounding prior to HAART (Fig. 6). Thus, a significant time period may be required to repair local tissue damage in the intestinal mucosal microenvironment before substantial CD4⁺ T-cell restoration could occur. Supporting evidence is provided by studies with the SIV-infected rhesus macaque model which demonstrated severe CD4⁺ T-cell depletion and histopathologic and functional changes in GALT during the early stages of SIV infection (14, 43). Our previous studies have also reported acute enteropathy in SIV-infected rhesus macaques during primary SIV infection accompanied by decreased nutrient digestive and absorptive functions that correlated with a high level of viral replication in GALT (14, 43, 45). Loss of epithelial growth and repair-associated gene expression was also observed in GALT during primary SIV infection (12). These findings suggested that enteropathogenic changes occurring early in viral infection might lead to a delay in the restoration during therapy.

Multiple mechanisms could contribute to the discordant results of CD4⁺ T-cell restoration between GALT and peripheral blood during therapy. These include incomplete suppression of viral replication and impaired immune functions in GALT compared to peripheral blood. Anton and colleagues have previously reported ongoing viral replication in GALT of
some HIV-infected patients receiving HAART, despite undetectable plasma viral loads (3). Thus, the ongoing viral replication may lead to infection and killing of CD4+ T cells trafficking to the gut mucosa during HAART. CD4+ T lymphocytes in GALT have dual expression of the HIV coreceptors CCR5 and CXCR4 and are naturally permissive to HIV infection by both CCR5-tropic and CXCR4-tropic variants (17, 25–27, 31, 40). In this study, we demonstrated that repopulating CD4+ T cells in GALT during HAART expressed low levels of the HIV coreceptors CCR5 and CXCR4, thus becoming potential targets for HIV infection. This poses a major challenge for the control of viral replication and immune restoration of the gut mucosa during HAART. The incomplete suppression of HIV replication in GALT following HAART may contribute to the ongoing infection of the repopulating CD4+ T cells, causing the delay in the restoration of T-cell homeostasis in GALT.

In the SIV model, initiation of antiretroviral therapy during early SIV infection led to effective viral suppression and near complete restoration of CD4+ T cells in GALT that correlated

![Figure 7: Mucosal gene expression profiles in patients with divergent CD4+ T-cell restoration during HAART. Gene expression profiles were determined in GALT biopsy samples pre-HAART (Pre) and at 3 months post-HAART (3 mo) by DNA microarray analysis and genes associated with inflammation and immune activation (A) and lipid metabolism and nutrient digestive and absorption (B) were identified.](http://jvi.asm.org/)

![Figure 8: Suppression of viral replication and increased gene expression of mucosal repair and regeneration during CD4+ T-cell repopulation in GALT. Data are presented at 6 months post-HAART for patient 116 (efficient CD4+ T-cell restoration) and patient 123 (poor CD4+ T-cell restoration).](http://jvi.asm.org/)
with increased expression of genes associated with mucosal regeneration and repair (11). In animals with incomplete suppression of viral replication in GALT during therapy, mucosal CD4⁺ T cells were found to be functionally impaired (23). Thus, suppression of viral replication and dampening of immune activation and inflammatory processes in GALT during therapy may be crucial to achieve effective restoration of the gut mucosal immune system in HIV and SIV infections. Our recent studies showed that decreased expression of genes associated with absorptive and digestive functions and drug metabolism were detected in chronically HIV-infected patients (11, 12, 34). The access to antiretrovirals in the gut mucosal tissue may be less efficient than in peripheral blood or other lymphoid compartments, and this may partly contribute to the incomplete suppression of viral replication in GALT during therapy.

Expression of mucosal homing markers on T lymphocytes is crucial for their trafficking to gut mucosal tissue. Previous studies have also demonstrated depletion of CD4⁺ T cells expressing αEβ7 in GALT of chronically infected HIV patients (37). In the SIV model, CD4 T cells expressing αEβ7 have primarily a memory phenotype and become rapidly depleted, as early as 1 week postinfection, and remain depleted during chronic stages in the absence of antiviral therapy (22). Evaluation of the phenotypic characteristics of CD4⁺ T cells repopulating GALT during HAART showed that the majority of these cells were of memory phenotype and expressed lymphocyte trafficking markers αEβ7 and LFA-1. Our results demonstrated that the levels of memory CD4⁺ T cells expressing LFA-1 in PHI patients was higher than that observed in CHI patients. However, both CHI and PHI patients had decreased levels of LFA-1⁺ CD4⁺ T cells in comparison to healthy uninfected individuals, demonstrating that impairment in mucosal homing may occur early in HIV infection. These data suggested that initiation of therapy before the immune system becomes severely compromised may help preserve the homing potential of memory T cells to mucosal tissues.

Antiviral CD8⁺ cytotoxic T-cell responses in mucosal tissues of HIV-infected patients have been shown to produce high levels of granzyme A but low levels of perforin (36), suggesting that HIV-specific CD8⁺ T-cell responses may not be fully functional in chronically infected patients (4, 5, 9, 20, 30). In agreement with previous studies, gene expression profiling indicated that although granzymes, T-cell receptors, and major histocompatibility complex class I molecules were up regulated, we could not detect up regulation of perforin in our HIV-infected patient cohorts. These data suggest that elevated, nonspecific CD8⁺ T-cell responses may contribute to the impairment of CD4⁺ T-cell restoration. We observed the presence of HIV Gag-specific CD8⁺ T-cell immune responses in both the peripheral blood and GALT of PHI and CHI patients during HAART. It was interesting that, in the PHI group, patient 116 did not have detectable HIV-specific CD8⁺ T-cell responses despite the complete restoration of mucosal CD4⁺ T cells and T-cell homeostasis (normal levels of CD8⁺ T cells) and undetectable viral loads in both peripheral blood and GALT. In contrast, patients 134 and 140 had relatively high Gag-specific CD8⁺ T-cell responses but did not experience CD4⁺ T-cell restoration and showed elevated CD8⁺ T-cell percentages in GALT. These data suggested that despite effective suppression of viral loads in the peripheral blood of PHI during HAART, a similar level of viral suppression might not have been achieved in GALT of these two patients, as reflected by the slow to modest increase in CD4⁺ T cells and high levels of viral antigen-specific responses. However, efficient viral suppression in GALT during HAART may lead to rapid restoration of mucosal CD4⁺ T cells, as was seen in patient 116. We hypothesized that a very low level of viral replication in GALT that was not detected by real-time reverse transcription-PCR was sufficient to induce antiviral responses in these patients. Thus, the high levels of HIV Gag-specific mucosal CD8⁺ T-cell responses might be indicative of incomplete viral suppression in GALT.

The gene expression analysis of mucosal samples in patients with efficient CD4⁺ T-cell restoration in GALT showed a correlation between decreased gene expression associated with inflammation and cell activation and better CD4⁺ T-cell restoration. In addition, the expression of genes involved in inflammatory responses, such as NF-κB, were largely normalized when HAART was initiated during PHI. Recent studies have demonstrated that control of HIV replication in the peripheral blood of HIV-infected patients receiving HAART was associated with high levels of HIV-specific interleukin-2 and IFN-γ-producing CD4⁺ T cells and low levels of T-cell activation (10). The low level of activation state could support the expansion of the cell populations in response to HIV but not deplete them due to hyperimmune activation. Thus, a relatively low level of cell activation state may support the survival of the quiescent pool of T cells. Our recent studies showed that drug-naive long-term HIV-infected patients had maintained normal CD4⁺ T-cell percentages and undetectable viral loads in GALT, which correlated with control of gene expression related to inflammation and cell activation (34). These results provided further evidence that chronic immune activation and inflammation and failure to control viral replication in mucosal tissues may be associated with impaired ability to restore the CD4⁺ T cells in GALT.

Our findings suggested that suppression of viral replication and control of inflammatory responses in GALT determine the level of restoration of the mucosal immune system during HAART. Previous studies in both peripheral blood and lymph nodes of HIV-infected patients have suggested that early HAART may avert the progressive immunologic damage associated with untreated HIV infection (1, 15, 21, 29, 32, 33, 42). However, limited information is available about the effects of HAART on viral suppression and restoration of T-cell homeostasis in gut mucosal tissues during HIV infection. In this study, we have demonstrated that pathological processes that occur in GALT during primary HIV infection may profoundly influence the characteristics of host antiviral responses and the integrity of the gut microenvironment. This may play a significant role in the degree of mucosal CD4⁺ T-cell restoration during the course of therapy. Our results emphasize the importance of monitoring GALT in the evaluation of immune restoration during therapy as well as the need for developing novel therapeutic strategies to enhance the repair and regeneration of the mucosal immune system.
ACKNOWLEDGMENTS

We thank all of our patients for their participation in the study and the nursing staff at the gastroenterology clinic, especially Tresha Reed for her invaluable help with patient management and scheduling. We also thank Thomas Ndolo for critical review of the manuscript.

This study was supported by the National Institutes of Health grants DK-61297, AI-43274, AI-057200, and the Universitywide AIDS Research Project CH05-D-006.

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