Evidence for Translocation of Microbial Products in Patients with Idiopathic CD4 Lymphocytopenia

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Translocation of microbial products has been described in chronic human immunodeficiency virus (HIV) infection and correlates with activation of the immune system. We investigated the potential translocation of microbial products in idiopathic CD4 lymphocytopenia (ICL), a rare disorder characterized by low CD4 T cell counts in the absence of HIV infection. Plasma lipopolysaccharide (LPS) levels and T cell activation were measured in a cross-sectional cohort study of patients with ICL and HIV infection and healthy control subjects. Increases in CD4 T cell proliferation but not CD8 T cell proliferation were observed in patients with ICL. LPS levels were significantly elevated both in patients with ICL and in patients with HIV infection, and they were strongly correlated with the proportion of proliferating CD4 T cells in the cohort of patients with ICL (r = 0.88; P = .003). The proportions of T helper (Th) 17 and Th1 CD4 cells in peripheral blood were similar between patients with ICL, patients with HIV infection, and control subjects. These findings suggest a potential association of translocation of microbial products with perturbed CD4 T cell homeostasis in individuals with CD4 lymphopenic states other than HIV infection.

Idiopathic CD4 lymphocytopenia (ICL) is a rare syndrome of unclear etiology [1, 2]. The working definition of ICL, as determined by the US Centers for Disease Control and Prevention in 1992, is repeated peripheral CD4 T lymphocyte counts of <300 cells/µL or <20% of total lymphocytes in the absence of human immunodeficiency virus (HIV) type 1 (HIV-1) or HIV type 2 (HIV-2) infections or other known causes of immunodeficiencies [3]. The characteristics of ICL are similar to those of HIV infection and include patient susceptibility to opportunistic infections, perturbations of T cell homeostasis with evidence of chronic immune activation, and loss of naive T cells confined predominantly to the CD4 T cell subset [4].

Translocation of microbial products from the gastrointestinal tract, or “leaky” gut, has been implicated as one potential cause of chronic immune activation in HIV-infected patients [5–8]. Previous studies have shown increased lipopolysaccharide (LPS) levels (a quantitative measurement of the degree of translocation) in patients with HIV viremia, compared with healthy volunteers [8, 9]. This finding has also been described in individuals with other disease processes, such as graft-versus-host disease and inflammatory bowel disease, and in persons who have undergone gastrointestinal surgery [10, 11]. LPS is a component of the gram-negative bacterial cell wall that is thought to induce immune activation in the periphery by binding to CD14+ monocytes and macrophages, thereby causing stimulation through the TLR4/MD2 pathway and producing soluble CD14 (sCD14) consequentially [12–14].
Although there is significant CD4 T cell depletion in the gastrointestinal mucosa during acute and chronic HIV infection [15–19], the phenomena that allow microbial translocation to occur remain unclear [8]. Interestingly, in mouse models of lymphocytopenia and lymphocytopenia-induced proliferation, it has been shown that endogenous flora can play a significant role in enhancing T cell expansion in the chronically immunodeficient host [20]. In the present study, we hypothesized that microbial products in plasma may be the result of CD4 T cell depletion in the gastrointestinal tract and/or lymph nodes or may be the by-product of endogenous flora–stimulated lymphocytopenia-induced T cell proliferation, regardless of HIV infection. To address this hypothesis, we studied whether evidence of translocation of microbial products was present in individuals with ICL.

**PATIENTS, MATERIALS, AND METHODS**

**Patients.** A cross-sectional study was implemented that included 8 HIV-negative volunteers (control subjects), 10 HIV-infected patients not receiving antiretroviral therapy, and 11 patients with ICL. This research study was approved by the institutional review board of the National Institute of Allergy and Infectious Diseases, and written informed consent was obtained from all participants. Characteristics of the study participants are shown in table 1. Patients with ICL were recruited between 1992 and 2006 nationwide. Inclusion criteria were (1) at least 2 confirmed CD4 T cell counts of $\geq 300$ cells/mm³ or (2) $<20\%$ of total lymphocytes, no serologic evidence of HIV-1 infection, and no coexisting condition thought to be a likely cause of lymphocytopenia [4]. Only participants with ICL for whom nonheparinized stored plasma samples were available were included in the study. Study participants with ICL were not acutely ill and were receiving a stable antimicrobial regimen if they had an underlying infection. Two patients with ICL did not have any known infection, 1 had a history of esophageal candidiasis, 2 had a history of cryptococcal disease, 2 had disseminated human papillomavirus disease, 2 had disseminated infection with *Mycobacterium avium* complex (1 of these 2 patients also had a history of esophageal candidiasis), 1 had disseminated histoplasmosis, and 1 had pulmonary infection due to *Mycobacterium chelonae*. CD4 T cell counts were measured in a Clinical Laboratory Improvement Amendments–approved laboratory, and HIV RNA levels were measured using an ultrasensitive branched DNA assay (Versant HIV-1, version 3.0; Siemens).

**Lymphocyte immunophenotyping.** Immunophenotyping by flow cytometry was conducted on viable cryopreserved peripheral blood mononuclear cells (PBMCs). The fluorochrome-conjugated antibodies used were anti-CD3 fluorescein isothiocyanate (FITC), anti-CD4 peridinin chlorophyll protein (PerCP) or allophycocyanin (APC), anti-CD8 PerCP, anti-CD45RO APC, anti-CD27 FITC, anti-CD14 PE, anti-Ki67 PE or FITC, anti–HLA-DR FITC, anti-CD25 PE, anti-CD27 FITC (all from BD Biosciences) and were analyzed using FlowJo software (version 8; Treestar).

**LPS and sCD14 assays.** LPS levels were quantified by taking fasting plasma samples that had been collected in EDTA tubes and diluting them to 10% with endotoxin-free water; plasma proteins were subsequently heat inactivated at 80°C for 15 min. Measurements of the samples were done using a Limulus amebocyte assay (Lanza). Along with LPS levels, soluble CD14 levels were measured to establish chronic LPS stimulation [12]. Soluble CD14 plasma levels were quantified using commercially available ELISA assays (R&D Systems). Samples were measured in duplicate, and background was subtracted.

**Intracellular cytokine T cell assays.** Cryopreserved PBMCs were thawed and resuspended at $10^6$ cells/mL in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (Gemini Bio-tech). Benzonase Nuclease (25 U/mL; Novagen) was used while washing the cells. T cells were stimulated using 50 ng/mL phorbol myristate acetate (Sigma) and 1 μg/mL ionomycin (Sigma) for 6 h in the presence of 1 μg/mL Brefeldin A (Sigma) and anti-

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**Table 1. Clinical characteristics of study participants.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control subjects (n = 8)</th>
<th>HIV-infected patients (n = 10)</th>
<th>Patients with ICL (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>45 (25–55)</td>
<td>48 (36–51)</td>
<td>38 (25–66)</td>
</tr>
<tr>
<td>Sex, male subjects:female subjects</td>
<td>3:5</td>
<td>8:2</td>
<td>6:5</td>
</tr>
<tr>
<td>CD4 T cell count, cells/μL</td>
<td>634 (335–1377)</td>
<td>217 (6–774)</td>
<td>116 (12–297)</td>
</tr>
<tr>
<td>CD4 cells, %</td>
<td>43 (34–58)</td>
<td>14 (1–32)</td>
<td>16 (7–38)</td>
</tr>
<tr>
<td>CD8 T cell count, cells/μL</td>
<td>365 (173–598)</td>
<td>983 (217–1742)</td>
<td>148 (101–382)</td>
</tr>
<tr>
<td>CD8 cells, %</td>
<td>24 (14–28)</td>
<td>60 (36–79)</td>
<td>39 (20–59)</td>
</tr>
<tr>
<td>CD4:CD8 ratio</td>
<td>1.74 (1.36–4.15)</td>
<td>0.23 (0.08–0.58)</td>
<td>0.78 (0.39–1.84)</td>
</tr>
<tr>
<td>HIV RNA level, copies/mL</td>
<td>NA</td>
<td>15,899 (2082–381,717)</td>
<td>NA</td>
</tr>
</tbody>
</table>

*NOTE.* Data are the median value (interquartile range), unless otherwise indicated. NA, not applicable.
CD4 (eBioscience) PE-Cy5.5. All cells were surface stained for phenotypic markers (anti-CD8 [BD] Pacific Blue, anti-CD27 [Beckman Coulter] Cy5 PE, and anti-CD45RO [Beckman Coulter] Texas Red PE), fixed, and permeabilized. Next, the cells were stained intracellularly for cytokines (anti–interleukin [IL]–17 [eBioscience] PE, anti–tumor necrosis factor [TNF] [BD] Cy7 PE, anti-CD3 [BD] Cy7 APC, anti–IL-2 [BD] APC, and anti–interferon [IFN]–γ [BD] FITC) and then were stained (with aqua-blue amine reactive dye) for viability. Stained samples were acquired on a FACSAria flow cytometer operated using FACS DiVa software, version 6 (both from BD Biosciences) and were analyzed using FlowJo software (version 8; Treestar). Frequencies of live memory CD4\(^+\) T cells (CD3\(^+\)aqua-blue CD4\(^+\)) that responded to stimulation were measured after subtraction of background values. Production of individual cytokines by all live CD3\(^+\)CD4\(^+\) memory T cells (excluding CD27\(^-\)CD45RO\(^-\) cells) was measured.

**Statistical analysis.** The median values of all measured variables for the 3 groups were compared using nonparametric methods (the Kruskal-Wallis test, for 3-group comparisons, and the Mann-Whitney U test, for 2-group comparisons), with the use of Prism software (version 4.0c; GraphPad). Associations were assessed by Spearman’s correlation. Because of the exploratory nature of the study, there was no correction for multiple comparisons, and calculated P values are reported.

### RESULTS

#### Study participants.

The characteristics of the study participants are shown in table 1. CD4 T cell counts both in HIV-infected patients and in patients with ICL were lower than those in control subjects (P = .002 and P < .001, respectively) (table 1). CD8 T cell counts in HIV-infected patients were significantly higher than those in control subjects (P < .001). In addition, patients with ICL had CD8 T cell counts that were lower than those of control subjects (P = .002).

**T cell immunophenotyping.** CD4 T cells from HIV-infected individuals had higher levels of cell activation (as measured by co-expression of HLA-DR and CD38) and proliferation (as measured by Ki67\(^+\)) than did CD4 T cells from control subjects (P < .001 [figure 1A] and P = .003 [figure 1C], respectively). Similarly, patients with ICL had higher frequencies of activated and proliferating CD4 T cells than did control subjects (P = .002 [figure 1A] and P < .001 [figure 1C], respectively). CD4 T cell proliferation and activation did not differ significantly between HIV-infected patients and patients with ICL. Patients with ICL had a lower percentage of naive CD4\(^+\) T cells than did control subjects (P = .019) (figure 1D). Higher levels of CD8 T cell activation were seen in HIV-infected patients but not in patients with ICL, compared with control subjects (P < .001 and P = .127, respectively) (figure 1B).

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**Figure 1.** Increases in activated T cells and decreases in naive CD4 T cells in idiopathic CD4 lymphocytopenia (ICL). Proportions of activated CD4\(^+\) T cells (A) and CD8\(^+\) T cells (B) are shown. Activated T cells were identified as HLA-DR\(^-\)CD38\(^+\) cells. C, Percentages of cycling CD4\(^+\) T cells (Ki67\(^+\)) were higher in HIV-infected (HIV\(^+\)) patients and patients with ICL than in control subjects. D, Patients with ICL had a lower proportion of naive (CD45RO\(^-\)CD27\(^+\)) CD4\(^+\) T cells, compared with control subjects and HIV-infected patients. Horizontal bars denote median values for each group. P values denote the difference between 2 groups, as determined using the Mann-Whitney U test.
Increased plasma levels of LPS in HIV-infected patients and patients with ICL. Plasma LPS levels were measured in the 3 study groups. Both HIV-infected patients and patients with ICL had significantly higher plasma LPS levels than did healthy control subjects (P = 0.009 and P = 0.021, respectively) (figure 2A). However, plasma LPS levels were not significantly different between HIV-infected patients and patients with ICL (P = 0.436) (figure 2A). Plasma samples obtained from HIV-infected patients and patients with ICL also had increased sCD14 levels, compared with those obtained

Figure 2. Increased lipopolysaccharide (LPS) and soluble CD14 (sCD14) levels in HIV-infected patients and in patients with idiopathic CD4 lymphocytopenia (ICL). Plasma levels of LPS (A) and sCD14 (B) were measured in control subjects, HIV-infected (HIV+) patients, and patients with ICL. Horizontal bars denote median values for each group. P values denote the difference between 2 groups, as determined by the Mann-Whitney U test.

Figure 3. Associations between plasma lipopolysaccharide (LPS) levels and the proportion of naive and proliferating CD4 T cells in peripheral blood. Correlations between plasma LPS levels and frequencies of naive CD4+ T cells in HIV-infected (HIV+) patients (A) and patients with ICL (B). Correlation between plasma LPS levels and cell cycling (CD4+Ki67+) in HIV-infected patients (C) and patients with ICL (D). P and r values were obtained using Spearman’s correlations.
from control subjects ($P = .009$ and $P = .015$, respectively) (figure 2B).

**Associations between LPS levels and CD4 T cell proliferation.** In HIV-infected patients, we observed a strong inverse correlation between the proportion of naive CD4 T cells and plasma LPS levels ($r = -0.68; P = .035$) (figure 3A). A strong correlation was observed between CD4 T cell proliferation and plasma LPS levels in patients with ICL ($r = 0.88; P = .003$) (figure 3D). There was no correlation between CD8 T cell activation and LPS in either the patients with ICL or the HIV-infected patients. LPS levels did not correlate with HIV RNA levels in HIV-infected participants ($r = -0.04; P = .918$).

**CD4 T cell cytokine production after mitogenic stimulation.** A lack of T helper (Th) 17 CD4$^+$ T cells in the peripheral blood of patients with a mutation in the signal transducer and activator of transcription 3 (STAT3), who are prone to recurrent bacterial infections (Job’s syndrome), recently has been reported elsewhere [21, 22]. In addition, association of a lack of Th17 CD4$^+$ T cells in the gastrointestinal mucosa with translocation of microbial products has been noted in HIV-infected individuals [23] and in simian immunodeficiency virus (SIV)–infected rhesus macaques (RMs) [24]. To study the potential association between Th17 CD4$^+$ T cells and elevated LPS levels in patients with ICL, production of IL-17, IFN-γ, IL-2, and TNF-α was evaluated after polyclonal T cell stimulation. Despite the low numbers of CD4 T cells in peripheral blood, the relative frequencies of CD4 T cells producing effector cytokines in patients with ICL and in HIV-infected patients were similar, compared with those in healthy control subjects (figure 4). Hence, translocation of microbial products in these individuals did not appear to be associated with a lack of functionality within the depleted CD4 T cell subset, at least as was seen in the peripheral blood. To our knowledge, to date, gastrointestinal biopsies to study lymphocyte subsets have not been performed for patients with ICL.

**DISCUSSION**

In the present study, both HIV-infected patients and patients with ICL showed evidence of translocation of microbial products, including elevated plasma levels of LPS and sCD14, compared with control subjects. Furthermore, increased activation
and cycling of peripheral CD4 T cells were observed in both CD4 lymphopenic states. Strong associations of LPS levels and T cell proliferation with loss of naive CD4 T cells were also observed. These data support the notion that, in individuals with CD4 lymphopenia, including HIV-infected individuals, translocation of microbial products may play an integral role in lymphopenia-induced proliferation and chronic immune activation, or it may simply be the result of CD4 T lymphopenia either in the gut or in the lymphoid tissue.

HIV or SIV infection causes massive CD4 T cell depletion in the gut mucosa [15, 18, 19, 25]. High levels of LPS have been observed both in chronic HIV infection and in pathogenic SIV infection of RMs [8]. The causal chain of events, however, is not entirely clear. In SIV infection of sooty mangabeys (SMs) or African green monkeys (AGMs), which are natural hosts for SIV that typically remain asymptomatic and do not experience progression to AIDS, there is evidence of CD4 T cell depletion in the gut [7, 26]. However, SMs and AGMs exhibit low levels of systemic T cell activation, proliferation, and bystander apoptosis during chronic infection. Furthermore, plasma LPS levels in SMs and AGMs are not elevated during chronic infection [7, 26]. More recently, the potential role of Th17-producing CD4 T cells in maintaining the integrity of the gut mucosa and/or clearance of bacterial products has been investigated. Both in SIV-infected RMs and in patients with chronic HIV infection, there is evidence of preferential depletion of Th17 CD4 T cells in the gut [23]. In contrast, Th17 production in the gut appears to be preserved during SIV infection of SMs, suggesting that, with regard to pathogenesis, there might be an association between CD4 T cells producing IL-17 and LPS in the plasma [23, 24]. There are no data regarding T lymphocyte composition in the lymphoid tissues and/or gastrointestinal mucosa of patients with ICL. However, it is unlikely that the loss of CD4 T cells is restricted to peripheral blood in patients with ICL.

The fact that translocation of antigenic stimuli may not always be a direct consequence of gut mucosal CD4 T cell depletion led us to study the potential role of CD4 Th17 cells in patients with ICL. Th17 cells express various cytokines, including IL-17, TNF-α, and IL-6, and possess antibacterial and antifungal properties. Both HIV-infected patients and patients with ICL, however, appear to maintain a similar Th17 cellular response to mitogenic stimuli in the periphery, compared with healthy control subjects. This hypothesis will need to be further studied by characterizing the immunophenotype and Th17 functional responses of T cells at the level of the gut and other mucosal sites.

In contrast to HIV-infected participants, patients with ICL did not have evidence of profound CD8 T cell activation. The finding of similar LPS levels in individuals with either of the 2 conditions (HIV infection and ICL) but very different levels of CD8 T cell activation suggests that factors in addition to microbial translocation lead to immune activation in HIV-infected individuals [8]. It is plausible that intermediary steps of the innate immune response may be vastly different in HIV-infected patients, compared with patients with ICL. Alternatively or in concert, tissue destruction, such as the collagen deposition that has been described in HIV infection [27], may lead to aberrations of the T cell responses because of restricted supply of cytokines or other proliferative stimuli. The lack of studies of lymph nodes or mucosal sites in patients with ICL significantly limits our ability to draw any firm conclusions.

It is possible that aberrant immune activation induced by microbial products in subjects with lymphopenia may be playing an important—and even beneficial—role in lymphocytopenia-induced proliferation. Previous studies have shown that polyclonal T cells injected into severe combined immunodeficiency (SCID) mice raised under conventional environments gave rise to a more prominent population of rapidly proliferating cells than did T cells injected into SCID mice raised under germ-free conditions [20]. Other studies involving murine models have shown that highly reactive exogenous agonist peptide–MHC complexes are required for activating naïve T cell differentiation in effector cell phenotypes in lymphopenic mice [28]. These findings suggest that, although self-peptides may be sufficient for T cell proliferation in lymphocytopenia-induced proliferation, exposure to foreign antigens, such as microbial products, could enhance the rate of homeostatic proliferation and activation.

In summary, our results suggest that translocation of microbial products is associated with perturbation of CD4 homeostasis in lymphopenic states other than HIV infection. Further definition of the mechanisms of immune activation in patients with ICL could help differentiate anticipated compensatory mechanisms of T cell turnover from the deleterious influence of HIV infection on T cell homeostasis.

Acknowledgments

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References