Progressive Human Immunodeficiency Virus–Specific Immune Recovery with Prolonged Viral Suppression

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Human immunodeficiency virus (HIV) infection results in impaired general and HIV-specific cellular immunity from the time of primary infection and progresses with the course of disease [1]. Intact HIV-specific immunity is important in the control of HIV infection, and it is likely that cytotoxic T lymphocyte (CTL)–mediated control of HIV infection requires HIV-specific CD4 T cell help, as is the case with other viral pathogens [2, 3]. This is supported by the observation of maintained lymphoproliferative responses to HIV antigens and strong Gag-specific CTL activity in HIV-infected long-term nonprogressors [4, 5]. A recent cross-sectional study of antiretroviral-naive persons found that levels of HIV p24 antigen induced proliferation were positively correlated with levels of Gag-specific CTL precursor frequency and were negatively correlated with plasma HIV RNA levels, further supporting this view [6].

Treatment of patients with potent antiretroviral therapy results in improved quantitative measures of cellular immunity, including increases in CD4 T cell count and percentage, proportion of naive T cells, CD4:CD8 T cell ratio, and expression of CD28, a costimulatory molecule required for optimal T cell function on CD8 T cells [7–13]. Improvements in T helper (Th) cell function, as determined by proliferative responses to mitogen, recall antigen, and especially HIV antigens, have been more variable [7, 9, 10, 14, 15]. This variability may be due to many factors, including the stage of disease at initiation of treatment (e.g., primary HIV infection, early asymptomatic disease, or advanced disease), characteristics of the antiretroviral therapy (e.g., potency or possible direct immunomodulatory effects), or technical aspects of the functional assays (e.g., specific antigens or conditions of antigen stimulation).

Although it appears that brisk proliferative responses to antigen can be readily restored in persons treated at the time of seroconversion [16], we demonstrated elsewhere only a modest improvement in such responses in a minority of patients with moderately advanced HIV deficiency after 6 months of therapy [10]. With the exception of 1 small case series, in which patients were treated with didanosine and hydroxyurea [17], other researchers have failed to observe such responses in the treatment of persons with established infection [9, 15, 18]. Because the immune recovery observed with antiretroviral therapy has been incomplete, it has been suggested that adjunctive immune-based therapies may be needed to restore optimal general and HIV-specific host immune function. However, it is possible that recovery of such responses has not been observed because of the
need for more potent antiretroviral agents or more prolonged suppression of viral replication and immune “healing.”

There are several possible ways by which suppression of viral replication leads to improved proliferative responses. HIV-associated impairment of peripheral blood mononuclear cell (PBMC) proliferative responses can be restored by in vivo or in vitro administration of interleukin (IL)–2 and IL-12 [19–23]. The production of these cytokines, which are instrumental in the generation of cell-mediated immunity, is impaired with progressive HIV infection [1, 24–28], and increased production of these cytokines may contribute to improved T cell proliferation in response to antiretroviral therapy. Similarly, as CD28 expression on CD8 T cells is progressively decreased during the course of HIV infection [29, 30], its restoration may also contribute to improved proliferative responses.

In the present study, we evaluated the functional consequences of prolonged use of effective anti-HIV therapy in patients with chronic HIV infection. Because transient increases in HIV RNA levels occurred in a small proportion of patients during this study, we studied the effect of this on host immune function. We also examined the potential for a direct effect of antiretroviral therapy on ex vivo measures of immune function.

**Patients and Methods**

**Patients.** Between April and July 1996, 42 patients were enrolled in the clinical trial, Safety and Efficacy of Ritonavir in Combination with Saquinavir in HIV-Infected Patients (Abbott Laboratories Protocol M96-462), at the Ottawa Hospital (Ottawa, Ontario, Canada). Inclusion criteria included CD4 cell count 100–500 cells/µL, discontinuation of nucleoside antiretroviral therapy for ≥2 weeks, and no prior use of protease inhibitors (PIs). Patients were randomly assigned to 1 of 4 doses of ritonavir plus saquinavir, and all patients received ≥400 mg of ritonavir twice daily and ≥400 mg of saquinavir twice daily. The addition of combination reverse-transcriptase (RT) inhibitor (RTI) therapy that a patient had not previously used was permitted 12 weeks after the start of ritonavir and saquinavir treatment at the discretion of the treating physician. For studies on PBMC of HIV-uninfected persons, volunteers documented as being HIV seronegative were recruited from among Ottawa General Hospital Research Institute staff members. We reported elsewhere the 60-week result of this protocol [31]. Preliminary data (24 weeks) on the immunologic responses in this patient cohort have been reported elsewhere also [10].

**HIV RNA level and CD4 lymphocyte counts.** At frequent intervals, including baseline and weeks 4, 24, 48, 72, and 96, blood was collected in sodium citrate-containing tubes, and PBMC were isolated by centrifugation over Ficoll-Paque (Pharmacia) and were resuspended at 3 × 10⁶ cells/mL in endotoxin-free RPMI 1640 with 5% pooled HIV-seronegative AB serum (Advanced Biotechnologies). Proliferation assays were performed as described elsewhere [1]. In brief, 100 µL of cell suspension was aliquoted into 96-well plates (Falcon) and was stimulated in triplicate wells with HIV p24 antigen (Chiron Biomaterials; supplied by the National Institutes of Health AIDS Research and Reference Reagent Program; final concentration, 1 µg/mL), phytohemagglutinin-M (PHA; Gibco BRL; final dilution, 1:200), or medium alone. After 6 days of incubation at 37°C with 5% CO₂, cells were pulsed with 1 µCi of [³H]thymidine. After a further 18-h incubation, cells were harvested (Harvester 96; Tomtec), and [³H]thymidine incorporation was measured as counts per minute (cpm) of radioactivity on a scintillation counter (1450 Microbeta Plus; Wallac Oy). Stimulation index (SI) was defined as the average cpm in the presence of a stimulus divided by average cpm in its absence. As have other investigators, we considered an SI >3 to represent a proliferative response [6, 18]. Thus, we term subjects with such responses “responders” at the time point the SI was >3. To determine the effect of the in vitro addition of ritonavir (Abbott Laboratories), saquinavir (Hoffman-LaRoche Laboratories), or lamivudine (Glaxo Wellcome) on proliferative responses, PBMC from HIV-seronegative subjects were cultured in the presence of PHA plus 0.01–10 µg/mL of ritonavir, saquinavir, or lamivudine in dimethyl sulfoxide (DMSO; Sigma Chemicals; final dilution, 1:1000) or with DMSO alone (1:1000) and were processed as above.

**Cytokine production measurement.** One milliliter of PBMC suspension was aliquoted into 12 × 75-mm polypropylene tubes (Falcon), was supplemented with 500 µl of PHA (final dilution, 1:100) or lipopolysaccharide (LPS; *Salmonella enteritidis*; Sigma L-2012; final concentration, 1 µg/mL), and was incubated at 37°C with 5% CO₂. After 24 h, cells were pelleted, and supernatants were collected and frozen at −70°C. Supernatants were subsequently thawed and assayed for IL-2 and IL-12 p40 (both from Biosource International) by commercial ELISA. The detection limits of the assay were 8.7 pg/mL for IL-2 and 1 pg/mL for IL-12 p40. To determine the direct effect of ritonavir, saquinavir, or lamivudine on cytokine production, PBMC from HIV-seronegative persons were cultured in the presence of PHA or LPS plus 0.01–10 µg/mL ritonavir, saquinavir, or lamivudine in DMSO (final dilution, 1:1000) or with DMSO alone (1:1000) and were processed as above.

**Flow cytometric analysis of CD28 expression.** At baseline and at weeks 4, 24, 48, 72, and 96, blood was collected in ACD-containing tubes from a subset of patients, and CD28 expression on CD4 and CD8 lymphocytes was analyzed by flow cytometry. One hundred microliters of whole blood was placed into 12 × 75-mm polystyrene tubes (Falcon) containing saturating amounts (5 µL) of phycoerythrin (PE)-conjugated mouse anti-CD4 antibody (Sigma) or PE-conjugated mouse anti-CD8 antibody (Becton Dickinson) and fluorochrome isothiocyanate-conjugated mouse anti-CD28 antibody (PharMingen). Red blood cells were lysed, and cells were fixed by use of Q-Prep equipment (Coulter Electronics). Blood samples processed in the absence of antibodies (autofluorescence) and isotype-matched fluorochrome-conjugated monoclonal antibody were included as negative controls. Data were acquired (10,000 events) on
an Excel flow cytometer (Coulter Electronics) and were saved as listmode files, and the percentage of CD4 and CD8 cells that expressed CD28 was determined.

Data analysis. Data are presented as median and interquartile range (IQR) or mean ± SE of cytokine concentration, as percentage of CD4 or CD8 T cells that express CD28, and as cpm, as appropriate. The proportion of patients with proliferative responses at each time point were compared with baseline by McNemar test. Changes in cytokine production and percentage of CD4 or CD8 lymphocytes that expressed CD28 and cpm relative to baseline at all time points were compared by analysis of variance (ANOVA) or Kruskal-Wallis test (ANOVA on ranks) with pairwise comparisons of on-treatment versus baseline levels (Dunnett or Dunn tests), as appropriate. Proliferation (cpm) and cytokine production were measured at baseline and after 4, 24, 48, 72, and 96 weeks of therapy. At baseline, PBMC from 2 (4.9%) of 41 subjects proliferated (SI > 3) in response to p24 antigen. In response to PHA, proliferation (SI > 3) was significantly greater than at baseline (P < .05, McNemar test). By 72 weeks, 53% and 94% of patients had proliferative responses to p24 antigen and PHA, respectively. At 96 weeks, 44% and 84% had proliferative responses. If the patients who had quantifiable HIV RNA levels were excluded from the analysis (2 patients at week 72 and 4 patients at week 96), 53% and 50% of patients had proliferative responses to p24 antigen at weeks 72 and 96, respectively. Of the patients with proliferative responses at week 48 who were followed-up through 96 weeks, 91% had proliferative responses at subsequent time points, indicating a degree of consistency within these responses. As expected, the amount of [3H]thymidine incorporation (cpm) was greater in response to PHA than to p24 antigen, as illustrated in figure 1B. Correction of cpm values for the number CD4 T cells in cultures had no significant impact on these results (data not shown).

The absolute cpm we observed for PHA-stimulated PBMC were relatively low for mitogen-induced proliferative responses. The modest responses to PHA reflect the culture conditions

<table>
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<th>Parameter</th>
<th>0</th>
<th>4</th>
<th>24</th>
<th>48</th>
<th>72</th>
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<td>No. of patients on study</td>
<td>41</td>
<td>41</td>
<td>40</td>
<td>38</td>
<td>36</td>
<td>35</td>
</tr>
<tr>
<td>No. of patients receiving 3TC and d4T</td>
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<td>1</td>
<td>9</td>
<td>10</td>
<td>12</td>
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<tr>
<td>Median (range) CD4 cell count, cells/µL</td>
<td>286 (60–492)</td>
<td>353 (177–892)</td>
<td>402 (204–746)</td>
<td>458 (177–876)</td>
<td>467 (294–913)</td>
<td>494 (269–1065)</td>
</tr>
<tr>
<td>Median (range) CD4:CD8 ratio</td>
<td>0.29 (0.10–1.26)</td>
<td>0.37 (0.14–1.31)</td>
<td>0.48 (0.18–1.57)</td>
<td>0.55 (0.18–1.61)</td>
<td>0.60 (0.21–2.04)</td>
<td>0.64 (0.29–2.21)</td>
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<tr>
<td>Median (range) HIV RNA level, log10 copies/mL</td>
<td>4.73 (3.31–5.75)</td>
<td>2.81 (&lt;2.30–3.88)</td>
<td>&lt;2.30 (&lt;2.30–4.47)</td>
<td>&lt;2.30 (&lt;2.30–4.00)</td>
<td>&lt;2.30 (&lt;2.30–3.65)</td>
<td>&lt;2.30 (&lt;2.30–4.21)</td>
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<tr>
<td>HIV RNA level &lt;200 log10 copies/mL, no. (%) of evaluable patients</td>
<td>0</td>
<td>15 (38)</td>
<td>31 (79)</td>
<td>33 (89)</td>
<td>33 (94)</td>
<td>30 (88)</td>
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NOTE. 3TC, lamivudine; d4T, stavudine; HIV, human immunodeficiency virus.

Table 1. CD4, CD8, and virus load measurements in response to therapy.

Results

Plasma HIV RNA levels and CD4 cell counts. Of the 42 patients enrolled, one withdrew from the study in the first week. All but one of the evaluable 41 patients had detectable plasma HIV RNA at baseline. The patient with undetectable HIV RNA at baseline was subsequently found to have HIV-1 subtype D and was excluded from virus load evaluations. Six of the 41 available patients withdrew from study before week 96. Reasons for withdrawal included adverse drug reaction or drug intolerance (3 patients), study noncompliance (2 patients), and moving from the country (1 patient). Twelve (34%) of the 35 patients remaining on study at week 96 received antiretroviral regimens intensified with lamivudine and stavudine between weeks 24 and 72, typically because of recurrent detectable plasma HIV RNA.

Table 1 shows HIV RNA levels, CD4 and CD8 T cell counts, and CD4:CD8 ratios for the 96-week study. These data demonstrate virologic suppression below the limit of quantitation in ~90% of patients and continuous increases in CD4 T cells and CD4:CD8 ratio. The transient increase in CD8 T cells is consistent with that observed in other studies of PI-based therapy [7–9, 32]. At 96 weeks, 4 patients (12%) had quantifiable HIV plasma viremia: 2 were associated with drug interruptions, and 2 were isolated transient viral “breakthroughs” that later resolved. No patients developed sustained virologic breakthrough in the absence of drug interruption.

Proliferative responses. To evaluate T cell function, PBMC proliferation in response to HIV p24 antigen and PHA was measured at baseline and after 4, 24, 48, 72, and 96 weeks of therapy. At baseline, PBMC from 2 (4.9%) of 41 subjects proliferated (SI > 3) in response to p24 antigen. In response to PHA, PBMC of 7 (17.1%) of 41 subjects proliferated at baseline. The proportion of patients with proliferative responses to p24 antigen and PHA progressively increased during the 96-week study (figure 1A) and at each time point was significantly greater than at baseline (P < .05, McNemar test). By 72 weeks, 53% and 94% of patients had proliferative responses to p24 antigen and PHA, respectively. At 96 weeks, 44% and 84% had proliferative responses. If the patients who had quantifiable HIV RNA levels were excluded from the analysis (2 patients at week 72 and 4 patients at week 96), 53% and 50% of patients had proliferative responses to p24 antigen at weeks 72 and 96, respectively. Of the patients with proliferative responses at week 48 who were followed-up through 96 weeks, 91% had proliferative responses at subsequent time points, indicating a degree of consistency within these responses. As expected, the amount of [3H]thymidine incorporation (cpm) was greater in response to PHA than to p24 antigen, as illustrated in figure 1B. Correction of cpm values for the number CD4 T cells in cultures had no significant impact on these results (data not shown).
Peripheral blood mononuclear cell (PBMC) proliferative responses. A. Proportion of patients with PBMC proliferative response (stimulation index [SI] >3) to human immunodeficiency virus (HIV) p24 antigen (Ag) or phytohemagglutinin (PHA) at baseline and at weeks 4, 24, 48, 72, and 96 of therapy. B. Proliferative responses (counts per minute [cpm]; median ± interquartile range) in presence of media alone (all patients) or HIV p24 Ag or PHA (responders only).

Association between proliferative responses and virus load increases. Increases in virus load, either due to discontinuation of antiretroviral therapy or due to virologic breakthrough during an antiretroviral regimen, are associated with loss of proliferative responses [10, 34]. It is not known whether transient increases in virus load have similar effects on proliferative responses. In the present study, at the time the immunologic studies were done, there were a number of transient increases in plasma HIV RNA levels to above the detection limit of the assay. Thus, we had the opportunity to examine the effects of this occurrence on proliferative responses. In 5 of 7 episodes that occurred beyond study week 24 week in patients who otherwise had sustained suppression of plasma viremia, the transient increase in plasma virus load was associated with the loss of proliferative responses to p24 antigen or PHA that had been present at the previous determination (figure 2). Of the 7 patients, one had a transient change in virus load from below the detection limit of the assay (signal equal to background) to 120 copies/mL; the other 6 patients had increases to ≥200 copies/mL. In 3 patients, the transient increase was associated with temporary drug discontinuation or lack of compliance. Two episodes appeared to be associated with an intercurrent viral illness, but for 2 episodes there was no clear explanation for the transient increase in virus load.

Cytokine production. To further assess T cell function, IL-2 production in response to stimulation with PHA was evaluated. PHA-stimulated PBMC produced a median of 268 (IQR, 105–803) pg/mL IL-2 at baseline. This increased to 716 (IQR, 302–1247) pg/mL at week 24 and 969 (IQR, 512–1594) pg/mL at week 48 (P < .001 for difference between groups, Kruskal-Wallis test; P < .05 vs. baseline, Dunn test). IL-2 production subsequently decreased to levels not significantly greater than at baseline at weeks 72 (341 pg/mL; IQR, 274–553 pg/mL) and 96 (354 pg/mL; IQR, 260–466 pg/mL; figure 3A).

To determine the effect of potent antiretroviral therapy on an aspect of monocyte function required for the generation of cellular immune function, IL-12 production in response to LPS stimulation was evaluated. LPS-stimulated PBMC, in which monocytes are the primary source of IL-12, produced a median of 543 (IQR, 276–1006) pg/mL IL-12 p40 at baseline. This was significantly greater at both 72 and 96 weeks (2192 [IQR, 1231–3524] pg/mL IL-12 p40 at baseline. This was significantly greater at both 72 and 96 weeks (2192 [IQR, 1231–3524] pg/mL at 72 and 96 weeks (2192 [IQR, 1231–3524] pg/mL at 72 and 96 weeks; P < .01 vs. baseline, Dunn test; P < .05 vs. baseline, Dunn test for each 72 and 96 weeks; figure 3B).

CD28 expression. Optimal T cell function requires T cell/antigen-presenting cell interactions that include signals mediated through CD28, a costimulatory molecule present on T cells [35, 36]. Because expression of CD28 is decreased in HIV infection [29, 30], the expression of CD28 on CD4 and CD8 T cells in response to potent anti-HIV therapy was evaluated. Whole blood was available from 13 patients at baseline for flow cytometric analysis of CD28 expression on CD4 and CD8 lymphocytes. In these persons, the mean ± SE percentage of CD8 lymphocytes expressing CD28 was 32.3% ± 2.7% at baseline. This continuously increased throughout the 96-week study to 56.8% ± 4.6%, approaching levels in HIV-seronegative persons (29) and data not shown; figure 3C). No change was observed in CD28 expression on CD4 lymphocytes.

Correlates of immunologic changes. Among possible predictors of immunologic improvement (i.e., baseline CD4 T cells, HIV RNA level, age, and RTI use), univariate analysis revealed that only age correlated positively with improved immune function. However, by multivariate analysis, no baseline characteristics correlated with improvements in immune function.

In vitro effects of antiretroviral agents on proliferative re-
Figure 2. Association between proliferative responses and transient increases in virus load. Seven patients had transient increase in virus load beyond 24 weeks of therapy, otherwise having achieved optimal suppression of plasma viremia. A, Proliferative responses (stimulation indices [SIs]) plotted with concurrent plasma human immunodeficiency virus (HIV) RNA level (copies/mL) for 5 patients with transient increase in HIV RNA level at time of proliferative assays who lost a proliferative response. In 4 patients, proliferative responses to p24 antigen (Ag) were lost concurrently with transient increase in virus load. Patient 2042 also lost proliferative response to phytohemagglutinin (PHA; data not shown). Patient 1008 lost proliferation response to PHA at week 72 but never generated proliferative response to p24 Ag. B, Summary data of 7 patients with transient increase in HIV RNA levels at time of proliferative assays. Time 0, time of increase in HIV RNA level; broken line, SI = 3. *P = .02; **P = .07 (Wilcoxon rank sum test vs. –24 weeks). NA, not available.
Figure 3. Cytokine production by stimulated peripheral blood mononuclear cells (PBMC) and CD28 expression on CD4 and CD8 T cells. A, Phytohemagglutinin-induced interleukin (IL)-2; B, lipopolysaccharide-induced IL-12 p40 production from PBMC (median ± interquartile range) at baseline and at weeks 4, 24, 48, 72, and 96 on therapy. C, CD28 expression on CD4 and CD8 T cells (mean ± SE) at baseline and at weeks 4, 24, 48, 72, and 96 on therapy. *P < .001 for difference between groups (Kruskal-Wallis analysis of variance [ANOVA] on rank test), and P < .05 vs. baseline (Dunn test). **P < .05 for difference between groups (ANOVA), and P < .05 vs. baseline (Dunn test).

Discussion

A number of studies have identified various degrees of immune recovery after 6–12 months of treatment of chronic HIV infection with highly active antiretroviral therapy [7–12].
Whether immune function continues to improve further, whether this improvement depends on the antiretroviral regimen used, and what other factors may predict the degree of immune recovery remain to be established. In patients receiving ritonavir plus saquinavir, of whom nearly all had plasma HIV RNA levels optimally suppressed at 96 weeks, CD4 T cell counts and CD4:CD8 ratios have yet to plateau. This suggests a continuing restoration of immune function throughout the study period and likely beyond. In parallel, IL-12 production, CD28 expression on CD8+ T cells, and PBMC proliferative responses and HIV-specific proliferative responses in particular also continue to improve.

Recovery of HIV-specific T helper cell function, as measured by proliferative responses to HIV antigens, occurs during treatment of primary infection with potent antiretroviral therapy [16] but has not been seen during treatment of chronic HIV infection [9, 15, 18]. However, in the present study, in which we evaluated patients with a prolonged duration of viral suppression, we found an improvement in HIV-specific proliferative responses in about half the patients studied. The proportion of patients in this study with levels of plasma viremia below the limit of quantitation not only exceeds that of other published studies, but a recent study demonstrated that, when used in combination with 2 RTIs, ritonavir plus saquinavir are more effective therapy than either ritonavir or indinavir [39], the PIs used in other studies of immune recovery. Thus, this particular therapy may be more potent than others studied for their effect on immune reconstitution. The duration of our study is also substantially longer than that of most other studies that examined the effects of antiretroviral therapy on host immune function. In one study that followed patients for >2 years and evaluated proliferative responses to HIV proteins, 9 patients received a PI (indinavir) plus 2 RTIs [15]. A proportion of these patients seemed to have proliferative responses to HIV antigens, but because the data were presented as mean (±SE) for the group and because the number of patients with proliferative responses to each HIV antigen was not reported, it is difficult to know whether these data are consistent with those in the present study.

Another explanation of why a substantial proportion of patients in our study developed HIV-specific proliferative responses may be the specific antiretroviral regimen used. In the present study, nucleoside analogue RTIs were avoided by most of the patients, and those who added RTIs to ritonavir and saquinavir avoided the use of zidovudine, which has immunosuppressive activity [40–42]. Controlled trials that compare the effect on immune function of RTI-containing versus RTI-sparing regimens with anticipated equivalent antiretroviral activity are currently addressing this specific question.

The proliferative responses to HIV p24 antigen in the present study were less brisk than those observed in the treatment of acute primary infection [16], possibly representing a lesser degree of reversibility of this defect at later stages of disease. IL-12 production and CD28 expression are both important in the generation of T helper cell function, and, therefore, increases in these may be responsible, in part, for the improvement in proliferative responses. The improved LPS-induced IL-12 production by PBMC is also suggestive of improved monocyte/macrophage function, an aspect of immune function critical in the immunopathogenesis of HIV disease that is yet to be well studied in the setting of treatment of HIV infection with potent antiretroviral therapy.

The transient nature of the increase in IL-2 production appears to be unique among measures of immune function that were evaluated. We reported elsewhere a significant increase in PHA-induced IL-2 production at 24 weeks [10], and this was sustained at 48 weeks, as we report here. By 72 weeks and at 96 weeks, IL-2 production decreased to levels not significantly greater than that observed at baseline. It is possible that the increase observed during the first 48 weeks resulted from suppression of plasma viremia. However, beyond 48 weeks, when lymphocyte function may be partially restored, a direct down-regulatory effect of ritonavir and/or saquinavir on IL-2 production, as observed in PBMC from HIV-seronegative subjects in vitro, may be occurring in vivo. Why this occurs with IL-2 production but not with PBMC proliferation may be because, although proliferative responses depend on IL-2 production, other factors, such as increased IL-12 production and improved costimulatory signaling, as a result of increased CD28 expression, contribute to improved proliferative response. Therefore, the proliferative responses seen may be the sum or net effect of several changing contributors: decreased virus load, time, differential effects of IL-2 and IL-12 production, and possible direct drug effects.

One factor that predicts a favorable immune response to antiretroviral therapy is a sustained virologic response [14, 34]. Pakker et al. [34] reported that, coincident with the loss of viral suppression, proliferative responses to anti-CD3 antibodies were lost, even though CD4 T cell counts remained elevated. This is similar to our previous observation of the loss of proliferative responses to p24 antigen and PHA shortly after discontinuation of effective antiretroviral therapy [10]. In the present study, 30 of 34 patients with detectable virus load at baseline had HIV RNA levels below the limit of quantitation at 96 weeks, and only 1 had likely drug-resistant virologic treatment failure. Given this homogeneity of response, it is not possible to determine from this study whether optimal viral suppression or the lack thereof was a significant predictor of immune recovery. The consistent loss of proliferative responses at times of transient increases in HIV RNA levels that we observed, however, suggests that immune restoration depends on suppression of viral replication.

A direct effect of PIs on immune function was proposed after a number of patients with sustained CD4 T cell increases were seen, despite the lack or loss of virologic responses to therapy [43, 44]. However, it may be difficult to determine whether PI
therapy results in changes in immune function independent of its effect on viral replication in HIV-infected patients. Even in persons who have CD4 T cell increases without decreased plasma HIV RNA levels, these drugs may sufficiently suppress viral replication in specific sites (e.g., lymphoid tissue), resulting in proliferation or redistribution of CD4 T cells, but this viral suppression is not sufficient to result in decreases in plasma HIV RNA levels. As recently observed, in patients with comparable degrees of plasma virus load suppression, lymph node viral replication and architecture may differ greatly, depending on the therapy used [45]. It is also possible that drug-resistant virus in persons with discordant CD4 T cell and virologic responses may be less virulent and, therefore, may not be associated with an otherwise expected decline in CD4 T cells. In addition, an effect on immune function, independent of suppression of viral replication, may still require the presence of virus or viral proteins as appears to be the case with the in vitro effect of PIs on apoptosis [46].

To address the possibility of a direct effect of PIs on immune function, we evaluated the effect of the in vitro addition of ritonavir, saquinavir, or lamivudine on PHA-induced proliferative responses, PHA-induced IL-2 production, and LPS-induced IL-12 production in PBMC from HIV-seronegative donors. The dose-dependent decrease in proliferation and IL-2 production that resulted from the addition of ritonavir or saquinavir suggest that these drugs may have immunomodulatory activity. This is consistent with findings by André et al. [47], who demonstrated that administration of ritonavir to mice resulted in impaired antigen presentation and CTL activity [47]. This observation and the loss of proliferative responses with even transient increases in plasma viremia in this study strongly support the argument that the immune restorative capacity of PI-based antiretroviral therapy is a result of suppression of viral replication, as opposed to a direct drug effect. The significance of the apparent immunosuppressive effects of ritonavir and saquinavir remain to be established.

Several recent studies that focused on T cell regeneration or repopulation found no or little improvement in T cell receptor (TCR) repertoire [9, 48, 49] and partial restoration of the proportion of naive T cells [7–9, 11, 13]. Although it is possible that full recovery of TCR repertoire perturbations and naive T cell number cannot be accomplished with antiretroviral therapy in chronic HIV infection, it is possible that such recovery requires more profound or prolonged (several years) suppression of viral replication. Regardless of quantitative and functional measures of immune competence, clinical immune deficiency is obviously reduced, as indicated by a decrease in opportunistic infections and the successful discontinuation of *Pneumocystis carinii* pneumonia prophylaxis and suppressive therapy for cytomegalovirus retinitis following the use of potent antiretroviral regimens [50–53]. The progressive and continuous improvement in measures of host immune function for 96 weeks, as demonstrated here, suggests that there is potential for further immune recovery.

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