The antimalarial drug hydroxychloroquine (HCQ) is endowed with immune modulatory effects including the reduction of inflammatory cytokine production and of IgG levels and a downmodulation of natural killer cell activity; these properties have warranted its use in autoimmune conditions including lupus erythematosus, Sjögren syndrome, and rheumatoid arthritis. In vitro data, as well as results obtained in a murine model, have shown that HCQ also modulates the intracellular TLR pathway as it reduces TLR9 of gut permeability. Hydroxychloroquine (HCQ) reduces endosomal TLR signaling; thus, we verified whether HCQ could dampen immune activation and be associated with an increase in CD4+ T cells. To this end, we enrolled in a prospective study 20 HIV-infected immunologic nonresponders (CD4 count < 200 cells/mL or CD4 increase < 5% in the last 12 months) who received 400 mg/day HCQ for 6 months. HCQ had a notable impact on immune activation as shown by significant modifications of the following parameters: (1) reduced plasma lipopolysaccharide; (2) decreased TLR4-expressing CD14+ cells, TLR4-mediated signal transduction, and mRNA synthesis; (3) reduced percentages of activated CD4+ (CD4+/Ki67+) and CD14+ (CD14+/CD69+) cells; (4) increased T-regulatory cells (Tregs), naive Tregs, and TLR4-expressing Tregs; (5) augmented plasmacytoid dendritic cells and reduced IFNα-secreting plasmacytoid dendritic cells; and (6) reduced IL-6 and TNFα production. HCQ-induced immune modulation was associated with increased percentages of circulating CD4+ T cells and was mostly retained 2 months after therapy interruption. HCQ reduces lipopolysaccharide/TLR-mediated immune activation; this compound could be a useful immunomodulant in HIV-infected patients. This study is registered at EutraCT as 2009-012499-28 with study number HLS01/2009-1-16-03-2009. (Blood. 2011;118(12):3263-3272)
or immunomodulation\textsuperscript{32} that, nevertheless, did not result in any significant effect.

We recently reported that lack of proper CD4\textsuperscript+ T-cell recovery in HIV-treated patients correlates with LPS-associated and TLR-mediated immune activation.\textsuperscript{33} On the basis of these observations and the ability of HCQ to downregulate TLR-mediated activation,\textsuperscript{18} we evaluated whether this compound would have an effect on immune activation and CD4\textsuperscript+ T-cell numbers in HIV-infected INRs.

**Methods**

**Study population**

Twenty ART-treated HIV-infected patients with an absolute CD4 count < 200 cells/\(\mu\)L during the last 12 months of therapy and with suppressed viremia (< 37 HIV RNA copies/mL) were consecutively enrolled in the study. All patients were treated with combined antiretroviral therapy according to the current guidelines (a nonnucleoside reverse transcriptase inhibitor plus a protease inhibitor or a nucleoside reverse transcriptase inhibitor plus a nonnucleoside reverse transcriptase inhibitor). Exclusion criteria included glucose-6-phosphate dehydrogenase deficit, pregnancy, breast feeding, acute infectious diseases, or CD4 T-cell count increase > 5% in the last year. All patients received HCQ (400 mg/day) for 6 months. Virologic and immunologic parameters were evaluated at baseline, at the end of treatment, and at 2 months after HCQ suspension. All individuals were enrolled by Infectious Diseases Units at Luigi Sacco Hospital, Milan, Italy, which approved this study; written informed consent was obtained before enrollment in accordance with the Declaration of Helsinki.

**Blood sample collection and PBMC separation**

Whole blood was collected by venipuncture in Vacutainer tubes containing EDTA (BD Biosciences). Plasma was stored and PBMCs were separated on lymphocyte separation medium (Cedarlane Laboratories Limited) and washed twice in PBS (PBI). The number of viable leukocytes was determined by trypan blue exclusion.

**Stimulation of PBMCs**

PBMCs were incubated for 18 hours in the presence or absence of a pool of env + gag peptides (HIV, 2.5\(\mu\)g/mL)\textsuperscript{34} kindly provided by Dr Renato Longhi (CNR Institute, Milan, Italy). For cytokine analyses, 1 \(\mu\)g/mL brefeldin A (Sigma-Aldrich) was added to the cell cultures 6 hours before cell analyses to block protein secretion.

**Immunophenotypic analysis**

Lymphocyte subsets were evaluated using 50 \(\mu\)L of EDTA-treated peripheral blood incubated for 10 minutes at room temperature with fluorochrome-labeled mAbs (anti-CD4, -CD8, and -CD14, PECy7; anti-CD14 and -CD25, PECy5; anti-CD69, allophycocyanine [APC]; anti-HLA-DR, -CD38, and -TLR4, PE; and anti-CD8, -CD16, -CD19, and -CD56), and anti-HLA DRII PE for 15 minutes, fixed with 1% saponin (Sigma-Aldrich) and stained for Ki-67 or mouse FITC-coupled IgG1 (isotype control). After a 45-minute incubation at 4\(^\circ\)C in the dark, cells were washed and fixed in 1% PFA.

Flow data were analyzed by first gating on the lymphocyte population as defined by forward and sidelight scatterers and on CD4\textsuperscript+ cells. Results were expressed as the percentage of CD4\textsuperscript+ Ki-67\textsuperscript+ cells on CD4\textsuperscript+ population.

**Identification of plasmacytoid dendritic cells and IFN\(\alpha\) production**

Analyses were performed both in basal and stimulated conditions as described above under “Stimulation of PBMCs.” PBMCs were washed in PBS, incubated with anti-CD123 PECy7, lineage – PECy5 (LIN’): anti-CD3, -CD14, -CD16, -CD19, and -CD56), and anti-HLA DRII PE for 15 minutes, fixed with 1% PFA, incubated for 15 minutes at 4\(^\circ\)C in the dark, washed with PBS, and permeabilized with 0.5% saponin. Anti-IFN\(\alpha\) mAbs were then added before a 45-minute incubation at 4\(^\circ\)C in the dark. Cells were then fixed with 1% PFA, and plasmacytoid dendritic cells (pDCs) were identified by flow cytometry (CD123\textsuperscript+ HLA-DR\textsuperscript- LIN’): IFN\(\alpha\) expression on these cells was evaluated.

**TLR4, TLR7, and TLR8 expression on stimulated monocytes**

PBMCs were incubated for 18 hours with PBS (TLR4 agonist; Sigma-Aldrich) or single-stranded RNA (ssRNA; TLR7 and TLR8 agonist; InvivoGen). For TLR4, PBMCs were washed with PBS, incubated with anti-CD14 PC5 and anti-TLR4 PE (eBioscience) mAbs for 15 minutes in the dark and were subsequently fixed with 1% PFA. For TLR7 and TLR8, PBMCs were washed with PBS and incubated with anti-CD14 PC5 for 15 minutes in the dark at room temperature. After a second washing, cells were permeabilized with 0.5% saponin and incubated with anti-TLR8 PE (Imgenex) and anti-TLR7 FITC (R&D Systems) for 45 minutes at 4\(^\circ\)C. At the end of this incubation, PBMCs were washed with PBS and fixed with 1% PFA.

**Plasma LPS concentration**

LPS concentration was measured in plasma with the LAL Chromogenic Endpoint Assay (Hycult Biotechnology). Samples, prepared according to the manufacturer’s instructions, were plated in a 96-well plate, followed by LAL reagent. After a 45-minute incubation at room temperature, absorbance was measured (405 nm) with a spectrophotometer. LPS concentration is expressed in endotoxin units per milliliter and was calculated relative to a standard curve.

**Plasma TNF\(\alpha\) and IL-6 concentration**

Prolinflammatory cytokines were evaluated in plasma using commercial ELISA kits (R&D Systems) following the manufacturer’s instructions. The plasma concentration of each protein was calculated relative to a standard curve.

**Identification of T-regulatory lymphocytes and of TLR-expressing T-regulatory lymphocytes**

PBMCs were incubated with anti-CD4 PC7, anti-CD25 ECD, and anti-PD-1 PE for 15 minutes at room temperature.\textsuperscript{35} The intracellular staining of PD1 and FoxP3 was performed according to the manufacturer’s instructions (eBioscience). To detect TLR-expressing T-regulatory cells (Tregs), PBMCs were stained with CD4 PC7, CD25 ECD, TLR4 PE, and TLR2 FITC mAbs.
Intracellular staining of FoxP3 was performed according to the manufacturer’s instructions (eBioscience).

Intracellular cytokine concentration

Antigen-stimulated PBMCs were stained for CD4 or CD14 expression. After a 15-minute incubation at room temperature in the dark, cells were fixed in 1% PFA (Sigma-Aldrich), incubated for 15 minutes at 4°C, and permeabilized with 0.5% saponin (Sigma-Aldrich). TNFα PE, TNFα FITC, and IL-6 mAbs were then added; after a 45-minute incubation at 4°C in the dark, cells were washed and fixed in 1% PFA (Sigma-Aldrich).

mAbs

The following mAbs were used: anti-CD123 (mouse IgG1 isotype) PECy7 (BioLegend); anti-CD4 (mouse IgG1 isotype), anti-CD8 (mouse IgG1 isotype), and anti-CD14 (mouse IgG2a isotype) PECy7; anti-CD3 (mouse IgG1 isotype), anti-CD14 (mouse IgG2a isotype), anti-CD16 (mouse IgG1 isotype), anti-CD19 (mouse IgG1 isotype), anti-CD25 (mouse IgG1 isotype), and anti-CD66 (mouse IgG1 isotype) all coupled to PE; anti-CD25 (mouse IgG1 isotype), anti-CD14 (mouse IgG2a isotype), anti-CD16 (mouse IgG1 isotype), and anti-CD45RO (mouse IgG1 isotype) coupled to PE; anti-CD8 (mouse IgG1 isotype), anti-CD14 (mouse IgG2a isotype), anti-CD16 (mouse IgG1 isotype), and anti-CD45RO (mouse IgG1 isotype) coupled to FITC (Beckman-Coulter); anti-TLR4 PE (mouse IgG2a isotype), anti-PDI PE (mouse IgG1 isotype), and anti-TLR2 FITC (mouse IgG2a isotype); anti-CD69 APC (mouse IgG1 isotype), anti-TLR2 APC (mouse IgG2a isotype), and anti-TLR4 FITC (mouse IgG2a isotype: eBioscience); and recombinant protein annexin V PE (Bender MedSystems). The intracellular staining detection mAbs were used anti-FoxP3 (rat IgG2a isotype) PECy5, anti-PDI1 FITC (mouse IgG1 isotype), anti-IL-6 APC (rat IgG1 isotype: BioLegend), anti-human IFNa FITC (mouse IgG1 isotype: PBL), anti-human Ki67 FITC (mouse IgG1 isotype: BD Biosciences); anti-TNFα PE (mouse IgG1 isotype: Beckman Coulter), anti-TNFα FITC (rat IgG1 isotype: Caltag Laboratories), anti-TLR7 FITC (mouse IgG2a isotype: R&D Systems); and anti-TLR8 PE (mouse IgG1 isotype: Imgenex).

TLR signaling pathway

On the basis of previously published data, intracellular TLR signaling pathway was evaluated in unstimulated and stimulated PBMCs. PBMCs were isolated from 20 HIV-positive and 10 HIV-negative patients and PBMCs were incubated for 3 hours with medium alone, LPS (2μg/mL), or ssRNA40 (2.5 μg/mL). mRNA was extracted from PBMCs by using the acid guanidinium thiocyanate-phenol-chloroform method, dissolved in RNase-free water, and purified from genomic DNA with RNase-free DNase acid guanidinium thiocyanate-phenol-chloroform method, dissolved in RNase-free water, and purified from genomic DNA with RNase-free DNase (RQ1 DNase, Promega). One milligram of RNA was reverse-transcribed to cDNA using M-oligo(dT) and 200 U of Moloney murine leukemia virus reverse transcriptase (Clontech). TLR signaling pathways were analyzed in a PCR array including a set of optimized real-time PCR primer assays on 96-well plates (SABiosciences Corporation) using a SYBR Green PCR mix (Finnzymes). This approach allows the monitoring of mRNA expression of 84 genes related to TLR pathway activation plus housekeeping genes. Controls were also included on each array for genomic DNA contamination, RNA quality, and general PCR performance.

Statistical analyses

Data were analyzed according to standard statistical tests; t tests were performed to compare patients during treatment. Procedures were based on parametric analyses. The rank-transformed variables were analyzed if distributions were not normal. Statistical analysis was performed using the SPSS statistical package (SPSS Inc).

Results

Study population

The clinical and demographic characterization of the patients enrolled in the study is presented in Table 1. HCQ was well tolerated in all patients with the exception of one individual who reported a maculopapular exanthema after 10 days of treatment. This individual was excluded from the final analyses. HIV viremia was undetectable throughout the study period.

### Table 1. Clinical and epidemiologic characteristics of HCQ-treated patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HCQ patients (n = 20)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>49.78 ± 2.92 (28-68)</td>
</tr>
<tr>
<td>Sex (male/female)</td>
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</tr>
<tr>
<td>HIV infection</td>
<td>5.33 ± 1.38 (1-15)</td>
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<tr>
<td>Duration of ART, y</td>
<td>4.43 ± 1.22 (1-15)</td>
</tr>
<tr>
<td>Viral load, copies HIV RNA/mL</td>
<td>&lt; 37</td>
</tr>
<tr>
<td>6 mo of HCQ</td>
<td>&lt; 37</td>
</tr>
<tr>
<td>2 mo after HCQ interruption</td>
<td>&lt; 37</td>
</tr>
<tr>
<td>Coinfections, n (%)</td>
<td></td>
</tr>
<tr>
<td>HBV</td>
<td>1/20 (5)</td>
</tr>
<tr>
<td>CD4+ T-cell count nadir, cells/mm³</td>
<td>54.57 ± 11.43 (6-112)</td>
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<tr>
<td>CD4+ T cells, %</td>
<td>10.68 ± 1.07 (3.8-18.2)</td>
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<tr>
<td>CD4+ T-cell counts, cells/mm³</td>
<td>143.70 ± 13.19 (6-200)</td>
</tr>
<tr>
<td>Total lymphocyte count, mean ± SE, cells/mm³</td>
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<tr>
<td>Baseline</td>
<td>1403.33 ± 171.07</td>
</tr>
<tr>
<td>6 mo of HCQ</td>
<td>1383.33 ± 145.25</td>
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<tr>
<td>2 mo after HCQ interruption</td>
<td>1304.45 ± 149.65</td>
</tr>
<tr>
<td>ART treatment, n</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>10</td>
</tr>
<tr>
<td>NNRTI</td>
<td>7</td>
</tr>
<tr>
<td>Other</td>
<td>3</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE (range) except where noted. HBV indicates hepatitis B virus; HIV, hepatitis C virus; NNRTI, nonnucleoside reverse transcriptase inhibitor; and PI, protease inhibitor

*No significant differences were detected.

CD4+ T cells

The percentage of CD4+ T cells was significantly augmented after 6 months of HCQ treatment (P < .001; Figure 1A) without changes in total lymphocytes counts (Table 1). This increase was retained 2 months after HCQ suspension (P = .001). A similar trend was observed in CD4+ T-cell counts, even if changes did not reach statistical significance (Figure 1B).

Subsets of activated immune cells: CD4+ and CD8+ T cells and CD14+ monocytes

Immune activation results in the detection in peripheral blood of increased quantities of subsets of immune cells expressing particular proteins. To evaluate a possible immunomodulatory effect of HCQ on this immune activation, we analyzed such subsets in all the individuals enrolled in the study.

CD4+ T-cell activation was evaluated in unstimulated cells and on HIV-specific stimulation. The percentage of Ki67-expressing CD4+ T cells (activated T cells) was significantly reduced after 6 months of HCQ in both unstimulated and in stimulated condition (unstimulated, P = .005; env + gag, P = .003; Figure 1C). This effect was retained 2 months after HCQ interruption (unstimulated, P = .024; env + gag, P = .044).

The percentage of HLADR II-, CD69-, and CD38/CD45RO-expressing CD8+ T cells decreased as well, albeit not significantly, after 6 months of HCQ treatment. A different pattern was seen in CD8+ T cells where changes did not reach statistical significance (Figure 1B).
and $P = .041$, respectively, Figure 2A), whereas (2) CD69$^+$CD8$^+$ and CD38$^+$CD45RO$^+$CD8$^+$ cells were significantly augmented compared with month 6 ($P = .043$ and $P = .039$, respectively, Figure 2B-C).

Finally, CD69-expressing CD14$^+$ cells were also significantly reduced after 6 months of HCQ treatment ($P = .04$). Percentages of these cells returned to the baseline values after 2 months of HCQ interruption (Figure 2D).

**TLR expression on monocytes**

HCQ downmodulates the expression of intracellular TLR in vitro and in the murine model. The effect of this compound in HIV infection was analyzed by evaluating TLR2, TLR4, TLR5, TLR7, and TLR8 expression on CD14$^+$ cells both in whole blood and on stimulation with specific antagonist. Results obtained in whole blood showed that HCQ induced a significant reduction in TLR2-, TLR4-, TLR5-expressing CD14$^+$ cells (TLR2, $P = .032$; TLR4, $P = .004$; TLR5, $P = .008$; Figure 3A-C). Results obtained when cell cultures were stimulated with TLR agonists showed a significant reduction in LPS-stimulated cells alone (TLR4, $P = .006$; Figure 3D) that vanished 2 months after interruption of HCQ ($P = .050$). In contrast, no changes in ssRNA-stimulated TLR7- and TLR8-expressing CD14$^+$ cells were detected (Figure 3E-F).

**Tregs and TLR-expressing Tregs**

Tregs were evaluated both in whole blood and with HIV-specific stimulation. Both naïve (PD1$^{neg}$) and activated (PD1$^{pos}$) Tregs were significantly increased after HCQ; this effect persisted 2 months after HCQ interruption (Figure 4A-B).

TLR2 and TLR4 expression on Tregs was evaluated in whole blood and with HIV-specific stimulation; TLR4-expressing Tregs were also analyzed on LPS stimulation. As shown in Figure 4C through E, TLR2- and TLR4-expressing Tregs were significantly augmented after HCQ, and this increase was maintained 2 months after HCQ suspension in all culture conditions (Figure 4C-E). Thus, the observed increase in Tregs induced by HCQ was at least partially supported by TLR2- and TLR4-expressing cells.

**pDCs**

pDCs represent a unique and crucial immune cell population capable of producing large amounts of type I IFNs in response to viral infection. The percentage of pDCs was significantly augmented after 6 months of HCQ treatment; this effect persisted 2 months after HCQ interruption ($P < .001$ in both cases; Figure 5A). Of note, IFN$\alpha$-secreting pDCs were diminished during HCQ treatment and 2 months after HCQ suspension ($P < .05$ in both cases; Figure 5B).

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**Figure 1.** CD4$^+$ T cells in HCQ-treated patients. Percentage of CD4$^+$ T cells (A) and CD4$^+$ T-cell counts (B) in HIV-infected INRs at baseline ( ), after 6 months of HCQ ( ), and 2 months after HCQ interruption ( ). Panel C shows unstimulated and env$^+$ gag$^+$-stimulated activated Ki67$^+$ CD4$^+$ T cells in HIV-infected INRs analyzed at the same time points. Mean values, SE, and $P$ values are indicated.
Proinflammatory cytokines

IL-6 and TNFα production by CD14+ and CD4+ T cells was analyzed in unstimulated and in LPS-, ssRNA-, and env + gag-stimulated conditions; the plasma concentration of these cytokines was evaluated as well. Results showed IL-6– and TNFα-secreting CD14+ and CD4+ cells to be diminished after HCQ treatment, reaching statistical significance only for ssRNA-stimulated CD14+ cells (Table 2). The plasma IL-6 concentration was significantly reduced after HCQ treatment as well (Table 2).

Plasma LPS levels

The plasma LPS concentration is an index of microbial translocation; an augmented LPS concentration is associated with alterations of the gut permeability. HCQ treatment induced a reduction in LPS plasma concentration that was maintained after interruption of therapy (baseline vs 2 months after interruption, \( P = .03 \); 6 months HCQ vs 2 months after interruption, \( P = .001 \); Figure 6).

TLR signaling pathway

Besides having an effect on TLR expression, HCQ reduces TLR-mediated signal transduction in vitro and in the murine model. To evaluate whether a similar effect could be seen in vivo in HIV-infected patients, TLR-mediated signaling pathways were evaluated using an RT-PCR array screening for the expression of 84 genes involved in such pathways. Engagement of TLR7/8 with specific agonists at baseline resulted in the activation of TLR-signaling pathways as demonstrated by increased expression of mediators involved in the transduction pathway (MAP2K3, MAP2K4, MAP3K1, MAP3K7, MAP3K7IP1, MAP4K4, MAPK8, MAPK8IP3, MYD88, NFKB1, NFKB2, NFKBIA, NFKBIL1, NFRK1, NR2C2, PELI1, PPARA, PRKRA, RELA, RIPK2, SARM1, SIGIRR, ECSIT, TBK1, TICAM2, and TIRAP) and of effector molecules (CSF2, CSF3, IL-12, IL-1, IL-6, IL-10, IL-12, and PTGS2). After 6 months of HCQ therapy, however, TLR7/8 responsiveness was markedly downmodulated, and it was not restored even after therapy suspension. Of note, TLR7 and TLR8 mRNA specific expression progressively decreased from baseline to suspension of HCQ treatment, suggesting a possible explanation for the decreased responsiveness observed after ssRNA stimulation (Figure 7).

Data collected after TLR4 stimulation resembled the immunologic profile observed in TLR7/8-stimulated PBMCs. Thus, at baseline PBMCs were highly responsive to LPS, and HCQ therapy resulted in a marked inhibition of the TLR4 pathway that persisted after treatment suspension. Similarly to what was observed for TLR7/8, the gradual decrease in cytokine/chemokine expression after LPS stimulation was accompanied by reduced expression of TLR4 mRNA (Figure 7).
Discussion

The underlying causes of the immune activation seen in HIV infection are still not fully clarified, but one of the most validated and solid hypotheses to explain this phenomenon stems from the observation that acute HIV infection is associated with a rapid and probably irreversible destruction of the CD4+ T cell population that resides in gut-associated lymphoid tissues. Loss of mucosal integrity results in impaired local cellular immunity and translocation of microbial products, including LPS, which contribute to persistent inflammation through TLR activation. Because LPS ligates TLR4, a molecule expressed on a variety of immune cells, the LPS/TRL4 axis has been postulated as being responsible for HIV-associated immune activation. Of note, the non-AIDS comorbidities that are more common in ART-treated patients with persistently reduced CD4+ T cells despite fully satisfactory suppression of viral replication (immunologic nonresponders) were recently suggested to be mainly associated with immune activation.

HCQ downregulates TLR signaling and reduces the production of inflammatory cytokines. On the basis of this assumption we verified whether this compound could modulate immune activation and CD4+ T-cell counts in INRs. Results herein indicate that HCQ has a significant effect on immune activation, as shown by the reduction of circulating activated immune cells, the downmodulation of TLR expression and of TLR-mediated signal transduction, and the decreased production of IL-6. This effect is probably associated with the increased concentration of Treg lymphocytes and the reduced quantities of serum LPS observed in HCQ-treated individuals and is accompanied by significantly augmented percentages of circulating pDC.

Of note, HCQ-associated reduction of immune activation was mostly retained after therapy interruption and was accompanied by a real increase in CD4+ T-cell percentage, because total lymphocyte counts did not change during the study period. This reduction of immune activation was also associated with a similar trend, albeit not reaching statistical significance, in CD4+ T-cell counts. These data suggest that HCQ is effective in reducing CD4+ T-cell activation, resulting in an increase in CD4+ T-cell percentage. We have shown the presence of upregulation of the LPS/TLR-dependent immune activation in HIV-infected individuals lacking CD4 normalization during ART, and recently a reduction in immune activation and a decrease in CD38+CD8+ T cells and of Ki-67 memory CD4+ T cells was reported in ART-treated and naive HIV-infected patients treated with HCQ. Data herein confirm and expand those results and indicate a possible therapeutic benefit of HCQ in ART-treated individuals in whom a satisfactory recovery of CD4+ T cells is not achieved.

HCQ was effective in reducing the percentage of proliferating CD4+ T lymphocytes and of activated (CD69+) monocytes, whereas its effect on CD8+ T cells was marginal, indicating a possible lack of effect of HCQ on this lymphocyte population. Of note, the use of this compound resulted in increased percentages of circulating Tregs. The role of these cells in HIV infection is still not totally clarified, but it is likely that the destruction or inactivation of Tregs by HIV would result in a lack of control over immune activation. Treg-mediated suppression of antigen-specific responses in vitro was shown to be more effective with cells isolated from relatively healthy HIV-infected individuals compared with those from patients with later-stage AIDS, suggesting that Tregs (total or HIV-specific) are depleted and/or dysfunctional later in HIV disease. HCQ treatment induced an increase in naive and
activated Tregs in both unstimulated and HIV-infected specific populations. These subpopulations of Tregs are endowed with strong immunosuppressive activity and probably play a role in the ability of HCQ to reduce immune activation. Of note, the increases in Tregs seen as a result of HCQ were at least partially supported by TLR2- and TLR4-expressing cells; these subpopulations of Tregs were shown to mediate a strong immunosuppressive activity.40

HCQ was also associated with an effect on pDC because these cells were augmented in HCQ-receiving individuals. pDCs are low-frequency cells found in peripheral blood and lymphoid tissues that are best known for their ability to produce large quantities of IFNα in response to viruses. An impairment in pDC function was described in HIV infection and in the pre-ART era pDCs were shown to inversely correlate with CD4+ T counts and viral loads.

Figure 4. Tregs and TLR-expressing Tregs are increased in HCQ-treated patients. Unstimulated (A) and env+ gag-stimulated (B) total, naive, and activated Treg (%) in HIV-infected INRs at baseline, after 6 months (ms) of HCQ treatment, and 2 months after HCQ interruption. Unstimulated (C) and env+ gag-stimulated (D) TLR2+ and TLR4+ Tregs and LPS-stimulated (E) TLR4+ Tregs (percentage) in the same patients. Mean values, SE, and P values are indicated.

Figure 5. pDCs are increased in HCQ-treated patients. pDCs (A) and IFNα-secreting pDCs (percentage; B) in HIV-infected INR patients at baseline (●), after 6 months (ms) of HCQ treatment (□), and 2 months after HCQ interruption (■). Mean values, SE, and P values are indicated.
These observations, together with the facts that ART increases pDC counts and that higher levels of pDC are seen in long-term nonprogressors, led to the suggestion that pDCs play a protective role against the progression of HIV infection.41-46 Although IFN{\textsubscript{α}} reduces viral burden in the acute phases of infection, elevated levels of this cytokine in advanced HIV diseases are associated with increased microbial translocation and could contribute to immune activation. Of note, despite increasing the number of pDCs, HCQ reduced IFN{\textsubscript{α}} production by these cells. IFN{\textsubscript{α}} production by pDCs is dependent on TLR9 engagement. 18 We did not directly examine the TLR9 pathway but rather concentrated on TLR7/8 and TLR4. Nevertheless, because we saw decreased mRNA synthesis for these 3 TLRs, as well as downregulation of TLR 7/8 and 4 signaling pathways, it is possible to speculate that reduced expression of TLR9 on pDCs and/or diminished efficacy of the TLR9-mediated signaling could justify the reduced IFN{\textsubscript{α}} production seen in pDCs of HCQ-treated individuals. In-depth analyses will be needed to clarify the exact mechanisms. Finally, whereas pDCs activated through TLR7 and TLR9 work as antiviral cells via IFN{\textsubscript{α}}, it was recently suggested that non–IFN{\textsubscript{α}}-producing pDCs can act as tolerogenic cells promoting Treg activation via the indoleamine 2,3-dioxygenase and programmed death 1 pathways.47 Data herein suggest that this phenomenon could take place in vivo in HCQ-treated individuals.

Table 2. IL6 and TNF\textsubscript{α} production in HCQ-treated patients

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>6 mo of HCQ</th>
<th>2 mo after HCQ interruption</th>
<th>P</th>
</tr>
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<tr>
<td><strong>LPS-stimulated PBMCs, % positive</strong></td>
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<tr>
<td>IL-6/CD14\textsuperscript{+} cells</td>
<td>63.94 ± 5.91</td>
<td>42.63 ± 4.95</td>
<td>62.00 ± 5.10</td>
<td>NS</td>
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<tr>
<td>TNF{\textsubscript{α}}/CD14\textsuperscript{+} cells</td>
<td>53.71 ± 3.87</td>
<td>40.56 ± 5.43</td>
<td>51.90 ± 4.10</td>
<td>NS</td>
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<tr>
<td>ssRNA-stimulated PBMCs, % positive</td>
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<td></td>
</tr>
<tr>
<td>IL-6/CD14\textsuperscript{+} cells</td>
<td>67.76 ± 3.70</td>
<td>58.99 ± 4.39</td>
<td>37.00 ± 5.00</td>
<td>6 mo vs 2 mo: 0.007</td>
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<tr>
<td>TNF{\textsubscript{α}}/CD14\textsuperscript{+} cells</td>
<td>78.30 ± 3.20</td>
<td>64.42 ± 3.32</td>
<td>69.39 ± 5.10</td>
<td>Baseline vs 6 mo: 0.038</td>
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<td><strong>Env + gag-stimulated PBMCs, % positive</strong></td>
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<tr>
<td>IL-6/CD14\textsuperscript{+} cells</td>
<td>1.71 ± 0.94</td>
<td>1.62 ± 0.58</td>
<td>3.37 ± 1.70</td>
<td>NS</td>
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<tr>
<td>TNF{\textsubscript{α}}/CD4\textsuperscript{+} cells</td>
<td>3.37 ± 1.70</td>
<td>2.66 ± 0.39</td>
<td>5.07 ± 1.24</td>
<td>NS</td>
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<td><strong>Plasma cytokine concentrations, pg/mL</strong></td>
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<tr>
<td>IL-6</td>
<td>5.78 ± 2.66</td>
<td>2.42 ± 0.80</td>
<td>1.18 ± 0.21</td>
<td>Baseline vs 2 mo: 0.048</td>
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<td>TNF{\textsubscript{α}}</td>
<td>8.55 ± 1.46</td>
<td>8.85 ± 2.42</td>
<td>27.41 ± 7.78</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. NS indicates not significant.

The use of HCQ resulted also in the downmodulation of IL6 production by immune cells, an effect that was previously shown to be associated with this drug. More interesting was the reduction of LPS plasma levels seen in HCQ-treated patients; this observation suggests an improvement in gut mucosa permeability. It will be interesting to determine whether this is a direct consequence of HCQ or rather a consequence of the reduced peripheral immune activation seen in these patients. These considerations notwithstanding, because LPS directly triggers TLR4-expressing immune cells, the HCQ-associated reduction of plasmatic LPS probably plays a major role in the effect on immune activation by HCQ described herein.

An important caveat of results herein is that the robust reduction of immune activation achieved by HCQ was not reflected in a similarly important increase in the absolute number of CD4\textsuperscript{+} T cells: a trend, not a significant amelioration, was observed. This discrepancy raises several interesting questions: (1) Is the effect of HCQ not potent enough to convincingly modify CD4\textsuperscript{+} T cells in patients who are not able to respond immunologically to potent ART? (2) Is immune activation the only cause responsible for lack of CD4\textsuperscript{+} T-cell recovery in these patients? And, finally, (3) Will the immune changes induced by HCQ be sufficient to positively affect the clinical outcome of these individuals? All the patients are
followed at regular time intervals; results from the follow-up will at least in part clarify these issues.

In conclusion, our data suggest that HCQ has beneficial effects on TLR/LPS-mediated immune activation, the possible cause of lack of CD4+ T-cell recovery and of associated comorbidity in INRs. HCQ could be considered to be a useful immunomodulating drug in particular groups of HIV-infected patients.

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Authorship

Contribution: S. Piconi, D.T., and M.C. designed the study and wrote the manuscript; S. Parisotto conducted cellular analyses; G.R., S. Passerini, R.T., B.A., P.M., and A.C. selected patients and performed clinical analyses; and M.B. conducted PCR analyses.

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