Increased Expression of Toll-Like Receptor 2 on Monocytes in HIV Infection: Possible Roles in Inflammation and Viral Replication

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Background. Toll-like receptors (TLRs) are key pattern-recognition receptors of the innate immune system, but their role in human immunodeficiency virus (HIV) infection is largely unknown.

Methods. In the present study, we examined the expression of TLR2 and TLR4 on monocytes from 48 HIV-infected patients and 21 healthy control subjects by flow cytometry.

Results. We found that freshly isolated monocytes from HIV-infected patients displayed enhanced expression of TLR2 but not TLR4, that TLR2 expression on the surface of monocytes was significantly increased upon stimulation of HIV type 1 envelope protein gp120, and that TLR2 stimulation in HIV-infected patients induced increased viral replication and TNF-α response.

Conclusion. Our findings suggest potential roles for TLR2 in chronic immune activation and viral replication in HIV infection.
PATIENTS, MATERIALS, AND METHODS

Patients and control subjects. Forty-eight HIV-infected patients (median age, 40 years; range, 23–64 years; 34 men and 14 women) and 21 healthy controls (median age, 36 years; range, 23–63 years; 12 men and 9 women) were included in the study. Individuals who had acute or chronic diseases and were using any drug were excluded from the control group. Twenty-nine patients were receiving HAART, 1 patient was receiving 2 nucleoside analogs, and 18 patients were not receiving antiretroviral treatment. Seven patients had antibodies against hepatitis C virus but no or minor biochemical signs of ongoing liver inflammation (alanine aminotransferase level, <100 U/L), 4 patients had hemophilia A, and no patients were using injection drugs. Median laboratory values (interquartile range [IQR]) for patients who were receiving HAART were as follows: CD4+ T cell count, 348 × 10^3 cells/L (217–720 × 10^3 cells/L); CD8+ T cell count, 1003 × 10^3 cells/L (740–1320 × 10^3 cells/L); and HIV RNA load, 26 copies/mL (1–3810 copies/mL). For patients who were not receiving antiretroviral therapy, the median laboratory values (IQR) were as follows: CD4+ T cell count, 442 × 10^3 cells/L (257–675 × 10^3 cells/L); CD8+ T cell count, 1139 × 10^3 cells/L (727–1566 × 10^3 cells/L); and HIV RNA load, 23,600 copies/mL (293–182,250 copies/mL). Blood samples were obtained when opportunistic infection was not present. The interval between the times at which blood samples were obtained and analyzed (<30 min) was the same for patients and control subjects. Samples from patients and control subjects were always handled in parallel. Informed consent for blood sampling was obtained from all study participants. The study was conducted in accordance with the ethical guidelines at our hospital (Rikshospitalet; Oslo, Norway) and the Helsinki Declaration and was approved by the hospital’s authorized representative.

Cell isolation, stimulation, and testing. Cell isolation was performed with Lymphoprep (Nycomed Pharma), and the culture media used were RPMI 1640 with 2 mmol/L l-glutamine and 25 mmol/L HEPES buffer (Gibco), PBS, and fetal calf serum (PAA Clone; PAA laboratories). Cell culture stimulants used were Pam, Cys-Ser-Lys, Pam, Cys; EM Microcollections) and recombinant HIV-1 gp120MN envelope protein (Protein Sciences). The antibodies used in flow cytometry were phycoerythrin (PE)-conjugated anti-CD14 (BD PharMingen), Alexa488-conjugated (Molecular Probes Europe) antibodies against TLR2 (TL2.1[18]) and TLR4 (HTA125; gift of Kensuke Miyake, Saga Medical School, Japan), and normal mouse IgG (isotype control; Dako). All stimulants and culture media were analyzed for endotoxin contamination using Limulus Amebocyte Lysate Assay (BioWhittaker/Cambrex Bio Science), with levels of endotoxin always determined to be <10 pg/mL.

PBMCs were obtained from heparinized blood by Isopaque-Ficoll gradient centrifugation. PBMCs were resuspended in RPMI 1640 with 10% fetal calf serum (2.5 × 10^5 cells/mL), seeded in 24-well plates (Costar; 1 mL/well), and stimulated with a TLR2 agonist (Pam, Cys; final concentration, 1 μg/mL), an HIV-1 envelope protein (gp120MN; final concentration, 1 or 10 μg/mL), or a negative control (PBS). In some experiments, goat anti-human TNF-α antibody or IgG from healthy goats (concentration, 1 μg/mL; R&D Systems) was added 30 min before addition of the TLR2 agonist. Cells and cell-free supernatants were harvested after a 20-h culturing period.

Flow cytometry was performed on freshly isolated PBMCs (ex vivo) and PBMCs cultured for 20 h (in vitro) using FACSCalibur and CellQuest software (BD Biosciences). In brief, 500,000 PBMCs were incubated for 45 min in the dark with antibodies against CD14 cells and with TLR2, TLR4, or an isotype control before washing and resuspension. List-mode files were collected for 50,000 cells from each sample. Monocytes were gated according to CD14 cell expression and side scatter. Flow cytometric analysis of a sample from each patient was performed consecutively in parallel with analysis of a sample from at least 1 healthy control subject using identical flow cytometer settings and immunostaining procedures. Overlap with the isotype control was not accepted for >5% of the cells. TLR2 and TLR4 were expressed by median percentages (IQRs) of 99% (77%–100%) and 66% (31%–82%), respectively, of positively gated monocytes. For continual control of flow cytometer settings, analysis was always performed with the LinearFlow Green Flow Cytometry Intensity Calibration Kit (Molecular Probes Europe) using 488-nm excitation and 515-nm emission. The coefficient of variation in the mean fluorescence intensity of the calibration beads was <10% at all 4 levels of intensity covering the relevant examination range. Concentrations of TNF-α and IL-10 in culture supernatants were determined using ELISA (Duoset; R&D Systems). Plasma HIV-1 RNA levels were measured by quantitative RT-PCR (Cobas AmpliPrep/Cobas Amplicor HIV-1 Monitor Test, version 1.5; Roche Diagnostic Systems), with a limit of detection of 400 copies/mL.

Statistical analysis. Differences between 2 groups were compared using the Mann-Whitney U test for unpaired data. The Wilcoxon signed rank test was used for comparison of paired data. Relations between variables were tested using the Spearman rank correlation test. Data are given as median values (IQRs). Probability values are 2-sided and considered to be significant when P < .05.

RESULTS

Ex vivo expression of TLR2 and TLR4. Compared with 21 control subjects, 48 patients had significantly increased TLR2 expression on freshly isolated monocytes (figure 1A), whereas
no significant difference was seen for TLR4 expression (figure 1B). We found no statistically significant correlations between peripheral CD4+ T cell counts or plasma HIV RNA levels and expression of TLR2 and TLR4. Furthermore, when dividing patients into groups according to receipt of antiretroviral therapy, CD4+ T cell count, and HIV RNA level, no differences in TLR2 or TLR4 expression were observed.

In vitro effects of HIV protein on TLR2 expression. To further study the possible relationship between HIV infection and TLR2 expression, PBMCs from 10 patients (peripheral CD4+ T-cell count of 253 × 10^3 to 370 × 10^3 cells/L) and plasma HIV RNA load of 1419 copies/mL (1–8295 copies/mL)), 7 of whom were receiving HAART and 3 of whom were not receiving antiretroviral treatment, and from 10 control subjects were stimulated with 2 different concentrations of HIV gp120 (1 or 10 µg/mL) or a negative control (PBS) and cultured for 20 h before flow cytometric analysis of TLR2 expression on monocytes. We found that gp120 (1 µg/mL) moderately but significantly increased TLR2 expression (12%–18% increase; \( P < .05 \)), with a similar pattern among HIV-infected patients and control subjects. However, when PBMCs were stimulated with a higher concentration of gp120 (10 µg/mL), no further increase in TLR2 expression was seen among control subjects, whereas a marked increase (>90% higher than unstimulated levels) was observed among patients (figure 2).

Cytokine responses after TLR2 stimulation. To examine whether the ex vivo differences in TLR2 expression could have functional consequences, PBMCs from all 48 patients and 24 healthy control subjects were stimulated with either a negative control (PBS) or a synthetic TLR2 agonist (Pam, Cys) and cultured for 20 h before measuring levels of TNF-α and IL-10 in PBMC supernatants. Although TLR2 stimulation significantly increased TNF-α levels (\( P < .001 \)) and IL-10 levels (\( P < .001 \)) in patients and control subjects, the increase in TNF-α was significantly more marked in the patient group (figure 3). In contrast, no differences between patients and control subjects were seen in the magnitude of IL-10 responses upon TLR2 stimulation (data not shown).

TLR2 stimulation and HIV replication. To explore any possible effects of TLR2 stimulation on HIV replication, HIV
Figure 3. Fold increases in TNF-α levels in supernatants of PBMCs stimulated with a toll-like receptor 2 agonist (Pam, Cys, 1 µg/mL) and cultured for 20 h. See Results for TNF-α levels in stimulated and unstimulated PBMCs. Bars, interquartile range (IQR). *P = .023, compared with control subjects.

RNA levels were measured using quantitative RT-PCR of culture supernatants of PBMCs from 16 patients (11 of whom were not receiving antiretroviral treatment and 5 of whom were receiving HAART) that were unstimulated or stimulated for 20 h with Pam, Cys. In a pilot study, HIV RNA loads were undetectable after 1 h of culture, but increasingly higher levels were seen after 20 and 120 h, which was interpreted to have been due to in vitro replication, although release of premade intracellular particles could not definitively be ruled out. Several findings were revealed. First, although 9 patients with low CD4+ T cell counts (<300 × 10^3 cells/L) showed a significant increase in HIV RNA levels after TLR2 stimulation [absolute increase, 37,000 copies/mL (6150–184,500 copies/mL); P = .008], this was not seen for 7 patients with higher CD4+T cell counts (≥300 × 10^3 cells/L) [absolute increase, 0 copies/mL (−19,350 to 5150 copies/mL)]. Indeed, the change in HIV RNA levels after TLR2 stimulation differed significantly between these 2 groups (figure 4A). Second, for the patient group as a whole, the change in HIV RNA levels after TLR2 stimulation was negatively correlated with CD4+ T cell counts in peripheral blood and positively correlated with TLR2 expression on monocytes (figure 4B and 4C). Third, we also found a positive correlation between TNF-α and HIV RNA levels in TLR2-stimulated PBMC supernatants (r = 0.63; P = .028). No statistically significant correlation was found between plasma HIV RNA levels and increases in HIV RNA loads after TLR2 stimulation (r = 0.35; P = .017).

To explore whether the observed increase in HIV RNA replication after TLR2 stimulation could be caused by increased TNF-α levels per se, PBMCs from 5 patients with low peripheral CD4+ T cell counts (<150 × 10^3 cells/L) and high plasma HIV RNA loads (≥90,000 copies/mL) were selected and stimulated with Pam, Cys alone or in combination with anti–human TNF-α antibodies.
α antibodies or goat IgG (isotype control). Although immunoassaying revealed that TNF-α levels were completely suppressed in the presence of anti–TNF-α antibodies, a robust TLR2 response with increased HIV RNA levels was observed in all cell cultures stimulated with Pam3Cys, with no impact of anti–TNF-α antibodies on viral replication (data not shown).

**DISCUSSION**

The discovery of mammalian TLRs has provided new insight into mechanisms of innate immunity to microbial pathogens [6, 7, 19]. Activation of TLRs leads to the induction of antimicrobial responses essential to innate immune defenses and to the upregulation of antigen molecules necessary for antigen presentation and secretion of cytokines influencing adaptive immune responses [9, 20]. Thus, defects in normal TLR activation might be expected to compromise antimicrobial defenses [21]. Indeed, recently described mutations in IL-2 receptor–associated kinase, an essential adapter molecule in TLR-activated intracellular pathways, appear to be associated with an increased risk of infection [22]. However, recent reports also suggest that abnormally upregulated TLRs may be associated with potentially harmful inflammatory responses [23, 24].

In this article, we report enhanced TLR2 expression on monocytes during HIV infection. We found no clear impact of HAART or peripheral CD4+ T cell counts on TLR2 expression. However, because the relatively high CD4+ T cell counts in patients who did or did not receive antiretroviral treatment may conceal a possible impact on TLR2 expression, the potential effect of HAART on TLR2 expression should be further examined during longitudinal testing. We also found no relation between plasma HIV levels and TLR2 expression. Nevertheless, our in vitro experiments showed that stimulation of PBMCs with gp120 significantly increases TLR2 expression on monocytes, especially among HIV-infected patients, supporting a possible link between HIV infection and TLR2 expression. These HIV-mediated effects on TLR2 activation may also be operative in individuals with very low or undetectable plasma HIV loads, because HIV-infected monocytes have been shown to be present in the blood of such patients [25].

The possible in vivo relevance of our findings is open to discussion. It could be argued that the enhanced TLR2 expression on monocytes freshly recovered from patients is rather small, compared with that for monocytes from healthy patients, and without biological or clinical significance. However, an increased TNF-α response upon TLR2 stimulation but no such difference in the IL-10 response was observed among patients, compared with control subjects, suggesting net inflammatory effects in the patient group. We and others have previously shown that persistent TNF activation may persist in HIV-infected patients even during HAART, possibly contributing to immunodeficiency and metabolic complications [26–28]. Our findings from the present study suggest that TLR2-related mechanisms could contribute to the HIV-related inflammation.

A major finding in this study was that TLR2 stimulation in vitro was accompanied by a significant increase in HIV replication in patients with low CD4+ T cell counts. Moreover, in the patient group as a whole, the increase in HIV RNA levels seen in vitro was positively correlated with the level of TLR2 expression on monocytes. It is well-known that microbes and microbial antigens can promote HIV replication in HIV-infected patients [29], and the results from the present study suggest that TLR2 activation by microbial agents may contribute to this process, especially in patients with marked immunodeficiency. Several studies have reported that TNF-α may induce HIV replication in vitro [26, 30, 31]. Our finding of a correlation between TNF-α and HIV RNA levels after TLR2 stimulation might suggest that such mechanisms could be involved in the TLR2-induced enhancement of HIV replication. However, we demonstrated increased HIV replication after neutralization of TNF-α by anti-TNF-α antibodies, as well, suggesting a direct effect of TLR2 stimulation on HIV replication that is independent of TNF-α. In accordance with our findings, transgenic mouse models also indicate that TLR2 may be of potential importance for HIV replication [16, 17].

Further studies are warranted to confirm our observations. However, if increased TLR2 expression on monocytes, as well as increased inflammatory response and viral replication after TLR2 stimulation, is occurring in vivo, it can possibly contribute to the immunopathogenesis of HIV infection and may ultimately offer novel targets for immunomodulatory therapy.

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