Human Immunodeficiency Virus Infection Alters Tumor Necrosis Factor Alpha Production via Toll-Like Receptor-Dependent Pathways in Alveolar Macrophages and U1 Cells

Marlynne Q. Nicol, Jean-Marie Mathys, Albertina Pereira, Kevin Ollington, Michael H. Ieong, and Paul R. Skolnik

Center for HIV/AIDS Care and Research, Boston University School of Medicine, Boston, Massachusetts; Molecular and Clinical Genetics, Royal Prince Alfred Hospital and University of Sydney, Sydney, Australia; and Pulmonary Center, Boston University School of Medicine, Boston, Massachusetts

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Human immunodeficiency virus (HIV)-positive persons are predisposed to pulmonary infections, even after receiving effective highly active antiretroviral therapy. The reasons for this are unclear but may involve changes in innate immune function. HIV type 1 infection of macrophages impairs effector functions, including cytokine production. We observed decreased constitutive tumor necrosis factor alpha (TNF-α) concentrations and increased soluble tumor necrosis factor receptor type II (sTNFRII) in bronchoalveolar lavage fluid samples from HIV-positive subjects compared to healthy controls. Moreover, net proinflammatory TNF-α activity, as measured by the TNF-α/sTNFRII ratio, decreased as HIV-related disease progressed, as manifested by decreasing CD4 cell count and increasing HIV RNA (viral load). Since TNF-α is an important component of the innate immune system and is produced upon activation of Toll-like receptor (TLR) pathways, we hypothesized that the mechanism associated with deficient TNF-α production in the lung involved altered TLR expression or a deficit in the TLR signaling cascade. We found decreased Toll-like receptor 1 (TLR1) and TLR4 surface expression in HIV-infected U1 monocytic cells compared to the uninfected parental U937 cell line and decreased TLR message in alveolar macrophages (AMs) from HIV-positive subjects. In addition, stimulation with TLR1/2 ligand (Pam3Cys) or TLR4 ligand (lipopolysaccharide) resulted in decreased intracellular phosphorylated extracellular signal-regulated kinase and subsequent decreased transcription and expression of TNF-α in U1 cells compared to U937 cells. AMs from HIV-positive subjects also showed decreased TNF-α production in response to these TLR2 and TLR4 ligands. We postulate that HIV infection alters expression of TLRs with subsequent changes in mitogen-activated protein kinase signaling and cytokine production that ultimately leads to deficiencies of innate immune responses that predispose HIV-positive subjects to infection.
tions, usually involving *Mycobacterium tuberculosis* (7) and bacterial pathogens, in patients treated with anti-TNF-α drugs, such as infliximab and etanercept (8, 38). Constitutive concentrations of TNF-α in the lungs of HIV-1-infected individuals or from AMs exposed ex vivo to bacterial ligands may vary based on the stage of disease or presence of OIs (17, 33). In this study, we examined asymptomatic HIV-positive subjects on stable HAART, HIV-positive individuals on stable HAART who were failing therapy (based on CD4 and viral load measurements), and healthy HIV-negative control subjects. We observed decreased constitutive TNF-α in bronchoalveolar lavage fluid (BALF) samples from HIV-positive subjects compared to HIV-negative controls. This was more pronounced in HIV-positive subjects with the lowest CD4 cell counts and the highest HIV RNA (viral load). We also observed that HIV-1-infected U1 monoblastoid cells, differentiated with phosphor 12-myristate 13-acetate (PMA) to produce an AM-like phenotype, showed deficient TNF-α production in response to LPS (the gram-negative bacterial TLR4 ligand) and Pam3Cys (the synthetic TLR1/TLR2 ligand) compared to uninfected U937 cells. This was associated with decreased TLR surface expression and changes in the signaling pathways involved in the secretion of TNF-α.

**MATERIALS AND METHODS**

**Study subjects.** The study was approved by the Institutional Review Boards of Boston Medical Center, New England Medical Center, and Beth Israel Deaconess Medical Center. All subjects were volunteers who gave informed consent before participating in the study. We used a comprehensive questionnaire regarding age, sex, smoking status, medical history, including HIV-1 infection, related infections, and medications. All subjects had no active pulmonary disease and normal spirometry. We obtained HIV risk factor information, peripheral blood CD4 count, HIV RNA (viral load) in plasma, history of HIV-related diseases, and antiretroviral therapy for the HIV-positive subjects who underwent blood CD4 count, HIV RNA (viral load) ranged from 27 to 51 years. At the time of evaluation, the CD4 counts for the HIV-positive subjects were further stratified for various analyses by CD4 cell count (<200 cells/μl), CD4+ T cell count (200–1,000 cells/μl), and by viral load (<10,000 copies/ml and >10,000 copies/ml). Similar results were ob-
tained when subjects were stratified by viral load using thresholds of 400 (the lower limit of detection of the assay), 1,000, and 5,000 copies/ml. Viral loads were obtained from peripheral blood (plasma) samples. CD4 cell counts and viral load measurements were obtained within 2 to 7.5 weeks of research bronchoscopy. All HIV-positive subjects were on HAART for at least the past 8 months. At the time of research bronchoscopy, some individuals were failing therapy, as indicated by lower CD4 counts and higher viral loads. The reasons for treatment failure were not documented but did not include any obvious difficulties with adherence, since stable HAART for at least the past 8 months was an inclusion requirement of the study. Control donors were balanced for age and gender, had negative HIV antibody at the time of participation, and no evidence of pulmonary or systemic disease.

**Altered TNF-α levels in BALF samples from HIV-1-infected individuals.** Proinflammatory responses are important in protection against opportunistic infections, and TNF-α is a critical inflammatory cytokine of the innate immune system. We examined BALF fluid samples from HIV-positive individuals and found a decrease in basal TNF-α compared to healthy controls ($P = 0.0135$; Fig. 1A). When stratified by measures of disease progression, TNF-α production in the lung decreased with increasing viral load and with decreasing CD4 cell counts in the blood. Moreover, sTNFRII levels in BALF samples were significantly greater in HIV-positive individuals ($P = 0.05$) and were greater with increasing viral load and decreasing CD4 cell count. No differences in levels of sTNFRI were observed between groups (data not shown). Compared to the HIV-negative control group, this resulted in a decrease of 2.3- to 2.6-fold in the ratio of TNF-α to sTNFRII antagonist activity in the group with less advanced HIV disease (≤10,000 copies/ml and ≥200 cells/mm$^3$, respectively), and a decrease of 3.2- to 4-fold in the group with more advanced HIV disease who were failing therapy (≥10,000 copies/ml and ≤200 cells/mm$^3$, respectively; Fig. 1B and C). These data show significantly altered perturbations of the inflammatory cytokine TNF-α and its antagonist sTNFRII in HIV-positive individuals that are associated with advancing HIV disease; these alterations in TNF-α and sTNFRII concentrations were noted even in subjects who had responded well to HAART, which suggests persistent defects in innate immune responses even in the group with successful anti-HIV therapy.

**HIV-1 infection impairs TLR-mediated TNF-α production.** To determine whether decreased basal TNF-α levels in BALF samples from HIV-positive patients were associated with altered TLR innate immune responses, we examined AMs from HIV-positive individuals stimulated ex vivo with either the TLR2 ligand Pam$_3$Cys or the TLR4 ligand LPS. The secretion of TNF-α by AMs from HIV-positive subjects in response to

![FIG. 1. (A) TNF-α levels in BALF samples from asymptomatic HIV-positive subjects and healthy controls. Data are means ± SEMs (error bars) for 11 HIV-negative (HIV−) controls and 21 HIV-positive (HIV+) subjects. (B) Molar ratio of TNF-α to sTNFRII in BALF samples from HIV-positive individuals compared to HIV-negative controls, stratified by HIV RNA (viral load). Data are mean ± SEMs (error bars) from 9 control subjects, 14 HIV-positive subjects with a viral load of ≤10,000 copies/ml, and 7 HIV-positive subjects with a viral load of >10,000 copies/ml. (C) Molar ratio of TNF-α to sTNFRII in BALF samples from HIV-negative controls compared to HIV-positive subjects, stratified by CD4 cell count (cells/mm$^3$). Data are means ± SEMs (error bars) from 11 HIV-negative controls, 12 HIV-positive subjects with a CD4 count of >200 cells/mm$^3$, and 5 HIV-positive subjects with a CD4 count of ≤200 cells/mm$^3$. Values that were significantly different ($P < 0.05$, Wilcoxon two-sample test) from the value for the HIV-negative control group are indicated with an asterisk.](image-url)
both TLR ligands was decreased 1.7-fold compared with secretion by AMs from healthy controls (Fig. 2). This was statistically significant for the LPS-treated group, but not the Pam3Cys-treated group, for which fewer subject samples were available for analysis, although a similar trend was observed.

To further examine the impairment of TLR-mediated TNF-α production, we compared TNF-α production by the chronically HIV-1-infected U1 cell line to the uninfected parental U937 cell line after stimulation with increasing doses of TLR ligand LPS or Pam3Cys for 24 h. To model AMs, U1 and U937 cells were differentiated to an AM phenotype with PMA. U937 cells had higher basal TNF-α concentrations and produced TNF-α in a dose-dependent manner in response to the TLR2 and TLR4 ligands Pam3Cys and LPS, respectively (Fig. 3A and B, solid bars); in contrast, the HIV-1-infected U1 cells produced minimal TNF-α in response to either TLR ligand (Fig. 3A and B, open bars). In addition, TLR-mediated TNF-α gene expression in the infected U1 cells was reduced compared to U937 cells, in response to either Pam3Cys (Fig. 3C) or LPS (Fig. 3D). Interestingly, there appeared to be higher basal TNF-α mRNA in U1 cells (no stimulation, P = 0.059 [Fig. 3C] and P = 0.037 [Fig. 3D] by Student’s t test), although there were lower basal protein concentrations (Fig. 3A and B, no stimulation). However, the magnitude of response in the HIV-1-infected U1 cells was less than that observed in the U937 cells and appeared to be TLR-ligand specific, with an increase of fivefold in TNF-α mRNA in response to the TLR2 ligand Pam3Cys (Fig. 3C) and no increase in TNF-α gene expression in response to the TLR4 ligand LPS (Fig. 3D). In the U937 cells, there was an increase of 20-fold in TNF-α mRNA in response to both the TLR2 and TLR4 ligands. Moreover, this TLR-induced TNF-α expression was inhibited by pretreatment of cells with the ERK inhibitor PD98059, as manifested by decreased TNF-α mRNA and subsequent protein production.
(protein data not shown). Taken together, the patient AM and cell line data confirm that HIV-1-infection alters the ability of TLR ligands to induce TNF-α production, which may result in a compromised innate immune response.

**Decreased expression of TLR1 and TLR4 on HIV-1-infected cells.** Because Pam3Cys and LPS led to reduced TNF-α production in AMs from HIV-positive subjects and in HIV-1-infected cells, we examined the cell surface expression of TLR1 (which forms heterodimers with TLR2), TLR2 (the receptor for Pam3Cys), and TLR4 (the receptor for LPS) on U937 and U1 cells. TLR1, TLR2, and TLR4 were present on both uninfected U937 and HIV-1-infected U1 cells; however, surface expression of TLR1 and TLR4, but not TLR2, were reduced in the U1 cells (Fig. 4B) compared to the uninfected parental cell line, U937 (Fig. 4A). We also examined TLR6, which signals with TLR2 in response to mycobacteria, and found no significant differences in expression between cell types (data not shown). Of note, TLRs were predominately detected on cells that showed an activated phenotype as determined by CD11b/CD18 staining. The majority of HIV-positive U1 cells (96%) were found to be CD11b/CD18 positive, compared to approximately 34% of the uninfected U937 cells. Both cell lines were pretreated with equal concentrations and duration of PMA; therefore, the presence of HIV is likely playing a role in the increased activation state of the U1 cells. Western blot analysis also showed decreased levels of TLR4 protein in HIV-positive U1 cells (Fig. 4C).

Reduced responses to TLR ligands could be explained by decreased surface expression of TLRs and decreased expression of TLR4 protein in the HIV-1-infected U1 cells. Consistent with these observations in a HIV-infected cell line, we also observed a downregulation of TLR gene expression in AMs from HIV-positive individuals compared to healthy controls (Fig. 4D). This difference was statistically significant for the TLR1 and TLR2 genes ($P \leq 0.05$), for which a difference of 1.5- to 1.8-fold was observed. Although the difference in TLR4 gene expression was not significantly different between HIV-positive and HIV-negative healthy controls, there was a trend toward less TLR4 in the HIV-positive AMs. We did not measure TLR surface expression on AMs by fluorescence-activated cell sorting (FACS) because of insufficient cell numbers for many study subjects after other experiments were performed. These data suggest that the decreased TNF-α response to TLR2 and TLR4 ligands in AMs from HIV-positive subjects may be due to reduced TLR gene and receptor expression.

**Reduced phosphorylated ERK in response to TLR ligands in infected promonocytic cells.** Phosphorylation of ERK is involved in many critical signaling pathways and is required for the production of TNF-α protein in response to TLR ligands (25). To assess the role of ERK in TLR2 and TLR4 signaling during HIV infection, we cultured AMs and U937 and U1 cells in the presence and absence of the MAP kinase inhibitor PD98059, an upstream inhibitor of ERK phosphorylation, followed by Pam3Cys or LPS treatment. Protein extracts were analyzed by Western blotting for phosphorylated ERK. We used the H3 protein as a control, since the expression of this protein was not influenced by TLR signaling or HIV-1 infection.

When we examined cytoplasmic protein extracts from HIV-positive AMs and healthy volunteers for the presence of phosphorylated ERK in response to the TLR4 ligand LPS, we found decreased activation of ERK in the HIV-positive AMs compared to uninfected AMs (Fig. 5A). In cell lines, Western blot data showed an increase of two- to threefold in phosphorylated ERK in the uninfected U937 cells detected up to 60 min in response to both TLR2 and TLR4 ligands. These responses were inhibited by pretreatment of the cells with PD98059 (Fig. 5B and C). In contrast, in the infected U1 cells, increased basal levels of phosphorylated ERK were observed, but there was no significant induction of phosphorylation through 60 min in response to either Pam3Cys (Fig. 5B) or LPS (Fig. 5C). U1 cells showed minimal activation of ERK in response to either ligand, and treatment with the inhibitor did not significantly decrease phosphorylation of ERK. Therefore, unlike U937 cells, the HIV-1-infected U1 cells did not induce further signaling upon treatment with TLR ligands. In U1 cells, there were higher basal levels of ERK, which was associated with dampened responses to TLR2 and TLR4 ligands; this may in part explain decreased TNF-α production. Therefore, altered signaling through ERK in the HIV-1-infected cell lines and AMs from HIV-positive subjects was associated with decreased inflammatory cytokine production in response to LPS and Pam3Cys.

**DISCUSSION**

Most studies of host immunity against HIV and opportunistic pathogens that affect HIV-positive individuals have focused on the adaptive immune response. Less is known about the more primitive innate immune response during HIV infection. In this study, we examined BALF samples and alveolar macrophages from HIV-positive subjects and healthy matched HIV-negative control subjects. We extended our investigation using the PMA-differentiated monocyte cell line U937 (23) and its HIV-1-infected subclone U1 to mimic an AM phenotype. We determined cell membrane expression of TLR1, TLR2, and TLR4 and found decreased surface expression of TLR1 and TLR4 on the HIV-1-infected U1 cell line as well as decreased TLR4 protein compared to the uninfected U937 cells. Due to low cell numbers obtained from bronchoalveolar lavage in this study group, we were unable to perform FACS for TLR surface expression on AMs, although results from real-time PCR showed decreased gene expression of a subset of TLRs (Fig. 4D). We also found significantly less production of TNF-α in response to TLR2 and TLR4 ligands in U1 cells and in AMs from HIV-positive subjects (TLR4 ligand) and a trend toward decreased responses to the TLR2 ligand in AMs from HIV-positive subjects, for which fewer samples from study subjects were available for analysis (Fig. 2).

Studies have shown that HIV infection quickly depletes intestinal lymphoid tissues and compromises mucosal integrity (6). This leads to “microbial translocation” and the presence of bacterial products in the peripheral circulation. These bacterial products include LPS from gram-negative bacteria and peptidoglycan, the naturally occurring TLR2 ligand analog of Pam3Cys from gram-positive bacteria that colonize the normal human intestine. Thus, the TLR ligand responses we studied have direct relevance to persons with HIV infection. Brenchley (5) and others have shown that plasma levels of LPS correlate with chronic immune activation of CD4 cells; this may lead to
FIG. 4. Flow cytometric analysis of TLR1, TLR2, and TLR4 constitutive surface expression on PMA-pretreated (10 nM for 48 h) U937 (A) and U1 (B) cells. Cells were labeled with PE-Cy5-conjugated CD11b/MAC-1 antibody, PE-conjugated TLR1 (left), fluorescein isothiocyanate-conjugated TLR2 (middle), or PE-conjugated TLR4-PE (right) antibody or matched isotype control. Cells were gated on CD11b/MAC-1-positive cells. Thirty-four percent of U937 cells and 97% of HIV-positive U1 cells were CD11b/MAC-1 positive. Representative histograms show isotype (shaded) or test antibody (black line). The percentage of positive cells for test antibody and the geometric mean fluorescence intensity in parentheses are shown for each graph in panels A and B. Samples were collected by FACS and analyzed with WinMDI version 2.9 software.

(C) Whole-cell protein extracts from cell lines pretreated with PMA were analyzed by Western blotting for the presence of TLR4 and control protein H3. We used densitometry and the Total Lab computer package to determine band intensities and normalized the TLR4 band with control H3 for no stimulation (NS) and LPS treatment (in minutes) for U937 (solid bars) and U1 (open bars) cells. The height of the triangle above the gel shows the increasing amount of time after LPS treatment. (D) Total RNA from HIV-negative healthy volunteers (n = 4; solid bars) and HIV-positive (n = 5; open bars) alveolar macrophages was used to perform two-step real-time PCR analysis. Data are mean relative mRNAs plus SEMs (error bars), normalized using the β-actin housekeeping gene. Values for HIV-positive AMs that were significantly different from the value for HIV-negative AMs are indicated as follows: *, P < 0.05 by t test.
T-cell activation, increased entry into the cell cycle, and ultimately increased death of central memory CD4 cells, which is one of the hallmarks of the immunodeficiency associated with HIV infection (N. T. Funderburg, A. Luciano, W. Jiang, S. Sieg, and M. Lederman, presented at the Fourth International Conference on HIV Pathogenesis, Treatment, and Prevention, Sydney, Australia, 22 to 25 July 2007). Our data concerning the effects of LPS on innate immune function through TLR4 in-
icate that similar mechanisms may be pertinent in vivo through microbial translocation.

Viral infection can cause alterations in TLR4 protein expression and membrane localization, altering the lung environment and innate immune responses, with a subsequent switch from an LPS-tolerant state to a LPS-responsive state (4, 22, 27, 34). Several HIV-1 accessory proteins have been implicated in the regulation of TLR expression and signaling (24). HIV-1 Nef protein altered TLR-dependent cytokine production in AMs from HIV-positive subjects through induction of cellular phosphatases and inhibition of intracellular signaling (35). HIV may utilize multiple strategies to alter TLR expression and signaling, and these effects may vary based on cell type and location. Heggelund et al. (14) showed enhanced expression of TLR2 (but not TLR4) on peripheral blood monocytes from HIV-positive subjects after exposure to the HIV-1 envelope protein gp120. Effects on TLR expression may vary in relation to the macrophage type (from different body compartments); specifically, AMs may differ from peripheral blood monocytes in terms of TLR expression during HIV infection. Reports of the expression of TLRs on U1 cells have also varied (29, 35), perhaps due to differences in experimental procedures and conditions, such as the concentration of the PMA used or the cell passage number. We have also shown that more U1 cells become activated, as measured by CD11b/MAC-1 expression, than U937 cells after PMA stimulation, and that the majority of TLR expression occurs on these activated cells. Further studies to determine the role of macrophage maturation on TLR expression and signaling will be of interest.

TNF-α is a cytokine which has pleiotropic biological actions, and since cytokines act locally, examining concentrations in specific organs, such as the lung, is important. The balance of TNF-α with antagonist moieties, such as sTNFRII, determines, in part, net agonist activity of TNF-α, since sTNFRII acts as a “scavenger” for TNF-α. The balance of TNF-α and sTNFRII in the lung likely determines innate immune function, rather than plasma concentrations as measured in other studies (15, 16, 31). In our study, the ratio of TNF-α to sTNFRII was lowest in the groups with the highest viral load and lowest CD4 cell count; higher ratios indicate more abundant agonist TNF-α activity, which we postulate is associated with normal innate immune function in the lung. Anti-TNF-α therapies highlight the importance of TNF-α in normal host innate immunity, since their use in Crohn’s disease (18) and rheumatoid arthritis (21) is associated with an increased incidence of mycobacterial and other bacterial infections, especially in the lung (32, 37).

Although sTNFRII “scavenges” soluble TNF-α and limits binding to surface receptors, thereby acting as an inhibitor, it has also been shown to stabilize TNF-α, preserving its activity and potentially augmenting longer-term effects (2). It may be that stabilizing TNF-α in compartments, such as alveolar spaces, where the clearance of TNF-α is already slow (28), may have significant biological consequences. The absolute amounts of TNF-α in BALF may not be as significant as the relationship between sTNFRII and its stability in the lung environment, which could lead to persistently abnormal innate immune function.

Cytokines also play a role in TLR signaling through alterations in expression of TLRs. TNF-α and IFN-γ regulate TLR4 expression in intestinal epithelial cells (1) and TLR4 in monocytes and macrophages (3). This makes biologic sense, since tight control of inflammatory responses at mucosal surfaces, which are exposed to commensal bacteria and exogenous agents, would be beneficial to host defense. In addition to decreased TNF-α production by AMs exposed to Pam3Cys or LPS, we also observed decreased responses in other pathways involved in TLR-induced TNF-α production, such as NF-κB p50 nuclear translocation (data not shown) and TNF-α gene expression. Moreover, there was decreased phosphorylation of ERK in AMs from HIV-positive subjects and infected cell lines. It is interesting to note there were increased basal (constitutive) levels of phosphorylated ERK in the U1 cell line and subsequent lower increases in response to TLR ligands, which may indicate that chronic stimulation of TLRs may “tolerize” their receptors and lead to defective TLR signaling and innate immune responses. This concept is supported by the observation of deficits in TLR-dependent TNF-α production in U1 cells that were almost all in the activated state as measured by CD11b/MAC-1 staining (Fig. 3). Further studies will be required to determine whether decreased phosphorylation of other MAP kinases, such as p38 and Jun N-terminal protein kinase, in response to HIV infection, is also evident.

Taken together, our studies show altered TLR-dependent innate immune responses of AMs from HIV-positive subjects and in HIV-1-infected cell lines. In future studies, it will be important to determine the effects of other TLR ligands on innate immune responses and whether altered surface receptor expression involves changes in transcription, translation, or membrane transport of TLRs. Our findings of altered pathogen recognition receptors and lower innate immune responses, through indirect cytokine or direct viral protein effects, may help explain the increased occurrence of OIs and other pulmonary infections in HIV-positive individuals.

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