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Diminished responsiveness to human β-defensin-3 and decreased TLR1 expression on monocytes and mDCs from HIV-1-infected patients

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ABSTRACT

hBD-3 is an antimicrobial peptide that may contribute to adaptive immune responses by activating professional APCs via a TLR1/2-dependent mechanism. Patients with HIV disease experience increased susceptibility to mucosal infections, which may, in part, stem from diminished APC function. Our current studies demonstrate a reduced capacity of hBD-3 to induce the expression of a costimulatory molecule, CD80, on monocytes and mDCs from HIV-infected persons compared with cells from healthy controls. Although the expression of TLR1 and TLR2 on monocytes was not a strong predictor of hBD-3 responsiveness in bivariate analyses, monocytes and mDCs from HIV-infected persons expressed significantly lower levels of TLR1. Monocyte expression of the activation marker CD69, in cells from HIV-infected persons with therapeutically controlled viremia, was correlated directly with TLR2 and TLR4 expression but not with TLR1 expression. Overall, these studies suggest that immune activation may affect TLR2 and TLR4 expression but may not fully account for reduced TLR1 expression in monocytes from HIV-infected persons. Impairments in hBD-3 responsiveness and TLR1 expression are likely to contribute to increased risk of mucosal infection in HIV disease. *J. Leukoc. Biol.* 92: 1103–1109; 2012.

Introduction

hBD-3 is an antimicrobial peptide with chemotactic and immunomodulatory properties [1–5]. hBD-3 can mediate activation of APCs, such as monocytes and mDCs, in a TLR1/2-dependent manner [6, 7]. As a consequence of activation, APCs express increased surface levels of costimulatory molecules, such as CD80. We have demonstrated previously that hBD-3 induction of CD80 on primary monocytes was blocked by a MyD88 inhibitor and by antagonistic TLR1/2 antibodies [6]. Thus, we have proposed that hBD-3 may activate APCs to link innate and adaptive defense mechanisms at mucosal sites and can mediate this effect, at least in part, by modulating the activation of PRRs.

HIV disease is characterized by monocyte activation and by altered functionality of these cells. For example, monocytes from HIV-infected persons express high levels of the activation marker CD69 and are less responsive to type I IFN [8–10]. Also, monocyte responses to LPS may be altered in HIV disease as a result of increased in vivo exposure to microbial products that gain access to circulation as a consequence of damage to the gut mucosal barrier [11–13]. It is possible that diminished monocyte function may contribute to generalized immunodeficiency in HIV-infected persons.

In this study, we examine monocyte responsiveness to hBD-3 and for comparison, LPS in cells from HIV-infected persons. As previous reports have suggested that TLR2 expression is increased in monocytes from HIV-infected persons [14], it was possible that responses to hBD-3 would be intact or even augmented in HIV disease. Surprisingly, monocytes from HIV-infected persons displayed defects in response to hBD-3 stimulation. We also assessed the relationship of these defects with TLR expression and found evidence of TLR1 deficiency in cells from HIV+ donors but absence of a clear correlation between TLR expression levels and responsiveness to hBD-3. Thus, mechanisms extending beyond TLR surface expression may play a significant role in the reduced monocyte responsiveness to hBD-3 in HIV disease. Overall, these observations suggest that diminished hBD-3 responsiveness could contribute to impaired mucosal immunity in HIV-infected persons.

Abbreviations: BD-3=β-defensin 3, BDCA-1=blood DC antigen 1, cART=combination antiretroviral therapy, hBD-3=human β-defensin 3, mDC=myeloid DC, MFI=mean fluorescence intensity, PB=Pacific Blue, shBD-3=synthetic human β-defensin 3, VL=viral load

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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MATERIALS AND METHODS

Reagents

shBD-3 was purchased from Peptides International (Louisville, KY, USA), and LPS from *Escherichia coli* 026:B6 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Complete medium consisted of RPMI 1640 (BioWhittaker, Walkersville, MD, USA), supplemented with antibiotics (penicillin/streptomycin; BioWhittaker), 2 mM L-glutamine (BioWhittaker), and 10% Human AB serum (Gemini Bioproducts, Woodland, CA, USA).

Cell preparation and culture

These studies were approved by the Institutional Review Board at Case Western Reserve University/University Hospitals/Case Medical Center (Cleveland, OH, USA). After informed consent was obtained, blood from 27 healthy donors and 45 HIV-1-infected patients was drawn into heparin-coated tubes. PBMCs were isolated over a Ficoll-Hypaque cushion. One million PBMCs were cultured in 0.5 mL medium with shBD-3 (20 μg/mL) or LPS (100 ng/mL) or in medium alone for 18 h. Expression of TLR1, -2, and -4 on monocytes and expression of activation markers on monocytes (CD69) and T cells (CD38 and HLA-DR) were also examined directly ex vivo in whole blood samples from HIV+ patients and uninfected controls.

PBMCs (1×10^6 cells/ml) were cultured in medium alone or in medium supplemented with LPS (100 ng/mL) or flagellin (*Salmonella typhimurium*, 0.1 μg/mL; Invivogen, San Diego, CA, USA) overnight to investigate the effects of transducin-like enhancer ligand exposure on TLR1 and -2 expression.

Flow cytometry

Cell populations were first identified by forward- and side-scatter and then by expression levels of surface proteins using fluorescently conjugated antibodies or appropriately matched isotype controls. Monocytes were electronically gated based on positive expression of CD14, using anti-CD14 FITC (BD PharMingen, San Diego, CA, USA). mDCs were electronically gated based on negative expression of CD14 and CD19, using anti-CD14 PerCP and anti-CD19-allophycocyanin-cy7 (BD PharMingen) and by positive expression of CD11c (allophycocyanin; BD PharMingen) and BDCA-1 (FITC; Miltenyi Biotec, Bergisch Gladbach, Germany). Expression of the activation markers CD80 and CD69 was measured using anti-CD80-PE and anti-CD69-PE-cy7.

T cell activation was measured using anti-CD38-PE, HLA-DR-FITC, and anti-CD3-allophycocyanin (BD Biosciences, San Jose, CA, USA); anti-CD8 (allophycocyanin-cy7) and anti-CD4-PB (BD PharMingen); and appropriate isotype control mAb.

Antibodies for TLR expression were purchased from eBioscience (San Diego, CA, USA): TLR1 (PE), TLR2 (PE-cy7), and TLR4 (PB).

Cells were stained for 15 min in the dark at room temperature, washed, and analyzed using an LSRII flow cytometer (Becton Dickinson, San Diego, CA, USA) and FACSDiva (Version 6.1, BD Biosciences) or FloJo software.

Statistical methods

Differences among variables between patients and controls were tested with a Mann-Whitney's U-test. We tested associations between pairs of continuous variables with Spearman's rank correlation. We processed the analyses using Graphpad Prism v. 5.02 (GraphPad Software, La Jolla, CA, USA). All tests are two-sided, and a P value of 0.05 was considered nominally significant.

RESULTS AND DISCUSSION

PBMCs were isolated from 27 healthy control donors and 45 HIV-infected patients. Among the controls, 63% of the controls were male, and the median age was 35 years (range=21–62). The normal range for $CD4^+$ T cell counts in HIV-uninfected patients is 700–1500 cells/μL [15]. Samples from the HIV+ patients were collected in two groups. Among the HIV+ patients in Group 1, 57% of the patients were male, and the median $CD4^+$ T cell count among this population was 551 cells/μL (range=32–1766 cells/μL). Fifty-six percent of the patients in Group 1 had undetectable viral replication (HIV RNA<400 c/mL) and were receiving cART. The median VL of the remaining patients in Group 1 was 22,090 c/mL (514–750,000 c/mL). Among the HIV+ patients in Group 2, 92% were male; all patients were on cART and had undetectable viremia. The median age of this group was 52 years (22–61years), and the median $CD4^+$ T cell count among these patients was 598 cells/μL (137–942). Demographic information for these patients is shown in Supplemental Table 1.

Reduced CD80 induction in monocytes and mDCs after stimulation with hBD-3

To assess APC responses to hBD-3, PBMCs were incubated in complete medium alone or in medium supplemented with an optimal concentration of hBD-3 (20 μg/mL), as determined in previous studies [6]. Monocyte responsiveness to LPS (100 ng/mL) was also measured. Monocytes and mDCs were assessed after overnight incubation for cell-surface expression of CD80 (**Fig. 1A** and **B**). Induction of CD80 by hBD-3 on monocytes (n=21; mean increase of 468.8±157 light units) and mDCs (n=7; 260.1±120 light units) from HIV-infected persons was reduced compared with the hBD-3-induced expression of CD80 on monocytes (n=15; 2555.3±879 light units; P=0.0039; Fig. 1C) and mDCs (n=6; 1545.8±338 light units; P=0.0023; Fig. 1D) from healthy control donors. We did not find a statistically significant relationship between $CD4^+$ T cell counts (r=−0.36; P=0.10) or viremia (r=−0.55; P=0.12) and hBD-3-mediated induction of CD80. Increases in the proportions of $CD80^+$ monocytes by hBD3 were also statistically different when comparing cells from HIV− (medium alone %$CD80^+$=8.4±2.2%; hBD-3=40.8±4.3%) versus HIV+ donors (medium alone=8.5±1.4%; hBD-3=19.9±3.4%; P=0.003; Supplemental Fig. 1A). Proportions of mDCs that express CD80 following hBD-3 exposure were also significantly different when comparing cells from HIV− (medium alone %$CD80^+$=24.9±3.6%; hBD-3=58.8±6.6%) versus HIV+ donors (medium alone=25.1±3.3%; hBD-3=31.7±3.5%; P=0.02; Supplemental Fig. 1B). Induction of CD80 by LPS on monocytes and mDCs also tended to be reduced when comparing results from HIV-infected patients and controls, but these differences did not reach statistical significance (Fig. 1C and D). The defects in hBD-3 responsiveness were observed in viremic and aviremic, cART-treated subjects, suggesting that antiretroviral therapy is not sufficient to correct these perturbations (induction of CD80 by hBD-3 on viremic patients=530.4±102 light units; aviremic patients=394.9±322 light units; P=0.25).

Diminished TLR1 expression in cells from HIV+ donors

Monocytes and mDCs from HIV-infected and HIV-uninfected donors were examined for TLR1 and TLR2 expression by flow cytometry immediately after PBMC isolation. As essentially all

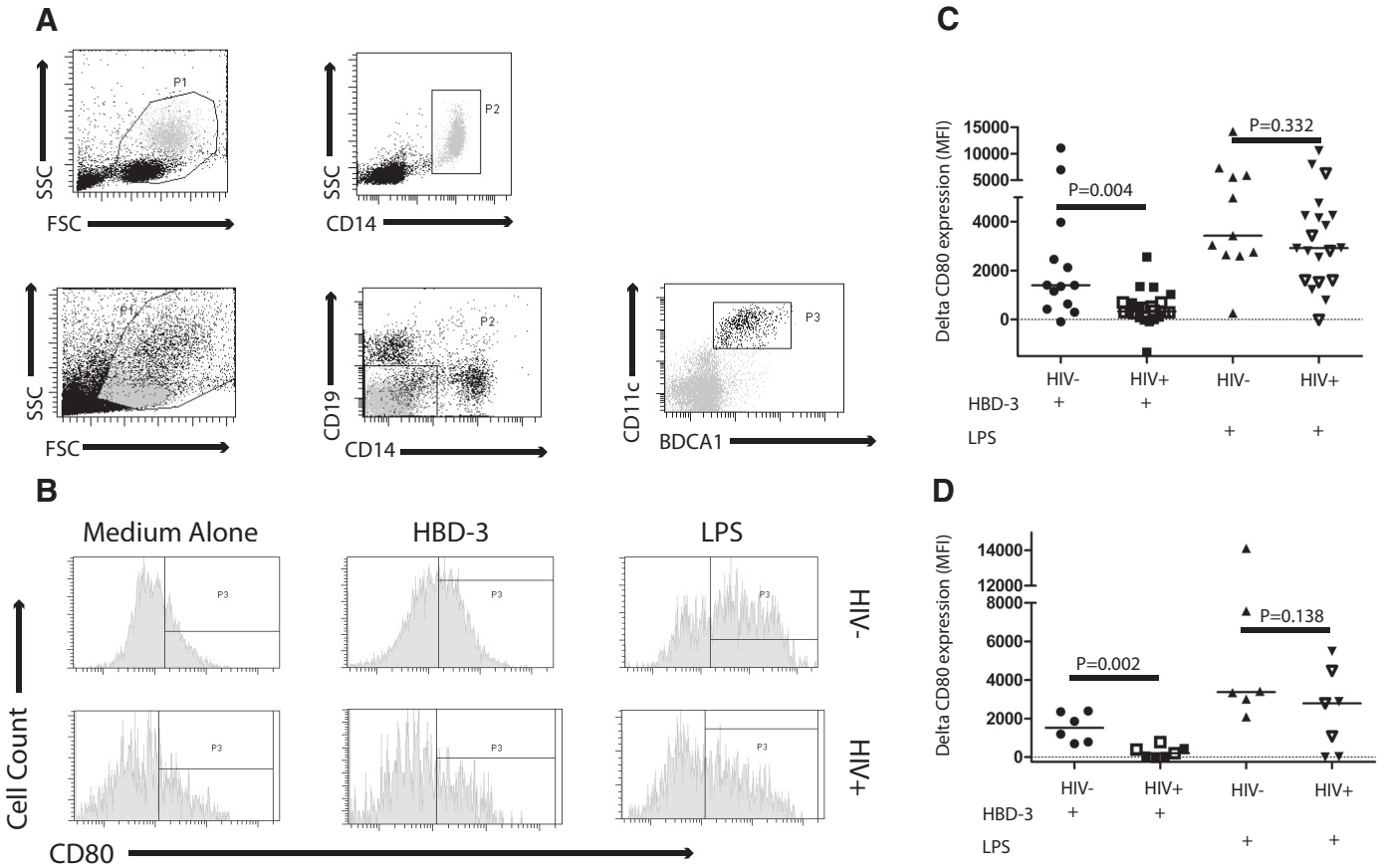


Figure 1. Monocytes and mDCs from HIV-infected patients are less responsive to hBD-3 than are cells from controls. PBMCs were isolated from 13 healthy controls and 21 HIV-infected patients. (A) Monocytes were identified by flow cytometry based on forward- and side-scatter (FSC and SSC, respectively) and by positive expression of CD14, and mDCs were identified by negative expression of CD14 and CD19 and positive expression of BDCA-1 and CD11c. PBMCs were incubated overnight in medium alone or in medium supplemented with 20 μg/mL hBD-3 or 100 ng/mL LPS. Expression of CD80 on monocytes and mDCs was measured on the surfaces of these cells. (B) Representative monocyte CD80 histograms from one control donor and one HIV-infected patient. Summary data of CD80 induction on (C) monocytes and (D) mDCs. The data are represented as the change in MFI of CD80 (MFI of CD80 expression in hBD-3-treated cells−MFI of CD80 expression in cells incubated in medium alone). Among the data from HIV+ patients, open symbols represent patients with uncontrolled viremia (VL>400 c/mL), whereas closed symbols represent patients with controlled viremia (VL<400 c/mL).

mDCs and monocytes expressed TLR1 and -2 in HIV+ and HIV− donors, we assessed the surface density of these receptors on APCs from infected and uninfected donors. Whereas TLR2 expression tended to be normal or slightly increased in monocytes and mDCs from HIV+ donors (**Fig. 2B**), TLR1 expression was diminished in cells from HIV-infected persons compared with expression on monocytes (mean MFI TLR1 1142±145 light units vs. 2075±351 light units; P=0.009; Fig. 2A) and mDCs (mean MFI TLR1 760±94 light units vs. 1550±386 light units; P=0.007; Fig. 2A) from control donors.

We next asked if TLR1 or TLR2 expression on monocytes was related to responses to hBD-3 in cells from HIV+ donors. The cell-surface expression of neither receptor demonstrated a significant relationship with hBD-3 responsiveness as measured by CD80 induction (r=−0.039, P=0.889 for TLR1; r=0.448, P=0.108 for TLR2; data not shown). These data suggest that although the reduction in TLR1 expression likely contributes to the poor responses to hBD-3, other mechanisms may play a more predominant role.

Relationship between TLR expression and monocyte activation in cells from HIV-infected persons

We next considered the possibility that immune activation could underlie the differences in TLR expression that we had observed in monocytes from HIV+ donors. This seemed feasible, as previous studies indicate that exposure to TLR agonists can influence TLR1/2 expression in monocytes [16]. We have found similar results by exposing PBMCs from healthy controls to various TLR agonists (LPS or flagellin) and assessing TLR1 and TLR 2 surface expression in $CD14^+$ cells. Exposure to LPS or flagellin resulted in increased expression of TLR2 (172±10% and 184±15%, respectively) and decreased expression of TLR 1 (54±13% and 45±11%, respectively; n=3; Supplemental Fig. 2). Thus, monocyte activation in vitro results in increased surface expression of TLR2 and decreased surface expression of TLR1, which is similar to the phenotype that we observed in freshly isolated monocytes from HIV+ donors.

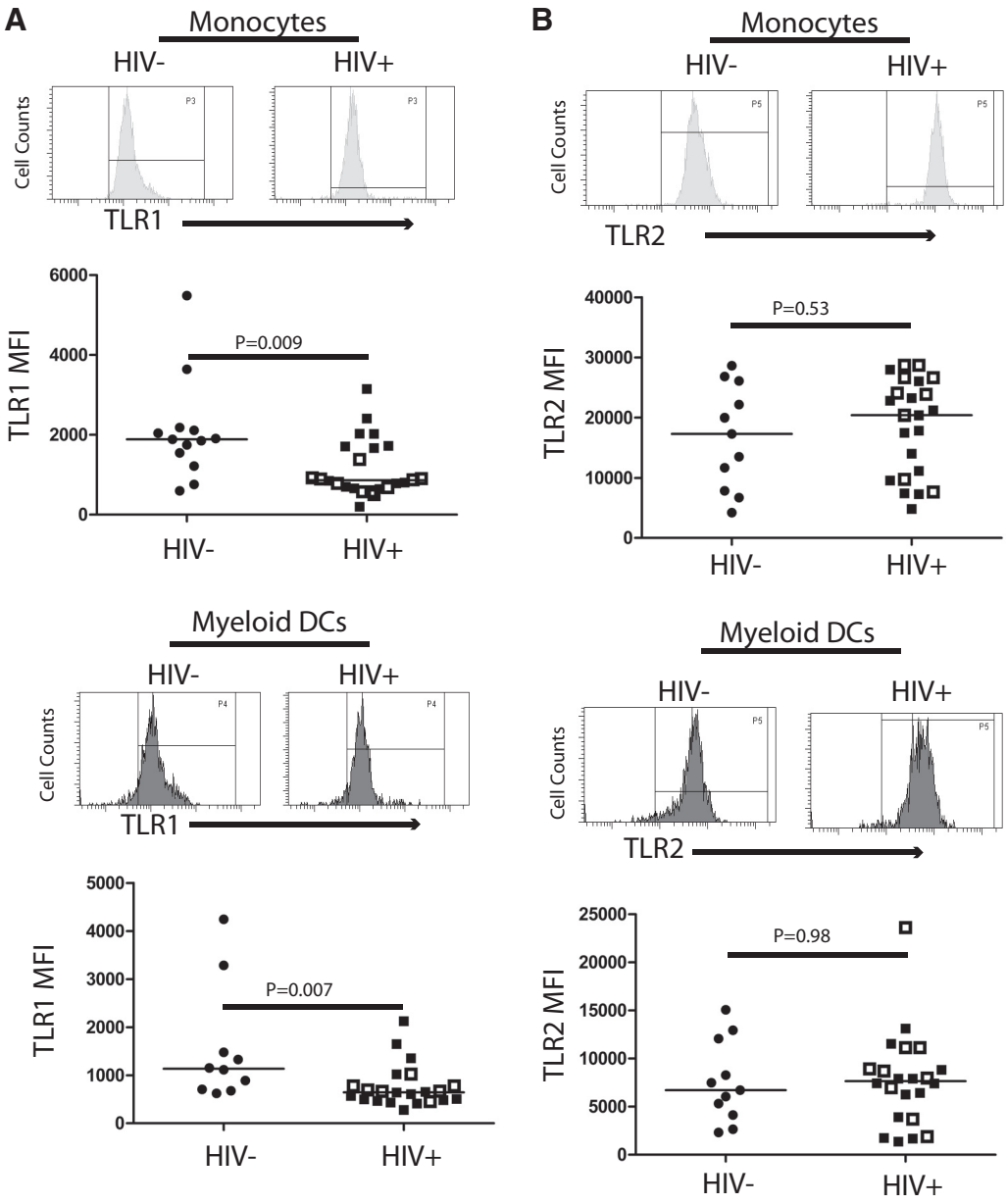


Figure 2. Expression of TLR1 is diminished on monocytes and mDCs in HIV-infected patients. PBMCs were isolated from healthy controls and HIV-infected patients, and expression levels of TLR1 and -2 were measured by flow cytometry directly ex vivo. Monocytes and mDCs were identified as described previously, and representative and summary data are shown for expression of (A) TLR1 and (B) TLR2 on monocytes and mDCs from HIV-infected and uninfected donors. Among the data from HIV+ patients, open symbols represent patients with uncontrolled viremia (VL>400 c/mL), whereas closed symbols represent patients with controlled viremia (VL<400 c/mL). Expression of TLR1 was reduced significantly on monocytes (P=0.009) and mDCs (P=0.007) from HIV-infected patients compared with expression on cells from controls.

To assess the role of immune activation on monocyte TLR expression in vivo among HIV-infected persons, we examined the expression of the activation marker CD69 and the expression of TLR1, -2, and -4 on monocytes from HIV+ donors. We also included an analysis of CD38 and HLA-DR expression on $CD8^+$ T cells as an indicator of T cell activation in these subjects. These studies were performed on a second group of HIV-infected patients, and we focused these studies on patients receiving antiretroviral therapy with suppression of virus below the limits of detection. Results from our initial HIV+ study group indicated that there were no differences in hBD-3 responsiveness among viremic and aviremic subjects, and we reasoned that by enlisting a second cohort of patients comprised of patients receiving successful cART, there would be less variability in immune activation parameters as a result of controlled viral replication. The proportion of activated $CD8^+$ Tcells was greater in this patient population (n=13; mean=24.1±3.5%) compared with this proportion in controls (n=9; 12.0±3.8%; P=0.0148; **Fig. 3A**). Monocytes from these patients were also enriched for cells that expressed CD69 (67.8±5.3%) compared with monocytes from controls (38.1±7.4%; P=0.0075; Fig. 3B), and as anticipated from the studies above, TLR1 expression was diminished (although not reaching statistical significance), whereas TLR2 and TLR4 tended to be increased in cells from HIV+ donors (Fig. 3C). TLR4 and TLR2 expression was correlated directly with CD69 expression in cells from HIV-infected persons, suggesting that activation of monocytes could be driving the expression of these TLRs (Fig. 3D). Surprisingly, TLR1 expression was not related to CD69 expression, suggesting that other mechanisms could play a more important role in regulation of TLR1 expression in HIV disease. Expression of TLR1, -2, or -4 on monocytes was not related to the proportion of activated $CD8^+$ T cells (not shown).

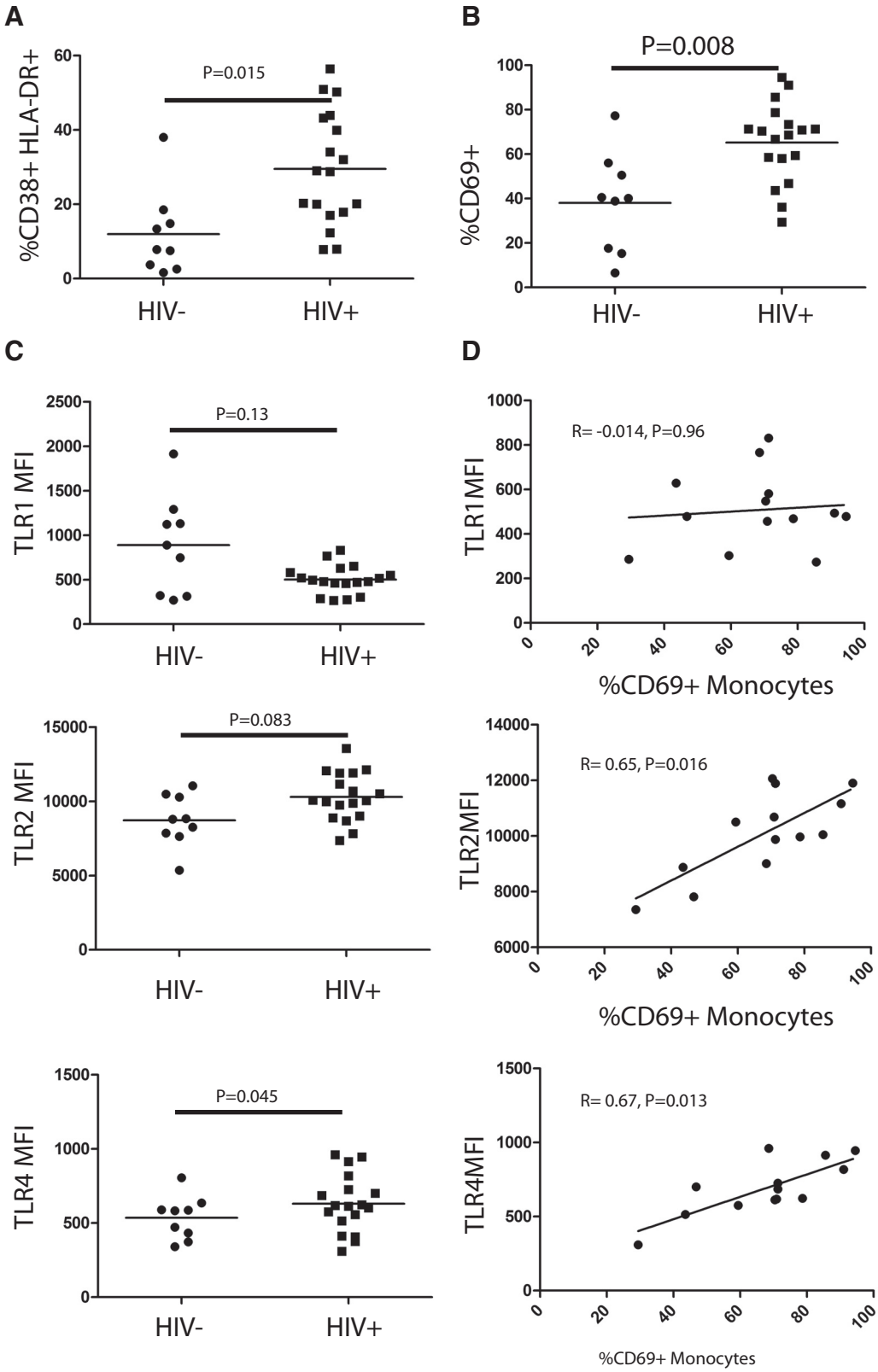


Figure 3. Expression of TLR2 and TLR4 is related to monocyte activation in HIV disease. Whole blood samples were obtained from separate populations of HIV-1-infected donors (n=13) and healthy controls (n=9), and the activation markers on $CD8^+$ T cells and $CD14^+$ monocytes were assessed by flow cytometery. $CD8^+$ T lymphocytes were identified by forward- and side-scatter characteristics and positive expression of CD3 and CD8. Activated $CD8^+$ T cells and monocytes were identified by dual expression of CD38 and HLA-DR and CD69, respectively. Summary data for the proportions of activated (A) $CD8^+$ T lymphocytes and (B) monocytes are shown. (C) Expression of TLR1, -2, and -4 was also measured on monocytes from uninfected and HIV-infected donors. Data from HIV− donors are represented as circles; data from HIV+ donors are represented as squares. (D) Among the HIV-infected patients, monocyte activation was related directly (Spearman correlation) to TLR2 (r=0.649; P=0.016) and -4 (r=0.667; P=0.013) but not TLR1 (r=−0.014; P=0.964) expression.

The role of antimicrobial peptides in bridging innate and adaptive defenses is becoming increasingly recognized. HBD-3 is expressed by cells in the basal layer of the epithelium, where it may have greater access to submucosal tissues. HBD-3 causes chemotaxis at nanomolar concentrations via CCR2 interactions [3, 4], and at higher concentrations, hBD-3 can mediate antimicrobial activity [1, 2, 5] and activate APCs [6, 7]. The data presented in this study suggest that monocytes and mDCs from HIV-infected persons may have diminished responses to hBD-3, as measured by induced expression of the CD80 costimulatory molecule. Notably, constitutive CD80 expression is relatively low in freshly isolated monocytes and is not significantly different when comparing monocytes with HIV-infected persons and healthy control donors [10]. The CD80 induced by hBD-3 requires 4–8 h of incubation prior to detection, suggesting that the molecule is induced rather than simply maintained by hBD-3 exposure [6]. Overall, these findings suggest that myeloid cells from HIV-infected persons may have a reduced capacity to induce immune responses at mucosal sites, but further studies are needed to fully assess this impairment.

The potential for hBD-3 to mediate APC activation may be important in bridging innate and adaptive defense mechanisms; moreover, hBD-3 also has antiviral effects, including an ability to cause down-modulation of HIV coreceptor (CXCR4) expression and to neutralize virions directly [17–19]. These functions of hBD-3 highlight the need to understand the activities of this molecule in mucosal and submucosal sites of HIV-infected persons. Important aspects of these studies will include not only assessment of APC responses to microbial peptides, as outlined in these experiments, but also future efforts to define the expression and regulation of hBD-3 in the setting of chronic and acute HIV infection.

Our studies are the first to demonstrate that TLR1 protein expression is diminished on monocytes and mDCs from HIV-infected persons; this may contribute to reduced responsiveness of these cells to hBD-3 stimulation or potentially to impaired recognition of potential pathogens in mucosal tissues. In contrast, TLR2 expression is not reduced in cells from HIV+ donors and may even be elevated. Nonetheless, neither TLR1 nor TLR2 expression predicts monocyte responsiveness to hBD-3 stimulation in cells from HIV+ donors, suggesting that other intracellular regulatory mechanisms may play a more predominant role in the impairment. We have reported previously that MyD88, NF-κB p65, and the MAPK signaling molecules p38 and ERK are involved in hBD-3-mediated cellular activation [6, 7]. Intracellular signaling pathways downstream of TLRs may be impaired in APCs from HIV-infected patients, potentially as a result of chronic immune activation. Experiments to assess signaling capabilities in purified APC populations from HIV-infected patients should be explored.

One possible explanation for our findings stems from the role of microbial translocation in HIV disease. Microbial products that cross the gut barrier are found at a higher concentration in plasma of HIV-infected persons compared with healthy donors and are directly related to measures of immune activation [11–13]. Thus, it is possible that chronic exposure to microbial products in circulation could cause activation of APCs and modify the ability of these cells to be activated further by TLR agonists. Such "tolerance" mechanisms are thought to stem from intracellular regulatory pathways and not necessarily from modulation of TLR surface expression [20]. This understanding is consistent with other findings, suggesting that TLR responsiveness is diminished in DCs from HIV+ donors and that ex vivo responses to LPS by monocytes from HIV+ donors are inversely related to LPS levels detected in circulation [11]. Our analyses of LPS stimulation also suggest that responsiveness to LPS tends to be diminished in cells from HIV+ donors. Under the conditions tested, however, the differences in LPS responsiveness between cells from healthy donors and HIV+ donors were relatively modest and not statistically significant. It is possible that deficiencies in LPS responsiveness could be demonstrated more readily with lower concentrations of reagent or with selection of HIV-infected persons experiencing more advanced disease.

The modulation of TLR1 and TLR2 expression following activation of monocytes in vitro is reflective of the phenotype of freshly isolated monocytes from HIV+ donors (decreased TLR1 expression and increased TLR2 expression). Furthermore, we found a strong correlation between TLR2 and TLR4 expression and an activated $CD69^+$ phenotype in freshly isolated monocytes from HIV patients receiving cART. In contrast, we did not find strong correlations between expression of CD69 on monocytes and levels of TLR1 nor did we find any evidence that immune activation measured by T cell expression of HLA-DR and CD38 was related to monocyte TLR expression levels. Overall, our studies suggest that immune activation of myeloid cells may play an important role in enhanced TLR2 and TLR4 expression in HIV disease, but the determinants of reduced TLR1 expression in myeloid cells of HIV-infected persons are likely to be more complex.

It is important to recognize that although we have previously shown a TLR-dependent mechanism involved in hBD-3 activation of APCs and specifically, CD80 induction [6], hBD-3 is also highly promiscuous in its interactions. For example, there is evidence that hBD-3 can interact with CXCR4 [17], MCR-1 [21], and CCR2 [3, 4]. Thus, although TLR1/2 is necessary for hBD-3-mediated APC activation, interactions with other receptors are also likely to contribute to the biological activities of hBD-3, and it is possible that such interactions could play a role in the diminished responses of APC to hBD-3 in HIV disease.

Our studies provide evidence of potential APC dysfunction in HIV disease. Notably, the defect in hBD-3 responsiveness and the defect in TLR1 expression are observed not only in viremic persons but also in persons with good control of viral replication, while on antiretroviral therapy. This raises the possibility that altered responsiveness to hBD-3 stimulation could contribute to increased risk of certain mucosal manifestations that persist in persons on antiretroviral therapy, such as the risk of oral warts [22]. Further studies to explore these relationships may help clarify the significance of impaired TLR responsiveness in HIV disease and in mucosal immunity.

AUTHORSHIP

N.T.F. performed experiments. S.F.S. and N.T.F. designed experiments, analyzed the data, and wrote the manuscript.

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