Published in final edited form as:

AIDS Res Hum Retroviruses. 2007 November ; 23(11): 1387–1395. doi:10.1089/aid.2007.0033.

Failure of TLR4-driven NF- κ B activation to stimulate virus replication in models of HIV-1 activation

Sushila K. Nordone¹, Glicerio A. Ignacio¹, Lishan Su², Gregory D. Sempowski³, Douglas T. Golenbock⁴, Liwu Li⁵, and Gregg A. Dean^{1,*}

¹Center for Comparative Medicine and Translational Research and Department of Molecular Biomedical Sciences, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606, USA

²Department of Microbiology and Immunology and The Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599, USA

³Departments of Medicine and Pathology, Duke Human Vaccine Institute, Duke University School of Medicine, Durham, North Carolina 27710, USA

⁴University of Massachusetts Medical School, Division of Infectious Diseases and Immunology, 364 Plantation Street, Lazare Research Building 308, Worcester, Massachusetts 01605, USA

⁵Department of Biology, Institute for Biomedical and Public Health Sciences, Virginia Tech, Blacksburg, VA, 24061, USA

Abstract

The interaction of HIV-1 with Toll-like receptors (TLR) on host target cells is incompletely understood. Data from several in vivo and in vitro model systems suggests TLR2, TLR4 and TLR9 remain functional and if stimulated, cause an upregulation of viral replication. In the present studies employing two different chronically HIV-1 infected cell lines and highly purified TLR agonists, we found ligation of TLR2 and TLR9, but not TLR4, resulted in significant up-regulation of HIV-1 production. This result was not due to a lack of TLR4 expression or impaired NF- κ B activation. Using 293T cells transfected with individual TLRs and an HIV-1 LTR reporter confirmed that TLR4 signaling does not directly activate the HIV-1 LTR. Finally, ultra-purified LPS did not enhance production of IL-1 β or IL-6 in chronically infected U1 cells, whereas significant cytokine production was observed in uninfected U937 cells. These results confirm the biological activity of ultra-purified LPS and raise the possibility that TLR4 signaling pathways may be altered during chronic HIV-1 infection. Collectively, these studies suggest that although several TLR can upregulate NF- κ B in HIV-1 infected cells, upregulation of NF- κ B alone is insufficient to activate the viral LTR. Further dissection of the TLR signaling pathways is necessary to determine how TLR stimulation leads to LTR activation and whether HIV-1 infection can alter signaling through TLR4.

*Correspondence and Request for Reprints: Gregg A. Dean, DVM, PhD, Center for Comparative Medicine and Translational Research, College of Veterinary Medicine, North Carolina State University, 4700 Hillsborough Street, Raleigh, North Carolina 27606, phone: 919 513 2819, Fax: 919 515 3044, Gregg_Dean@ncsu.edu.

Keywords

AIDS; lipopolysaccharide; transcription factors; HIV; Toll-like receptor

INTRODUCTION

It was recognized early in the AIDS epidemic that co-infection with microbial pathogens resulted in transient increases in plasma viremia and exacerbated disease^{1,2}. A direct mechanism for viral up-regulation by microbial molecules, most notably LPS, was subsequently demonstrated and shown to be mediated by NF- κ B activation of the viral long terminal repeat (LTR)^{3–5}. A decade passed before the discovery of Toll-like receptors (TLR) presented the obvious molecular link between co-infection and HIV-1 up-regulation. These germ-line encoded pattern recognition receptors recognize conserved bacterial, viral and protozoal molecular motifs such as lipoprotein (TLR2), double-stranded RNA (TLR3), lipopolysaccharide (LPS) (TLR4/MD2), flagellin (TLR5), single-stranded RNA (TLR7 and TLR8) and unmethylated CpG motifs (TLR9)⁶. Importantly, TLR are expressed on host cells known to be HIV-1 reservoirs⁷, including memory CD4 T cells, macrophages and dendritic cells^{8–10}. Several studies have investigated specific TLR for their capacity to increase HIV-1 viral replication. Study designs included the use of latently infected cell lines, HIV-1 LTR-transfected cell lines and the HIV-1 transgenic mouse model^{11–15}. Because of the early emphasis on LPS as a mediator of LTR activation, TLR4 was implicated as a direct link between co-infection and increased HIV-1 plasma virus levels^{14,16}. Collectively, data from these studies suggest that TLR4 ligation directly activates HIV-1 replication¹².

Here we show activation of TLR2 and TLR9 on HIV-1 infected cells upregulates virus replication but ligation of TLR4 with ultra-pure LPS does not. Furthermore, the effect through TLR9 is mediated by B class CpG but not A class CpG. We also show that induction of cytokines through TLR4 stimulation may be compromised by HIV-1 infection.

MATERIALS AND METHODS

Cell lines

293T human epithelial kidney (HEK) cells engineered to express TLR-YFP chimeras have been previously described^{17,18}. 293T cell lines were maintained in high glucose DMEM supplemented with 10% FBS, 2 mM L-glutamine and 1 μ g/ml ciprofloxacin. Expression of TLR-YFP was selected using 0.5 mg/ml G418-sulfate. The human monocytic cell line U937 and its HIV-1 infected subclone, U1¹⁹, were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol and 10 μ g/ml ciprofloxacin. THP₈₉₆GFP (generous gift of Dr. David N. Levy, NYU) were cultured under identical conditions²⁰. Experiments were conducted in the absence of ciprofloxacin and no overt differences were observed in proliferation or viability between cell lines.

Antibodies for detection of TLRs

Antibodies against human TLR2 (clone TL2.1), TLR4 (clone HTA125) and TLR9 (clone 26C593.2) were obtained from Imgenex Corporation (San Diego, CA). PE-labeled goat anti-mouse IgG (Caltag, Burlingame, CA) was used as the detection reagent. For detection of intracellular TLR9, cells were permeabilized using Cytotfix/Cytoperm (BD Bioscience, San Jose, CA) per manufacture's instructions with one modification. After initial permeabilization and prior to intracellular staining, cells were incubated in permeabilization buffer supplemented with 10% FBS for 20 minutes at room temperature to block non-specific staining. Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, La Jolla, CA) and data were further analyzed using CellQuest software (Becton Dickinson).

TLR agonists

Synthetic tripalmitoylated lipopeptide (PAM₃CSK₄), ultra-purified *E. coli* 011:B4 LPS, B class CpG-ODN 2006 (5'- tcg tcg ttt tgt cgt ttt gtc gtt -3'), control ODN 2006 (5'- tgc tgc ttg tgc ttt gtc gtt -3'), A class CpG-ODN 2216 (5'- ggG GGA CGA TCG TCg ggg gg -3') and control ODN 2216 (5'- ggG GGA GCA TGC TGg ggg gc -3') were purchased from Invivogen (San Diego, CA). Crude purified *E. coli* 011:B4 LPS (designated as WT LPS) was purchased from Sigma (L3024, St. Louis, MO). Dose response curves were performed to determine optimal TLR agonist concentrations for each cell line and concentrations were chosen based on maximal NF-κB reporter activity (data not shown) and/or p24 production (Table 1).

NF-κB reporter assay

293T HEK cells were transfected with pNF-κB-NIFTY-SEAP (Invivogen) using Eugene 6 transfection reagent (Roche Applied Science, Indianapolis, IN) and the subsequent NF-κB driven SEAP-reporter gene expression was assayed according to manufacturer's instructions (Roche Applied Science). U937 and U1 cells were transfected with pNF-κB-NIFTY-SEAP using the Amaxa system (Amaxa Inc., Gaithersburg, MD) and similarly assayed for SEAP reporter activity. Data were background subtracted from transfected, unactivated control wells of the respective cell line. All data were normalized to an external GFP control.

Quantification of HIV replication

Replication of HIV-1 in U1 cells was determined by quantification of p24 in culture supernatant by ELISA. Briefly, 96 well plates (Maxisorb flat bottom ELISA plates, Nalge NUNC, Rochester, NY) were coated overnight at 4°C with 10 µg/ml anti-HIV-1 p24 antibody (clone 183-H12-5C, NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Bruce Chesebro and Kathy Wehrly) in 0.5 M NaHCO₃ buffer, pH 8.6 (bicarbonate coating buffer). Plates were blocked with 2% w/v bovine serum albumin and 1% w/v nonfat dry milk in bicarbonate coating buffer for 2 h at room temperature. Plates were washed 3 times with PBS 0.05% Tween 20 wash buffer and gently tapped to remove excess liquid. Cell culture supernatant was serially 2-fold diluted in PBS 5% v/v FBS 1% v/v Triton X-100 and 100 µl of each dilution was added to wells. A previously quantified HIV-1 positive sample was diluted in a similar manner from 20 ng to 20 pg and served as the standard curve. Plates were incubated for 2 h at room temperature,

then washed 4 times with wash buffer. As a secondary detection reagent, HIV Ig (catalog # 3957, NIH AIDS Research and Reference Reagent Program, NABI and NHLBI) was diluted 1:2000 in PBS with 5% v/v normal mouse serum, 5% v/v normal goat serum, 5% v/v FBS, and 0.1% v/v Tween-20 (secondary diluent). 100 μ l was added to wells and allowed to incubate for 1 h at room temperature. Plates were again washed 4 times with wash buffer. Goat anti-human horse radish peroxidase-conjugated IgG (Jackson ImmunoResearch, West Grove, PA) was diluted 1:10,000 in secondary diluent and 100 μ l was added to each well. After 1 h, plates were washed six times with wash buffer and developed using TMB substrate (KPL, Gaithersburg, MD) following manufacturer's directions.

Replication of HIV-1 in THP_{896GFP} cells was measured by intensity of EGFP fluorescence using an IVIS imaging system (Xenogen, Alameda, CA) and secreted p24 by ELISA.

Quantification of secreted cytokines

IL1- β , IL-6 and TNF α in tissue culture supernatant were measured using Luminex technology per manufacturer's directions (Bio-Rad, Hercules, CA).

LTR luciferase reporter assay

293T TLR cell lines were co-transfected with a pNL4-3-luciferase R-E- plasmid (p-NL4-3-luc) (NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. He and Landau) and the pGL4-renilla luciferase reporter (Promega, Madison, WI) using the amaxa system (Gaithersburg, MD). Cells were harvested 12 h post-TLR activation and luciferase was developed using firefly and renilla reporter assay systems per manufacturer's instructions (Promega). All NL4-luciferase data were normalized to internal renilla controls and expressed as fold-increase in luciferase activity above un-stimulated controls from each respective time point.

Statistical analysis

Differences between treated and untreated cells were determined by analysis of variance and Duncan's multiple range test (SAS, Cary, NC). The level of significance used was $p < 0.05$. Error bars represent the SEM.

RESULTS

Validation of TLR ligands

To establish the purity and specificity of TLR agonists to be used in subsequent experiments, HEK 293T cells engineered to stably express human TLR2, TLR3, TLR4, TLR4/MD2 and TLR9 were transfected with an NF- κ B reporter and treated with known ligands for TLR2, TLR4 and TLR9. The synthetic lipopeptide PAM₃CSK₄ induced a 28-fold up-regulation of NF- κ B activity in TLR2⁺ 293T cells and failed to induce NF- κ B in TLR4 (data not shown), TLR4/MD2 or TLR9 expressing cells (Figure 1A, B and C), indicating the synthetic agonist was specific for TLR2. Similarly, ultra-purified LPS was specific for TLR4/MD2, and as previously reported²¹, required MD2 co-expression for signaling (data not shown). In contrast, WT LPS activated TLR2 and TLR9 in addition to TLR4/MD2, indicating contamination with bacterial lipopeptide and unmethylated B class

CpG-ODN (Figure 1A, B and C). B class CpG-ODN did not induce NF- κ B activation in either TLR2 or TLR4/MD2 293T cells, indicating specificity for TLR9 (Figure 1C). Interestingly, the magnitude of NF- κ B activation following TLR9 activation with B class CpG ODN was markedly lower than that of TLR2 and TLR4, resulting in a moderate 3–4 fold increase above background. None of the TLR agonists induced NF- κ B activation in HEK-TLR3 293T cells, indicating preparations were free of TLR3 agonist activity (data not shown).

TLR4 activation does not up-regulate HIV-1 replication in U1 and THP_{896GFP} cells

Chronically HIV-1-infected U1 and THP_{896GFP} cells were used to evaluate the role of TLR stimulation on virus replication. To avoid non-specific upregulation of virus replication, cells were not induced to mature with PMA prior to TLR agonist exposure.²² Forty-eight hours post-treatment with TLR ligands, culture supernatant from U1 cells was assayed for the presence of HIV-1 p24 by ELISA. Constitutive p24 levels averaged 188 ± 33 pg/ml in supernatants from untreated U1 cells (Figure 2A). Stimulation with Pam₃CSK₄ resulted in a significant increase of p24 to 2027 ± 557 pg/ml ($p < 0.05$) (Figure 2A), representing a 10.7-fold increase in virus production post-TLR2 engagement. In contrast, ultra-purified LPS exposure did not significantly increase p24 levels (477 ± 277 pg/ml, $p=0.21$). B class CpG-ODN was the most potent activator of new virus production in U1 cells, with p24 levels significantly increasing 30.7-fold to 5737 ± 2264 pg/ml ($p < 0.05$) (Figure 2A). The combination of TLR2, 4 and 9 agonists present in WT LPS moderately enhanced virus production, with a 7.4-fold increase to 1400 ± 227 pg/ml ($p < 0.05$). The A class CpG-ODN did not induce any measurable increase in HIV-1 p24 levels (data not shown).

To further validate our observations, we repeated this experiment with latently HIV-1 infected THP_{896GFP} cells in which EGFP is under control of the HIV LTR (Figure 2B–D). Similar to U1 cells, activation of TLR4 with ultra-purified LPS resulted in an insignificant increase in virus production ($1,695 \pm 1,763$ vs. 110 ± 192 pg/ml p24 in untreated cells, $p = 0.34$) as compared to Pam₃CSK₄ ($12,794 \pm 6,099$ pg/ml, $p = 0.04$), B class CpG-ODN ($6,344 \pm 5,576$ pg/ml, $p=0.06$), TNF- α ($24,308 \pm 12,046$ pg/ml, $p=0.0002$) or PMA ($5,274 \pm 3,764$ pg/ml, $p=0.07$) (Figure 2D). The crudely purified WT LPS that contains contaminating TLR2 and TLR9 agonists enhanced virus production 7.2-fold over that of unstimulated THP_{896GFP} cells (**2382 ± 1587 pg/ml**). The apparent disconnect between secreted HIV-1 p24 and EGFP expression following PMA or B class-CpG treatment of THP_{896GFP} cells was not due to reduced cell viability as determined by trypan blue exclusion (data not shown).

TLR4 is expressed and responsive to LPS ligation in U1 cells

To determine whether the lack of HIV-1 up-regulation after TLR4 ligation with ultra-purified LPS was due to a lack of TLR4 expression, extracellular TLR2 and TLR4 and intracellular TLR9 protein was assessed in U1 cells by flow cytometry. U1 cells expressed all three TLRs and the level of expression was comparable to the parental cell line U937 (Figure 3A–C). Furthermore, activation of TLR2 and TLR4 with their respective agonists induced an equivalent down-regulation of surface protein²³, suggesting both TLR2 and

TLR4 were responding to ligand binding in an expected and analogous manner (Figure 4A and B). TLR4 expression was similarly observed in THP_{896GFP} cells (data not shown).

Stimulation of TLRs activates NF- κ B but not cytokine production in chronically infected cells

NF- κ B activation is a well characterized outcome of TLR activation and an accepted mechanism for reactivation of virus production in HIV-1 infected cells²⁴. To determine whether a defect in TLR4-induced upregulation of virus production could be associated with a failure to activate NF- κ B, chronically infected U1 cells and the uninfected parental cell line U937 were transfected with an NF- κ B-SEAP reporter and exposed to TLR2, 4 and 9 agonists.

As has been previously described, background NF- κ B activity in unstimulated, chronically HIV-1 infected U1 cells was greater than that of uninfected U937 cells (Figure 5A)²⁵. The TLR2 agonist PAM₃CSK₄ and TLR4 agonist ultra-purified LPS induced activation of NF- κ B to similar levels in U937 and U1 (Figure 5A). Interestingly, B class CpG induced a higher level of NF- κ B activation in U1 cells ($p < 0.05$).

Secretion of cytokines is an important downstream event following activation of NF- κ B during an immune response and is also implicated in HIV-1 reactivation. Culture supernatant was assayed for IL-1 β , IL-6 and TNF α at 24 h post-treatment with TLR agonists using a Luminex bead-based cytokine multiplex assay. IL-1 β was below the level of detection (200 fg/ml) in both U937 and U1 cells in the absence of stimulation (Figure 5B). Treatment with TLR2 agonist PAM₃CSK₄ induced a low level of IL-1 β production in U937 cells (1510 ± 1409 fg/ml) while identically treated U1 cells produced approximately 3-fold less IL-1 β (550 ± 311 fg/ml). Ultra-purified LPS was a more effective inducer of IL-1 β from U937 cells, increasing the concentration of cytokine to 4950 ± 2851 fg/ml. In contrast, ligation of TLR4 on U1 cells failed to stimulate the production of IL-1 β . B class CpG did not induce measurable production of IL-1 β in either cell line. TNF α secretion was only observed following activation of TLR2 in U937 and U1 cells, and similar to IL-1 β , the concentration of secreted cytokine was greater from uninfected cells (Figure 5C). As previously reported, LPS did not induce TNF α production in the absence of PMA-preactivation²⁶.

Of the cytokines evaluated, IL-6 was produced at the greatest concentration following TLR activation (Figure 5D). Prior to TLR activation, U937 and U1 cells produced approximately 10 pg/ml. Stimulation of U937 cells with PAM₃CSK₄ increased IL-6 secretion to 341 ± 187 pg/ml whereas only 65 ± 32 pg/ml IL-6 was found in U1 cell supernatant (Figure 5D). Ultra-purified LPS was a more potent inducer of IL-6 in comparison, increasing IL-6 concentrations to 753 ± 198 pg/ml in U937 cells. Secretion of IL-6 in response to TLR4 activation in U1 cells was severely reduced, measuring only 36 ± 6 pg/ml ($p = 0.01$). Unexpectedly, B class CpG induced a 7-fold increase in IL-6 production in U1 cells but no increase in uninfected U937 cells (Figure 5D) ($p = 0.009$). The induction of IL-6 in U1 cells was specific for B class CpG in that A class CpG did not induce IL-6 production in either cell type (data not shown). Collectively, the relationship between NF- κ B activation and cytokine production was strong in uninfected U937 cells (IL-1 β $r = 0.63$, $p < 0.01$; IL-6 $r =$

0.78, $p < 0.0009$) but less evident in infected U1 cells (IL-1 β $r = 0.42$, $p = 0.11$; IL-6 $r = 0.38$, $p = 0.16$).

During natural infection with a pathogen multiple PAMPs would engage multiple TLRs, inducing additive or synergistic effects on cellular processes such as signaling and cytokine production²⁷. Recent data in murine macrophages suggest the ‘combinatorial code’²⁷ first identified to explain TLR synergy is due to concurrent activation of the MyD88-dependant (TLR2, TLR4, TLR9) and MyD88-independent pathways (TLR3 and TLR4)²⁸. In contrast, redundant activation of a single pathway does not result in additive or synergistic cytokine responses^{27,28}. We measured the effect of the combination of TLR2, TLR4 and TLR9 agonists found in WT LPS on both NF- κ B activation and cytokine production (Figure 5A–D). The magnitude of these responses was slightly greater, albeit not significantly, when uninfected cells were treated with WT LPS, suggesting that there may be a moderate augmentation of responses when agonists are combined during natural infection. There was no augmentation of responses when infected U1 cells were treated with WT LPS.

TLR4 engagement does not directly activate the HIV-1 LTR

The apparent disconnect between TLR4-induced NF- κ B activation and virus induction in chronically infected monocytic cells led us to ask whether TLR4 signaling would activate the viral LTR in a more controlled system. 293T cells expressing a single TLR were transfected with the HIV-1 LTR reporter construct **p-NL4-3-luc** and then were treated with TLR agonists. TLR2 agonist induced a significant up-regulation of **p-NL4-3-luc** activity in the TLR2 expressing 293T cells ($p < 0.05$) and B class CpG-ODN led to a significant, albeit lesser, increase in **p-NL4-3-luc** activity in the TLR9 expressing cells ($p < 0.05$). In contrast, ultra-purified LPS did not induce luciferase expression from the HIV LTR in TLR4/MD2 expressing 293T cells. These data suggest that TLR4 activation is not sufficient to activate the HIV-1 LTR.

DISCUSSION

The regulation of HIV-1 expression within infected cells is controlled at many levels by both viral factors and host-cell transcription and translation machinery. Toll-like receptors have been implicated in HIV-1 replication, however; a clear understanding of the molecular pathways involved has yet to be established. The first reports of pathogen-associated HIV-1 production from chronically infected cells were a decade prior to identification of the TLR family and the subsequent characterization of TLR ligand specificity. In those seminal reports, LPS from *E. coli* strains 0127:B8 and 026:B6 were shown to be potent inducers of HIV-1 from chronically infected U1 and THP-1 cells^{3,4}. Stimulation of virus induction by LPS was associated with LTR activation and a concomitant enhancement of NF- κ B activation⁴. Several reports have followed indicating stimulation through TLR2, 4 and 9 leads to activation of the HIV-LTR, production of virus and potentially AIDS progression¹². Confounding these conclusions is both the use of LPS of uncertain purity with respect to TLR2 and/or TLR9 agonists^{3,4,14,15,29} and the disparity between using an LTR reporter in uninfected cells and the actual functionality of TLR signaling pathways in HIV-1 infected cells^{4,11,14,15,30}. While others have gone to lengths to remove TLR2 agonist contamination

from LPS used in LTR activation studies¹⁴, our study is the first to use an LPS preparation that is confirmed to be free of both TLR2 and TLR9 agonists. Importantly, the ultra-purified LPS preparation retained the ability to stimulate cytokine production in uninfected cells, verifying its biological activity.

In the present study, we initially validated purity of all agonists using 293T cells stably transfected with individual TLR gene constructs. The synthetic lipoprotein PAM₃CSK₄ was a potent and highly specific agonist for TLR2. Similarly, ultra-purified LPS and B class CpG- ODN were specific for TLR4 and 9, respectively. However, incubation of TLR cell lines with WT LPS resulted in up-regulation of NF-κB in cell lines expressing TLR2 and TLR9, suggesting contamination of the commonly used *E. coli* O11:B4 LPS with lipoprotein and CpG-ODN.

In agreement with previous reports^{11,15,31–34}, we found TLR2 and TLR9 agonists are potent inducers of HIV-1 virus production in chronically infected U1 and THP_{896GFP} cells. The induction of virus correlated with the ability of these two agonists to up-regulate NL4-LTR activity in their respective TLR-specific 293T cell line. In contrast to previous reports is our finding that TLR4 stimulation does not induce HIV-1 production, nor does it appear to directly activate the HIV-1 LTR in a TLR4/MD2-expressing 293T cell line. Despite the conflicting conclusions, our observations can be reconciled with published reports that conclude LPS activates the HIV-1 LTR. First there is the possibility that LPS preparations, particularly those used before human TLR were discovered, were contaminated with other TLR agonists. Second, it is possible that LPS up-regulation of HIV is an indirect effect due to the induction of cytokines. Third, it is possible that LPS from different bacteria may vary in their interaction with TLR4. For example, it is known that LPS from *E. coli* species have a conical conformation while LPS from *Salmonella* has a cylindrical conformation³⁵. Whether different conformations of LPS can lead to unique signaling and subsequent cellular outcomes is a topic of intense research. Finally, different cell types may respond differently to LPS ligation of TLR4.

Our results are not at variance with the seminal report on LPS/LTR interaction. In 1990 Pomerantz *et al.* similarly reported that LPS did not up-regulate virus in chronically HIV infected U1 or THP-1 cells. Only when U1 cells were pretreated with GM-CSF did LPS stimulation increase HIV p24 production. The authors hypothesized that either U1 cells are immature and lack the LPS receptor or possibly a component of proximal NF-κB pathway. We have shown that undifferentiated U1 cells express TLR4 and up-regulate NF-κB in response to LPS. Pomerantz *et al.* (1990) and more recently Equils *et al.* (2001) have clearly shown that NF-κB binding of the HIV LTR is essential to induce viral replication. Furthermore, Equils *et al.* have elegantly demonstrated in human dermal microvessel endothelial cells that the response is mediated through TLR4 and the TLR4 signal transduction molecules. As the authors suggest, whether TLR4 stimulation alone is adequate to activate the HIV LTR may depend on the cell type being studied. We suspect that in the monocytic cell lines used here and in TLR4/MD2-transfected 293T cells, a transcription factor (or factors) that are required for LTR activation are not activated in response to ultra-purified LPS. Candidate transcription factors beyond NF-κB include Sp1, C/EBPβ/NF-IL-6, PU.1, CREB, USF and AP-1 as all have been reported to play a role in HIV-1 LTR

activation in other primary cells and cell lines^{17,36}. Along these same lines, we observed that TLR9 activation by B class CpG ODN resulted in virus production despite relatively minor NF- κ B activation. This supports the idea that TLR-induced virus production relies on multiple transcription factors. Clarification of these signaling pathways and identification of critical additional transcription factors may inform chemotherapeutic strategies to inhibit pathogen-induced virus production without the potential negative impact of global NF- κ B impairment.

Alternately, there may be a point in the TLR4 signaling pathway (unrelated to NF- κ B activation) that is disrupted during chronic HIV-1 infection. The failure of ultra-purified LPS to induce IL-1 β and IL-6 in chronically infected U1 cells is suggestive of such a signaling defect; although we cannot rule out that these observations are particular to this cell line and/or its inherent state of differentiation. This possibility is supported by work of Tachado and colleagues who reported normal TLR4 expression but impaired TNF α production in alveolar macrophages from HIV-1 infected patients and PMA-differentiated U1 cells³⁷. In those studies it was shown that reduced ERK1/2 phosphorylation may be responsible, at least in part, for the observed defects in TNF α induction. When Tachado *et al.*'s observations are combined with our observation that TLR4 does not directly signal to the HIV-1 LTR, it could be hypothesized that virus-induced impairment of the TRAM/Trif and/or MAPK arm of the TLR4 signaling cascade³⁸ sacrifices TLR4-mediated virus propagation but enhances virus survival through negation of TLR4-induced IFN α and IFN β production. Studies designed to investigate the multiple arms of TLR4 signaling during HIV-1 infection are warranted.

We explored the effects of TLR activation during acute infection of U937, THP-1 and primary macrophages and found that these cells were capable of generating a strong anti-viral response early in infection. Activation of TLR2, TLR4 and TLR9 seven days post-HIV-1 infection resulted in down-regulation or complete abrogation of virus production (data not shown). These observations are in agreement with previous studies in which LPS or mycobacterial exposure inhibited virus replication when HIV-1 infection of monocyte/macrophages was initiated immediately before or simultaneously with microbial stimulation³⁹⁻⁴¹. Therefore, we surmise that TLR4 signaling inhibits viral replication early in infection and does not enhance viral replication from latency. In contrast, our data and those of others clearly demonstrate TLR2 and TLR9 are implicated in reactivation of virus during chronic infection. Agonists of these TLR are ubiquitously expressed by parasites and/or Gram positive and negative bacteria and have the potential to be important contributors to HIV pathogenesis.

The relevant issue here is whether TLR4 agonists are detrimental to HIV-1 infected patients. The concern is that TLR4 ligation whether by LPS from Gram negative bacteria or a vaccine adjuvant, could lead to increased viral replication. Immunomodulation through the Toll-like receptors is under consideration as a means to improve the immunocompetence of AIDS patients. If TLR ligation results in greater viral replication, this approach may be detrimental. Conversely, if the objective is to reactivate HIV-1 from cellular sources of latent activation as a means of facilitating clearance during highly active antiretroviral therapy, the use of TLR ligands may be beneficial. Either way, a clear understanding of how

each TLR affects viral replication is needed on a cellular level and ultimately in the patient. Complicating this understanding may be changes in TLR function from the acute to chronic stage of HIV infection. In support of this concept, we found activation of TLR2, TLR4 and TLR9 resulted in a potent antiviral response during acute infection that was either lost (TLR4) or supportive of viral replication during chronic infection (TLR2 and TLR9). Similar results have been reported for TLR7/8 activation during acute vs. chronic HIV-1 infection⁴², suggesting progressive loss of TLR-mediated anti viral function may be a hallmark of HIV disease. Clearly there is much to learn, but the wide range of TLR expression and potential for cellular interactions may require evaluation *ex vivo* in samples from HIV-1 infected patients and *in vivo* using animal models. Important questions to address are whether TLR4-mediated effects during HIV-1 infection are different during chronic vs. acute infection, are specific to the LPS of Gram negative bacterial species, and are particular to individual cell types.

Acknowledgments

The authors thank Dr. David N. Levy (NYU) for critical review of the manuscript. This work was supported by the National Institutes of Health (R03 AI058855 and R01 AI47707).

Reference List

1. Blanchard A, Montagnier L, Gougeon ML. Influence of microbial infections on the progression of HIV disease. *Trends Microbiol.* 1997; 5(8):326–331. [PubMed: 9263412]
2. Wahl SM, Orenstein JM. Immune stimulation and HIV-1 viral replication. *J Leukoc Biol.* 1997; 62(1):67–71. [PubMed: 9225995]
3. Molina JM, Scadden DT, Byrn R, Dinarello CA, Groopman JE. Production of tumor necrosis factor alpha and interleukin 1 beta by monocytic cells infected with human immunodeficiency virus. *J Clin Invest.* 1989; 84(3):733–737. [PubMed: 2474573]
4. Pomerantz RJ, Feinberg MB, Trono D, Baltimore D. Lipopolysaccharide is a potent monocyte/macrophage-specific stimulator of human immunodeficiency virus type 1 expression. *J Exp Med.* 1990; 172(1):253–261. [PubMed: 2193097]
5. Moriuchi H, Moriuchi M, Mizell SB, Ehler LA, Fauci AS. In vitro reactivation of human immunodeficiency virus 1 from latently infected, resting CD4+ T cells after bacterial stimulation. *J Infect Dis.* 2000; 181(6):2041–2044. [PubMed: 10837189]
6. Takeda K, Akira S. Toll-like receptors in innate immunity. *Int Immunol.* 2005; 17(1):1–14. [PubMed: 15585605]
7. Zarembka KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol.* 2002; 168(2):554–561. [PubMed: 11777946]
8. Caron G, Duluc D, Fremaux I, et al. Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells. *J Immunol.* 2005; 175(3):1551–1557. [PubMed: 16034093]
9. Pasare C, Medzhitov R. Toll-dependent control mechanisms of CD4 T cell activation. *Immunity.* 2004; 21(5):733–741. [PubMed: 15539158]
10. Steinman RM, Hemmi H. Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immunol.* 2006; 311:17–58. [PubMed: 17048704]
11. Bafica A, Scanga CA, Schito ML, Hieny S, Sher A. Cutting edge: in vivo induction of integrated HIV-1 expression by mycobacteria is critically dependent on Toll-like receptor 2. *J Immunol.* 2003; 171(3):1123–1127. [PubMed: 12874196]

12. Bafica A, Scanga CA, Schito M, Chaussabel D, Sher A. Influence of coinfecting pathogens on HIV expression: evidence for a role of Toll-like receptors. *J Immunol.* 2004; 172(12):7229–7234. [PubMed: 15187096]
13. Bafica A, Scanga CA, Equils O, Sher A. The induction of Toll-like receptor tolerance enhances rather than suppresses HIV-1 gene expression in transgenic mice. *J Leukoc Biol.* 2004; 75(3):460–466. [PubMed: 14657211]
14. Equils O, Faure E, Thomas L, et al. Bacterial lipopolysaccharide activates HIV long terminal repeat through Toll-like receptor 4. *J Immunol.* 2001; 166(4):2342–2347. [PubMed: 11160291]
15. Equils O, Schito ML, Karahashi H, et al. Toll-like receptor 2 (TLR2) and TLR9 signaling results in HIV-long terminal repeat trans-activation and HIV replication in HIV-1 transgenic mouse spleen cells: implications of simultaneous activation of TLRs on HIV replication. *J Immunol.* 2003; 170(10):5159–5164. [PubMed: 12734363]
16. Equils O, Shapiro A, Madak Z, Liu C, Lu D. Human immunodeficiency virus type 1 protease inhibitors block toll-like receptor 2 (TLR2)- and TLR4-Induced NF-kappaB activation. *Antimicrob Agents Chemother.* 2004; 48(10):3905–3911. [PubMed: 15388451]
17. Rohr O, Marban C, Aunis D, Schaeffer E. Regulation of HIV-1 gene transcription: from lymphocytes to microglial cells. *J Leukoc Biol.* 2003; 74(5):736–749. [PubMed: 12960235]
18. Latz E, Schoenemeyer A, Visintin A, et al. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat Immunol.* 2004; 5(2):190–198. [PubMed: 14716310]
19. Folks TM, Justement J, Kinter A, et al. Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. *J Immunol.* 1988; 140(4):1117–1122. [PubMed: 2449497]
20. Kutsch O, Benveniste EN, Shaw GM, Levy DN. Direct and quantitative single-cell analysis of human immunodeficiency virus type 1 reactivation from latency. *J Virol.* 2002; 76(17):8776–8786. [PubMed: 12163598]
21. Re F, Strominger JL. Monomeric recombinant MD-2 binds toll-like receptor 4 tightly and confers lipopolysaccharide responsiveness. *J Biol Chem.* 2002; 277(26):23427–23432. [PubMed: 11976338]
22. Biswas P, Poli G, Kinter AL, et al. Interferon gamma induces the expression of human immunodeficiency virus in persistently infected promonocytic cells (U1) and redirects the production of virions to intracytoplasmic vacuoles in phorbol myristate acetate-differentiated U1 cells. *J Exp Med.* 1992; 176(3):739–750. [PubMed: 1512539]
23. Dunzendorfer S, Lee HK, Soldau K, Tobias PS. TLR4 is the signaling but not the lipopolysaccharide uptake receptor. *J Immunol.* 2004; 173(2):1166–1170. [PubMed: 15240706]
24. Nabel G, Baltimore D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature.* 1987; 326(6114):711–713. [PubMed: 3031512]
25. Hiscott J, Kwon H, Genin P. Hostile takeovers: viral appropriation of the NF-kappaB pathway. *J Clin Invest.* 2001; 107(2):143–151. [PubMed: 11160127]
26. Pradines-Figueres A, Raetz CR. Processing and secretion of tumor necrosis factor alpha in endotoxin-treated Mono Mac 6 cells are dependent on phorbol myristate acetate. *J Biol Chem.* 1992; 267(32):23261–23268. [PubMed: 1429672]
27. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol.* 2005; 6(8):769–776. [PubMed: 15995707]
28. Bagchi A, Herrup EA, Warren HS, et al. MyD88-dependent and MyD88-independent pathways in synergy, priming, and tolerance between TLR agonists. *J Immunol.* 2007; 178(2):1164–1171. [PubMed: 17202381]
29. Sundstrom JB, Little DM, Villinger F, Ellis JE, Ansari AA. Signaling through Toll-like receptors triggers HIV-1 replication in latently infected mast cells. *J Immunol.* 2004; 172(7):4391–4401. [PubMed: 15034054]
30. Osiecki K, Xie L, Zheng JH, et al. Identification of granulocyte-macrophage colony-stimulating factor and lipopolysaccharide-induced signal transduction pathways that synergize to stimulate HIV type 1 production by monocytes from HIV type 1 transgenic mice. *AIDS Res Hum Retroviruses.* 2005; 21(2):125–139. [PubMed: 15725751]

31. Scheller C, Ullrich A, McPherson K, et al. CpG oligodeoxynucleotides activate HIV replication in latently infected human T cells. *J Biol Chem.* 2004; 279(21):21897–21902. [PubMed: 15016800]
32. Heggelund L, Flo T, Berg K, et al. Soluble toll-like receptor 2 in HIV infection: association with disease progression. *AIDS.* 2004; 18(18):2437–2439. [PubMed: 15622324]
33. Heggelund L, Damas JK, Yndestad A, et al. Stimulation of toll-like receptor 2 in mononuclear cells from HIV-infected patients induces chemokine responses: possible pathogenic consequences. *Clin Exp Immunol.* 2004; 138(1):116–121. [PubMed: 15373913]
34. Heggelund L, Muller F, Lien E, et al. Increased expression of toll-like receptor 2 on monocytes in HIV infection: possible roles in inflammation and viral replication. *Clin Infect Dis.* 2004; 39(2): 264–269. [PubMed: 15307037]
35. Netea MG, van Deuren M, Kullberg BJ, Cavaillon JM, Van der Meer JW. Does the shape of lipid A determine the interaction of LPS with Toll-like receptors? *Trends Immunol.* 2002; 23(3):135–139. [PubMed: 11864841]
36. Lodie TA, Reiner M, Coniglio S, Viglianti G, Fenton MJ. Both PU.1 and nuclear factor-kappa B mediate lipopolysaccharide- induced HIV-1 long terminal repeat transcription in macrophages. *J Immunol.* 1998; 161(1):268–276. [PubMed: 9647233]
37. Tachado SD, Zhang J, Zhu J, Patel N, Koziel H. HIV impairs TNF-alpha release in response to Toll-like receptor 4 stimulation in human macrophages in vitro. *Am J Respir Cell Mol Biol.* 2005; 33(6):610–621. [PubMed: 16109884]
38. Yamamoto M, Sato S, Hemmi H, et al. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nat Immunol.* 2003; 4(11):1144–1150. [PubMed: 14556004]
39. Goletti D, Carrara S, Vincenti D, et al. Inhibition of HIV-1 replication in monocyte-derived macrophages by *Mycobacterium tuberculosis*. *J Infect Dis.* 2004; 189(4):624–633. [PubMed: 14767815]
40. Verani A, Scarlatti G, Comar M, et al. C–C chemokines released by lipopolysaccharide (LPS)-stimulated human macrophages suppress HIV-1 infection in both macrophages and T cells. *J Exp Med.* 1997; 185(5):805–816. [PubMed: 9120386]
41. Zybarth G, Reiling N, Schmidtmayerova H, Sherry B, Bukrinsky M. Activation-induced resistance of human macrophages to HIV-1 infection in vitro. *J Immunol.* 1999; 162(1):400–406. [PubMed: 9886413]
42. Schlaepfer E, Audige A, Joller H, Speck RF. TLR7/8 triggering exerts opposing effects in acute versus latent HIV infection. *J Immunol.* 2006; 176(5):2888–2895. [PubMed: 16493046]

Figure 1. Unpurified LPS activates TLR2, 4 and 9

HEK 293T cells stably transfected with (A) TLR2, (B) TLR4/MD2 or (C) TLR9 gene constructs were transfected with an NF-κB- SEAP reporter and treated with 500 ng/ml Pam₃CSK₄, 5 μg/ml ultra-purified LPS, 5 μg/ml WT LPS, 1 μM B class CpG-ODN or B class control ODN. Cell culture supernatant was collected 24 h post-treatment and assayed for SEAP. Data are expressed as fold-increase over unstimulated cells within each respective cell line and are the mean and SEM of three separate experiments.

B:

Figure 2. Activation of TLR4 does not significantly enhance HIV production

(A) 1×10^6 U1 cells were treated with TLR agonists as described in Figure 1. Cell culture supernatants were collected 48 h post-treatment and assayed for HIV p24 by ELISA. Data represent the mean and SEM of 3 separate experiments. (*) $p < 0.05$, treatment of U1 cells with the specified agonists resulted in a significant increase in HIV p24 production over that of untreated cells. (B) 5×10^5 THP₈₉₆GFP cells were seeded into wells of a 96 well plate and stimulated with TLR agonists as described in Figure 1. Cells were photographed 48 h post-TLR activation using a fluorescent microscope under $100 \times$ magnification. (C) EGFP from

TLR-stimulated THP_{896GFP} cells was quantified using an IVIS imaging system. Data are expressed as relative fluorescent units (RFU) after subtraction of background from untreated controls and represent the mean and SEM of 3 separate experiments. (D) Cell culture supernatants from 1×10^6 THP_{896GFP} cells were collected 48 h post-TLR activation and assayed for HIV p24 by ELISA. Data represent the mean and SEM of 3 separate experiments. (*) $p < 0.05$, treatment of THP_{896GFP} cells with the specified agonists resulted in a significant increase in HIV p24 production over that of untreated cells.

Figure 3. Constitutive expression of TLR2, TLR4 and TLR9 is not altered by chronic HIV infection

Flow cytometric histograms representative of 3 separate experiments in which U937 and U1 cells were stained for surface expression of (A) TLR2 and (B) TLR4, and intracellular expression of (C) TLR9.

Figure 4. HIV infected cells down-regulate TLR2 and TLR4 in response to specific agonists
Flow cytometric histograms representative of 3 separate experiments in which U937 and U1 cells were either left untreated or stimulated with 500 ng Pam₃CSK₄ or 5 µg/ml ultra-purified LPS and analyzed for (A) TLR2 and (B) TLR4 expression 8 hours later.

Figure 5. TLR agonists activate NF-κB in infected cells

(A) 1×10^6 U937 and U1 cells were transfected with an NF-κB-SEAP reporter and exposed to 500 ng Pam₃CSK₄, 5 μg/ml ultra-purified LPS, 5 μg/ml WT LPS or 1 μM CpG DNA. Cell culture supernatant was collected 48 h post-treatment and assayed for SEAP activity. Data represent the mean and SD of 3 separate experiments. (*) $p < 0.05$, treatment of U937 cells with the specified TLR ligand resulted in a significantly higher induction of activated NF-κB when compared with U1 cells. (B–D) 1×10^6 U937 and U1 cells were treated as described above. Cell culture supernatant was collected 24 h post-treatment and assayed for (B) IL-1β, (C) TNFα and (D) IL-6 using Luminex fluorescent bead technology. (*) $p < 0.05$, treatment with the specified agonists resulted in a significant difference in cytokine secretion between U937 and U1 cells.

Figure 6. TLR4 engagement does not activate the HIV-LTR

1×10^6 TLR2, TLR4/MD2 or TLR9 293T cells were transfected with an attenuated NL4-luciferase reporter and stimulated for 12 h as described in Figure 1. Data represent the fold-increase in NL4 activity relative to un-stimulated cells and represent the mean and SEM of 3 separate experiments. (*) $p < 0.05$, treatment of cell lines with the specified agonists resulted in a significant increase in NL4-luciferase activity compared with the respective un-stimulated cells.

Table 1

1×10^5 THP₈₉₆GFP cells were treated with TLR agonists in varying concentrations listed in Table 1. Cell culture supernatants were collected 48 h post-treatment and assayed for HIV-1 p24 by ELISA. Data represent the mean and SD of 3 separate experiments.

Pam ₃ CSK ₄ (ng/ml)	p24 (pg/ml)
50	529 ± 22
100	1091 ± 33
500	3275 ± 243
1000	1881 ± 69
Purified LPS (µg/ml)	
0.5	314 ± 445
1	340 ± 480
5	586 ± 142
10	339 ± 491
CpG (µM/ml)	
0.1	776 ± 46
0.5	4300 ± 2285
1	4850 ± 921
5	1793 ± 45
WT LPS (µg/ml)	
0.5	457 ± 396
1	659 ± 10
5	807 ± 50
10	711 ± 8