Increased Expression of TLR3 in Lymph Nodes during Simian Immunodeficiency Virus Infection: Implications for Inflammation and Immunodeficiency

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As pattern recognition receptors, TLRs signal and induce expression of multiple host defense genes including proinflammatory cytokines and chemokines. To investigate the mechanisms of up-regulation of proinflammatory cytokines and chemokines during SIV infection in rhesus macaques, we measured the relative levels of expression of TLRs 1–10 in lymphoid tissues during different stages of SIV infection. By real-time RT-PCR, TLR3 was determined to be up-regulated in macaque lymph nodes (LN) throughout the course of infection, whereas TLR9 was down-regulated during early stages of infection. CXCL9/Mig, CXCL10/IP-10, IFN-γ, and IFN-α mRNAs were also increased during acute SIV infection and AIDS. Treatment of macaque spleen and LN cells with TLR3 and TLR9 ligands led to the induction of these same genes. TLR3 stimulation had disparate effects on viral transcription and viral replication, because poly(I:C), a model TLR3 ligand, stimulated the viral promoter but potently inhibited SIV replication in primary cultures of macaque spleen and LN cells. These findings identify roles for TLR3 inflammation in lymphoid tissues and in the immunopathogenesis of HIV-1/SIV, and suggest that TLR3 ligands could potentially be used to flush out latently infected cells that persist during antiretroviral therapies. *The Journal of Immunology, 2005, 175: 5314–5323.*

Infection with HIV-1 and subsequent development of AIDS continues to be a major worldwide public health problem (1). However, our understanding of how HIV-1 infection leads to the development of AIDS is incomplete. Events early after infection of nonhuman primates with the related SIV in intestinal and lymphoid tissues include rapid loss of CD4+ T lymphocytes (2). These anatomic compartments provide a suitable environment for viral propagation (3). These early events occur before the development of a robust adaptive immune response and therefore could involve innate host responses at the cellular and tissue level. Among the possible mechanisms contributing to early and/or cumulative losses of immune cells and the development of immunodeficiency, diverse roles could be played by changes in the magnitude, responsiveness, and/or nature of innate immune responses.

TLRs are signaling pattern recognition receptors of the innate immune system that play a crucial role in sensing microbial infection and initiating a signaling cascade that results in induction of innate and adaptive immune responses (4). It has become clear that viral molecular patterns are recognized by multiple TLRs leading to induction of inflammatory cytokines (5, 6). Viral surface glycoproteins activate signaling through TLRs 2 and 4 (5, 6), and viral nucleic acid ligands such as dsRNA, ssRNA, and unmethylated CpG-containing DNA activate signaling through TLR3 (7), TLRs 7 and 8 (9, 10), and TLR9 (10), respectively. The potential responsiveness of a tissue microenvironment to microbial or endogenous molecules could be altered due to changes in TLR gene expression patterns at either the cellular or tissue level. Changes in TLR gene expression patterns have been observed, and can result from TLR engagement and signaling (11), the action of proinflammatory cytokines (12), and local fluxes in the relative proportions of specific cells expressing TLRs (13).

Proinflammatory molecules, including IFN-γ and the chemokine CXCL9/Mig, are up-regulated in lymphoid tissues of SIV-infected rhesus macaques (14, 15). This IFN-γ/CXCL9-ligand axis comprises a positive feedback loop that potently contributes to disease progression by ectopically recruiting cells into lymphoid tissues, resulting in a continually renewed source of susceptible cells, such as activated T lymphocytes, for ongoing viral replication, loss of trafficking of effector cells to sites of needed peripheral immune effector activity, and increased recruitment of immunomodulatory cells such as NK cells and plasmacytoid dendritic cells (pDCs).4

We hypothesized that TLR signaling might contribute to the inflammation observed in lymphoid tissues during HIV/SIV infection. To further understand the underlying mechanisms leading to the up-regulation of proinflammatory mediators in lymphoid tissues, we measured the expression of TLRs 1–10 as well as downstream effectors in rhesus macaque lymph nodes (LNs) during multiple stages of SIV infection. In this study we report that TLR3, IFN-α, and CXCL10/interferon-inducible protein (IP)-10 mRNAs are up-regulated, whereas TLR9 mRNA is down-regulated in macaque LN after SIV infection. Functional characterization of macaque TLR3 revealed that signaling through rhesus TLR3 (rhTLR3) by dsRNA ligands such as poly(I:C) and SIV gag RNA lead to stimulation of NF-κB-mediated transcription and induction

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4 Abbreviations used in this paper: PDC, plasmacytoid dendritic cell; LN, lymph node; IP, interferon-inducible protein; rhTLR3, rhesus TLR3; PI, postinfection; Ct, threshold cycle; βm, β2-microglobulin; IVT, in vitro transcript; TCID50, 50% tissue culture infectious dose; LTR, long terminal repeat; ODN, oligonucleotide.
of IFNs and IFN-induced genes in macaque spleen and LN cell populations. In stark contrast, however, TLR3 or TLR9 stimulation caused a significant reduction in SIV replication in these same primary cell populations. These data suggest that increased TLR3 expression and signaling play disparate roles during SIV infection, by inducing inflammatory networks that could contribute to recruitment of cells susceptible to ongoing viral replication, cellular sequestration in LN and loss of peripheral immune function, and by stimulating viral transcription while simultaneously contributing to the inhibition of viral replication.

Materials and Methods

Animal tissue and RNA isolation

The study was performed with the approval of the University of Pittsburgh Institutional Animal Care and Use Committee. RNAs were isolated from axillary LNs from 13 previously characterized and described rhesus macaques (Macaca mulatta) infected with SIV/DeltaB670 of which eight were obtained during acute infection (2 wk postinfection (PI)), four were obtained upon progression to AIDS, one was a long-term nonprogressor, and one served as uninfected controls (14, 16, 17). Total RNAs were treated with DNase (Ambion) and further purified with RNeasy columns (Qiagen).

Real-time RT-PCR

Four hundred nanograms of RNA from each specimen was reverse transcribed as described (14). Reverse transcription-negative controls were included for each RNA sample. Primers and probes used for the real-time RT-PCR were either a subset of previously published primer sequences (18–20) or were designed using the Primer Express (Applied Biosystems) software package (primer sequences are available from the authors upon request). Real-time RT-PCR (SYBR-Green; Applied Biosystems) was used to quantify relative mRNA expression levels by the comparative threshold cycle (Ct) method of relative quantitation (PerkinElmer User Bulletin no. 2). Each RT-PCR was performed in duplicate in 50-μl reactions consisting of 1× SYBR-Green PCR master mix (Applied Biosystems). PCR cycling was performed as follows: 50°C for 2 min, 95°C for 12 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Threshold values were set on the amplification plots, and the calculated Ct values were exported to Microsoft Excel for analysis. The Ct values for each gene were normalized to the endogenous control gene β2-microglobulin (β2m). The specific amplification of targets was validated by examining the thermal dissociation curves (PerkinElmer User Bulletin no. 2). TLRs 1–9 consistently showed specific amplification, whereas amplification of TLR10 was not as specific based on the broader thermal dissociation curve. Real-time RT-PCR was performed in duplicate in two independent experiments. The relative expression for each gene was calculated using the mean value obtained from all ΔΔCt values. For quantification of tissue-associated SIV gag RNA, we developed a modified absolute real-time RT-PCR assay for SIV gag RNA using published primer and probe sequences (19). We modified the template for in vitro transcription used for generation of external RNA standards and in vitro cell culture studies, by generating a PCR product with a T7 RNA polymerase promoter at 5′ to the gag sequence, thereby abrogating any potential readthrough of vector sequences into the population of RNA standards. This in vitro transcript (IVT) was DNase I treated and run on an agarose gel to confirm its size and intactness. Secondary structure analysis of SIV gag RNA was performed using the mFOLD 3.1 program (www.bioinfo.rpi.edu/applications/mfold). The dynamic range of the SIV gag absolute quantitation in our hands was 1.7 × 10^3–1.7 × 10^4 copies/ng of input total RNA. External standards for the endogenous control β2m were generated in the same way as the SIV standards, and served to normalize the viral RNA copy numbers for amount and integrity of input RNA. TaqMan RT-PCR were performed as described (14). For each experimental sample, the Ct values obtained for both SIV gag and β2m were extrapolated on the respective standard curves to obtain raw target copy numbers for each gene (PerkinElmer User Bulletin no. 2), and SIV gag copy numbers were then normalized to β2m copy numbers.

Immunohistochemistry

Immunohistochemical staining of axillary LN tissue sections was performed as described (18–20). Monoclonal anti-TLR3 Ab (clone TLR3-7; eBioscience) or isotype control Ab (BD Biosciences) was incubated with tissue sections in PBS overnight at 4°C (21). After washing in PBS twice, the tissue sections were incubated with HRP-conjugated secondary Ab (Zymed Laboratories) for 15 min, and 3,3′-diaminobenzidine substrate was used for the detection of TLR3+ cells.

Enzymatic digestion of spleen and axillary LN tissues

The axillary LN tissues from cynomolgus macaques (Macaca fascicularis) were minced in digestion medium containing DNase I (20 mg/ml; Sigma-Aldrich) and collagenase A (1 mg/ml; Roche). After incubation for 60 min at 37°C, the minced tissue was passed through a 100-mm cell strainer and centrifuged at 1200 rpm for 5 min at room temperature. The pellet was resuspended in 1× RBC lysing solution (155 mM NH4Cl, 10 mM NaHCO3, 0.1 mM EDTA; pH 7.4) and centrifuged for 1200 rpm for 5 min at room temperature. The pellet obtained was then resuspended in 1× PBS, and aliquots of cell suspensions were frozen in freezing medium (90% FCS and 10% DMSO) and stored in liquid nitrogen until used.

Treatment of macaque spleen and axillary LN cells and TZM-bl cells with TLR3 and TLR9 ligands

TZM-bl cells expressing CD4 and CCR5 (kindly provided by Drs. J. Kappes and X. Wu and Tranzyme through the National Institutes of Health AIDS Research and Reference Reagent Program, Germantown, MD: Ref. 22) and macaque spleen and axillary LN cells were exposed to poly(LC) (InvivoGen; 50 μg/ml), CpG-C (InvivoGen; 5 μM), SIVmac251 (2 × 10^3 TCID50) (50% tissue culture infectious dose), recombinant IFN-α (R&D Systems; 100 U/ml), or medium alone. Total RNAs were isolated at 24 h poststimulation, and real-time RT-PCR was performed to determine cytokine and chemokine gene expression levels using the comparative Ct method as described (PerkinElmer User Bulletin no. 2).

Cloning of rhesus macaque TLR3 and TLR4

Using the following primer pairs: SRRhTLR3F6, 5′-AAAAGGAA AGGCTGACGCATCACCAACAGACTA-3′ and SRRhTLR3R6, 5′-AAATTATTTAAATATCTAATAGGAAAGAACTTCTTCA-3′; and SRRhTLR4F3, 5′-ACACAAGGAGTGGAGGATGGCAGGGA-3′ and SRRhTLR4R3, 5′-AAGAGAAGAAAAAATACCTGCTGGCAGTTTCTTCT-3′, rhesus macaque TLR3 and TLR4 cDNAs were amplified from axillary LN tissues. The resulting cDNAs were cloned into the TOPO cloning pcDNA3.1 (Invitrogen Life Technologies) and DNA sequenced. GenBank accession numbers for these sequences are AY864734 and AY864735.

Flow cytometric analysis of rhesus macaque TLR3/7-transfected HEK293T cells

HEK293T cells transiently transfected with plasmid-expressing rhesus macaque TLR3 were stained with PE-labeled anti-TLR3 mAb (clone TLR3.7; eBioscience) for surface expression as well as for intracellular expression after fixation and permeabilization with CytoFix-Cytoperm (BD Biosciences). Stained cells were analyzed using an XL cytometer (Beckman Coulter). Staining with isotype control Abs was performed in parallel. Flow cytometry data were analyzed using the Expo32 software package (Beckman Coulter).

NF-κB luciferase reporter assays

HEK293T cells (American Type Culture Collection; 1.5 × 10^6 cells/well) were seeded in six-well plates 24 h before transfection with Lipofectamine (Invitrogen Life Technologies). pcDNA3.1 expression plasmids (2 μg) encoding rhesus macaque TLR3, TLR4, or empty vector were cotransfected along with reporter construct pNF-κB-luc (2.5 μg; BD Biosciences) and an internal control vector pcDNA3.1/V5/His/LacZ expressing β-galactosidase (Invitrogen Life Technologies). Cells were transfected with Tnf-α (25 ng/ml; Sigma-Aldrich), LPS (2.5 μg/ml; Sigma-Aldrich), poly(I:C) (50 μg/ml; InvivoGen), or SIV gag IVT (5 μg/ml). Cells were lysed using reporter cell lysis buffer (Promega) 24 h posttransfection and assayed for NF-κB-driven luciferase levels (Luciferase Assay System; Promega) using a luminometer (Veritas). Luciferase levels were normalized to levels of β-galactosidase activity as measured using colorimetric analysis.

HIV-1 long terminal repeat (LTR) luciferase assay

TZM-bl cells, which harbor an integrated copy of the luciferase gene under the control of the HIV-1 promoter (22), were seeded into six-well plates and 24 h later were stimulated with Tnf-α (25 ng/ml). SIVmac251 (2 × 10^3 TCID50), poly(I:C) (25 μg/ml), SIV gag RNA (5 μg/ml), LPS (2.5 μg/ml), or medium only. Cells were lysed 24 h poststimulation, and luciferase activity was measured as described above.
Measurement of the effects of TLR ligands on SIV replication

A3.01, TZM-bl, and macaque spleen and axillary LN cell populations were infected with SIVmac251 (2 × 10^3/ml TCID50) and stimulated with poly(I:C) (50 μg/ml) and CpG-C (5 mM) or medium only. RNA was isolated 24 h PI and examined for SIV gag RNA copy numbers as described above.

Statistical analysis

Student’s t test was used to compare differences between disease states. A p value of <0.05 was considered significant. Pearson’s correlation coefficient (r values) was used to measure the strength of association between the mRNA relative expression levels of two genes.

Results

TLR3 mRNA is up-regulated in axillary LN during SIV infection

To determine the effects of SIV infection on expression of TLRs 1–10 in lymphoid tissues, we used real-time RT-PCR to measure TLR mRNA levels in total RNA samples from macaque axillary LN. These LN were obtained from rhesus macaques inoculated i.v. with the pathogenic SIV/DeltaB670 isolate (14, 16, 17) and sacrificed during the early, acute phase of infection (2 wk PI) or upon development of AIDS, in addition to two uninfected controls and one SIV/DeltaB670 long-term nonprogressor.

Of the 10 TLRs, only TLR3 and TLR9 were differentially expressed (Fig. 1). TLR3 mRNA was up-regulated to the highest levels during acute infection (mean fold increase, 4.9-fold; p < 0.05) and AIDS (3.7-fold; not significantly different), whereas TLR9 mRNA expression levels were lower in acutely infected animals (4.8-fold decrease; p < 0.001) and in animals with AIDS (2.3-fold decrease; p < 0.01). Tissue-associated viral loads in these axillary LN ranged from 1 × 10^3 to 1.7 × 10^6 copies/μg of total RNA and were positively correlated with TLR3 mRNA levels (r = 0.65; p < 0.01). Immunohistochemical staining of tissue sections revealed levels of TLR3 expression that were generally concordant with the relative levels of expression of TLR3 mRNA (Fig. 2). Cells expressing TLR3 were present in all microanatomic compartments, including germinal centers, paracortices, and medullary sinuses, suggesting that before and throughout the course of SIV infection, TLR3 mRNA is up-regulated in axillary LN during SIV infection.

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Effects of SIV infection on the relative expression levels of TLRs 1–10 in macaque LN. Real-time RT-PCR was used to measure the changes in mRNA levels of TLRs 1–10 following SIV/DeltaB670 infection of rhesus macaques. The expression levels were normalized to the endogenous control gene βm, and the relative expression levels were calculated using the uninfected animal M6600 as a calibrator.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Immunohistochemical detection of TLR3 in axillary LN tissue sections from SIV-infected and uninfected rhesus macaques. Immunohistochemical staining was performed using an anti-TLR3 mAb. Axillary LN tissue sections were obtained from uninfected (A), acutely SIV/DeltaB670 infected (B), and immunodeficient animals (C). The fold increase in TLR3 mRNA expression observed by real-time RT-PCR are shown for each animal on each image. The inset in (B) represents a region (same magnification) of a subjacent tissue section stained in parallel with an isotype control mAb. Original magnifications, ×400; size bar in A, 50 μm.
infection, TLR3 was expressed by multiple cell types. The widespread and up-regulated expression of TLR3, which recognizes dsRNA, an intermediate of viral replication (7), suggests that lymphoid tissues are poised to sense such dsRNAs throughout the tissue.

Host defense genes are differentially expressed in axillary LN during SIV infection

As sensing receptors of the innate immune system, TLR signaling leads to the induction of inflammatory molecules. Among downstream host defense genes, we observed up-regulation of IFN-α (AIDS, 23.7-fold increase, not significantly different; acute infection, 31.7-fold, not significantly different) and CXCL10/IF-10 (AIDS, 3.6-fold, not significantly different; acute infection, 8.3-fold, not significantly different) mRNA levels, and down-regulation of IL-12 p40 (AIDS, 17.6-fold decrease; p < 0.05; acute infection, 9.0-fold decrease; p < 0.05) mRNA levels (Fig. 3). We have previously reported an up-regulation of CXCL9/Mig and IFN-γ in these same tissues (14). As antimicrobial peptides, defensins are innate effectors induced by TLR signaling (23, 24). We therefore measured mRNA expression levels of β-defensin 2, as well as α-defensins 1–3, which can inhibit HIV-1 replication (25). Overall, mRNAs encoding β-defensin 2 and α-defensins 1–3 did not consistently change during SIV infection, although there was a trend toward down-regulation of β-defensin 2 mRNA and a trend toward up-regulation of α-defensin 3 (Fig. 3). Notably, a few SIV-infected animals contained high levels of expression of α-defensin 3 mRNA.

Pairwise comparisons (n = 420) between gene expression levels among uninfected and SIV-infected animals revealed 19 (4.5%) correlations with r > 0.60. Among these, only TLR3 mRNA levels were significantly correlated with SIV RNA levels (r = 0.65; p < 0.01). A small number of correlations were also observed between multiple TLRs (e.g., TLR3 vs TLR8; r = −0.77; and TLR7 vs TLR9; r = 0.69) and downstream inflammatory molecules (e.g., CXCL10/IF-10 vs IFN-α; r = 0.75). These associations suggest that there are shared regulatory mechanisms for positively correlated genes, or that there are positive (or in some instances, negative) cross-regulatory mechanisms.

TLR3 and TLR9 ligands increase expression of proinflammatory molecules in macaque LN and spleen cell populations

Signaling through TLR3 or TLR9 is known to induce proinflammatory cytokines and chemokines (7, 10), including those we identified as differentially expressed in vivo during SIV infection (Fig. 3). To better understand the possible mechanisms leading to increased expression of proinflammatory mediators in macaque lymphoid tissues during SIV infection, we treated macaque LN and spleen cells with the following: poly(I:C), which is a synthetic analog of dsRNA and a ligand for TLR3; CpG-C, which is a synthetic DNA oligonucleotide (ODN) ligand for TLR9; SIVmac251; or recombinant IFN-α. We reasoned that these populations of primary cells would model complex cell-cell interactions that occur in dynamic lymphoid tissues more so than established cell lines. For comparative purposes, though, we included the TZM-bl HeLa cell line derivative, which expresses CD4 and CCR5 entry receptors and is susceptible to SIV infection (22). These cells express TLR3 and TLR9 (data not shown). The effects of these stimulations on the mRNA expression levels of proinflammatory molecules were measured using real-time RT-PCR (Fig. 4, A–C).

Poly(I:C) stimulation of these cell populations led to the largest number and most dramatic increases in expression of the proinflammatory mediators examined (Fig. 4). In spleen cells, poly(I:C) stimulation resulted in significant increases (p < 0.05) in CXCL9/ Mig (mean-fold increase, 21.3-fold), CXCL10/IF-10 (3.2-fold), IFN-γ (6.0-fold), IFN-α (5.2-fold), and IFN-β (7.8-fold). In contrast, poly(I:C) stimulation of axillary LN cells resulted in significant increases in expression only of IFN-α (29.4-fold) and IFN-β (29.0-fold). In the TZM-bl cell line, poly(I:C) led to a different pattern of gene expression that included increased expression of...
CXCL10/IP-10 (29.0-fold), IL-6 (12.5-fold), and IFN-β (4.3-fold) mRNAs.

The effects of the other treatments were fewer and less dramatic (Fig. 4). CpG-C ODN stimulation led to moderate but significant increases in IFN-α mRNA in spleen (2.1-fold) and axillary LN (2.1-fold) cells, as well as in the TZM-bl cell line (3.2-fold). CpG-C ODN stimulation of the TZM-bl cell line also led to increased expression of CXCL10/IP-10 (6.3-fold) and IL-6 (2.4-fold) mRNAs. IFN-α treatment led to significant increases in CXCL10/IP-10 in all cell populations, ranging from 1.9-fold in spleen cells to 39.8-fold in the TZM-bl cell line. Finally, infection with SIVmac251 led to increased expression of CXCL10/IP-10 mRNA in axillary LN cells (3.0-fold) and in the TZM-bl cell line (10.5-fold). Therefore, these findings demonstrate that proinflammatory mediators found to be up-regulated in lymphoid tissues during SIV infection can be induced by ligands for TLR3 and TLR9, by SIV infection, and by IFN-α.

IFN-α up-regulates TLR3 mRNA expression in multiple cell populations

To investigate possible mechanisms leading to increased TLR3 expression during SIV infection in lymphoid tissues, we examined the effects of poly(I:C), recombinant IFN-α and SIVmac251 infection on TLR3 mRNA expression in macaque LN and spleen cells, and the TZM-bl cell line (Fig. 5). Only treatment with
poly(I:C) as well as an SIV siveness of rhTLR3 was measured using the experimental ligands A), similar to huTLR3 (26). The signaling respon- siently transfected cells revealed that rhTLR3 is expressed pre- blication and signaling properties. Flow cytometric analysis of tran- siently transfected a mammalian expression vector encoding caque TLR3 and characterized its functional properties. To iden- tify properties that could impact SIV-associated disease, we tran- siently transfected a mammalian expression vector encoding rhTLR3 into HEK293T cells and examined its subcellular distri- bution and signaling properties. Flow cytometric analysis of tran- siently transfected cells revealed that rhTLR3 is expressed pre- dominantly intracellularly with only limited expression on the cell surface (Fig. 6A), similar to huTLR3 (26). The signaling responsiv- eness of rhTLR3 was measured using the experimental ligands poly(I:C) as well as an SIV gag IVT. The SIV gag IVT was pre- dicted by the mFOLD 3.1 program to contain extensive secondary structure, and we therefore reasoned that it could potentially serve as a model viral ligand that is recognized by rhTLR3 during in- fection in vivo. Responsiveness of rhTLR3-expressing cells to these ligands was determined by measuring the induction of lucif- erase activity in cells from a cotransfected NF-κB-driven luciferase reporter plasmid (Fig. 6B). TNF-α was included as a posi- tive control for induction of luciferase, and LPS was included to control for TLR ligand specificity, given that it is a ligand for TLR4. Treatment of cells with poly(I:C) or the SIV gag IVT led to a TLR3-dependent increase in NF-κB-driven luciferase activity (mean fold increases of 23.5 and 12.7, respectively; p < 0.05) relative to media only controls (Fig. 6B), whereas no induction was observed in cells that were mock transfected, transfected with the luciferase reporter only, or cotransfected with the rhTLR4 expres- sion plasmid. LPS treatment did not increase luciferase activity in cells expressing rhTLR3, or cells expressing rhTLR4, possibly due in the latter population of cells to the lack of coexpression of CD14 and/or MD2, which are coreceptors for LPS (27). The positive control of TNF-α treatment significantly increased NF-κB-driven luciferase activity in all cell populations. These data indicate that similar to huTLR3, rhTLR3 recognizes dsRNA ligands, as well as a model SIV RNA sequence, leading to NF-κB-mediated signal transduction.

Contrasting effects of poly(I:C) on viral transcription and viral replication

Our findings that signaling through rhTLR3 increased NF-κB-me- diated transcription (Fig. 6B), prompted us to examine whether TLR3 signaling would also increase expression from the viral promotor/enhancer. Exposure of TZM-bl cells, which harbor an HIV-1 LTR-driven luciferase expression cassette, to TNF-α or SIVmac251, led to an increase in luciferase activity of 2.8- to 4.9-fold over the media control (Fig. 7). Similarly, treatment with poly(I:C) caused a 2.1-fold increase in luciferase activity over the media control, whereas treatment with SIV gag IVT caused only a slight increase (1.3-fold). Because increased transcription of the viral LTR would be expected to contribute to increased viral replic- ation, we next examined the effects of TLR ligands on SIV replication in primary macaque LN and spleen cells, as well as the TZM-bl and A3.01 (human T cell) cell lines. To do this, we quanti- tated cell-associated SIV gag RNA levels following infection with SIVmac251 and treatment with poly(I:C) or CpG-C ODN (Fig. 8). SIV RNA levels were significantly reduced in all of the cell populations treated with poly(I:C), relative to untreated controls (p < 0.0002; Fig. 8A). CpG-C ODN treatment also inhibited SIV RNA levels in spleen, axillary LN, and TZM-bl cells (p < 0.003; Fig. 8B), but less dramatically so compared with poly(I:C). As assessed by trypan blue exclusion, there were no discernible differences in cell viabilities in the TLR ligand-treated and un- treated cultures. Taken together, these data indicate that activation of NF-κB by TLR3 signaling can result in stimulation of the viral promotor/enhancer, although treatment of susceptible cell popula- tions results in inhibition of viral replication.

Discussion

The inflammatory response that occurs in lymphoid tissues during HIV-1 and SIV infection prompted us to examine potential roles for TLRs in the production of inflammatory mediators in vivo and in vitro. In this study, we present data for the first time on the effects of immunodeficiency virus infection on the in vivo expres- sion patterns of TLRs in lymphoid tissues using the best animal model system currently available for HIV-1 infection and disease induction. Following pathogenic SIV infection of rhesus ma- caques, TLR3 mRNA expression was increased in LNs during acute infection and AIDS, whereas TLR9 mRNA expression was decreased prominently during acute infection and less so during AIDS. These findings, coupled with the additional data we present on the effects of TLR3 and TLR9 signaling on viral and inflam- matory cytokine expression levels, define potential roles for these TLRs in HIV-1 and SIV-associated disease through the activation and cellular recruitment that comprise part of the inflammation.

There is only limited information available currently on the ef- fects of HIV-1 infection on the patterns of expression of TLRs in infected individuals, and our study provides the first comprehen- sive analysis of TLR expression in lymphoid tissues—key anat- omic compartments in which viral replication occurs, immune re- sponses to the virus are generated, and effector responses to the virus are delivered. The involvement of TLRs in HIV-1 infection

FIGURE 5. Increased expression of TLR3 mRNA in macaque spleen and LN cells by recombinant IFN-α. Rhesus macaque spleen and axillary LN cells, and the TZM-bl cell line, were treated with poly(I:C), recombi- nant IFN-α, or SIVmac251 infection and examined for the expression of TLR3 mRNA by real-time RT-PCR. Two independent experiments were performed in duplicate, and the means ± SD are shown. *, p < 0.05; **, p < 0.01.
and AIDS has been primarily studied using in vitro systems or HIV-1 transgenic mice, as recently reviewed by Bafica et al. (28). Emphasis has been given primarily to the positive effects of TLR signaling on the HIV-1 transcriptional elements present in the viral LTR. Ligands for TLR1, TLR2, TLR4, TLR6, and TLR9 have all been shown to have positive effects on the HIV-1 LTR (29, 30). Our studies have provided additional insight into this issue, as we have found that poly(I:C), a synthetic TLR3 ligand, can be added to the list of TLR ligands that stimulate the viral LTR. These findings suggest that activation of TLR3 signaling in vivo can enhance viral transcription via NF-κB action on the HIV-1/SIV LTR. Our understanding of TLR expression patterns during HIV-1 infection is limited, but it was recently revealed that TLR2 levels are increased by ~2-fold on blood monocytes in HIV-1-infected individuals relative to uninfected controls, whereas TLR4 levels are not different (31).

TLR3 recognizes dsRNA, which is an intermediate formed during viral replication (7), and due to (1) its up-regulation as opposed to down-regulation, (2) the positive correlation between TLR3 and SIV RNA levels, and (3) the fact that HIV and SIV are RNA viruses and likely generate TLR3 ligands, we gave emphasis to analysis of its potential effects on the virologic and immunologic environments in lymphoid tissues. Recent in vitro and in vivo studies have demonstrated a role for TLR3 in sensing infection by both RNA and DNA viruses, although the relative contributions of TLR3 to limitation or augmentation of disease can differ. In mice, TLR3 plays a protective role during CMV (10) and HSV (32) infections, whereas TLR3 enhanced the pathogenesis of West Nile virus infection (33) by increasing brain vascular permeability and viral entry into the brain in a TNF-dependent manner.

TLR3 is an inducible gene (34) that can be up-regulated upon treatment of cells with poly(I:C) (27, 35), IFN-α (36–38), and recently by IFN-γ (38). In vitro stimulation with recombinant IFN-α resulted in increasing TLR3 expression in all of the cell populations we examined (Fig. 5). Our observation that IFN-α (Fig. 3) and IFN-γ (14) mRNA levels are up-regulated in LNs after SIV infection suggests that this cytokine milieu likely contributes to the local up-regulation of TLR3.

**FIGURE 6.** Characterization of rhesus macaque TLR3 subcellular localization and signaling. A, HEK293T cells were transiently transfected with a plasmid (pcDNA3.1)-expressing rhesus macaque TLR3. Cells were stained using a TLR3-specific mAb (black histograms) before or after permeabilization, and examined by flow cytometry. White histograms represent staining with an isotype control Ab. B, HEK293T cells were transiently transfected with plasmids encoding rhTLR3, rhTLR4, or vector only, in combination with a pNF-κB-luciferase reporter plasmid. Mock-transfected cells were included as controls. The transfected cells were exposed to the indicated stimuli, and luciferase activity was measured 24 h later. Fold changes in luciferase activity were calculated relative to the media control for each transfection. Three independent experiments were performed in duplicate, and the means ± SD are shown. *, p < 0.05.
We also have shown in this study that in addition to TLR3 up-regulation, TLR9 mRNA is down-regulated during acute SIV infection. Unmethylated CpG motifs in microbial DNA are recognized by TLR9 (10) resulting in induction of IFN-α/β, especially from PDCs. TLR9 contributes to protective immune responses during viral infections (10) and has recently been shown to inhibit HIV-1 replication in primary cultures of human tonsil cell populations (39). Signaling through TLR9 expressed on PDCs leads to production of IFN-α/β, which was also found to be up-regulated in lymphoid tissues by us and others (15). The mechanism by which TLR9 is down-regulated at the same time that TLR3 is up-regulated in macaque lymphoid tissues during SIV infection is not clear. However, stimulation through TLR9 by CpG-containing oligodeoxynucleotides (ODNs) can lead to down-modulation of TLR9 gene expression in B cells or PDC (40, 41). Therefore, it is possible that CpG DNA from coinfecting bacteria or viruses during SIV infection can lead to down-regulation of TLR9. Alternatively, the local cytokine milieu could also contribute to the changes in TLR9 expression, as we propose it does for TLR3 expression. Down-modulation of TLR9 could contribute to immunopathogenesis due to a resultant gap in the innate immune repertoire. Further studies are required to address this complex issue.

Increased expression of TLR3 in lymphoid tissues during SIV infection suggests that the immunologic milieu in these anatomic compartments will be highly responsive to TLR3 ligands. In addition to a known, synthetic TLR3 ligand, we demonstrated that a model viral dsRNA ligand, which was an IVT spanning a portion of the SIV gag region, also signaled through TLR3. Our in silico analysis of the SIV gag IVT revealed extensive secondary structure, which was also a characteristic of the comparable region of HIV-1 RNA (not shown). Interestingly, a recent report indicates that TLR3 also responds to cellular RNAs associated with apoptotic bodies (42). The increased apoptosis in HIV-1- (43) and SIV (44)-infected LN relative to uninfected controls could provide an abundant source of host-derived TLR3 ligands, in addition to viral dsRNA.

The consequences of increased TLR3 expression in lymphoid tissues would be expected to include increased signaling leading to induction of a local proinflammatory response. Data presented in this study indicate that TLR3 stimulation of populations of primary lymphoid tissue cells leads to a robust increase in IFN-α/β and to a lesser extent IFN-γ mRNAs. In turn, recombinant IFN-α led to increased expression of CXCL10/IP-10 in axillary LN cells and TZM-bl cells, and of IFN-γ in axillary LN cells, in addition to increasing TLR3 expression in all of the cell populations examined. Furthermore, TLR3 ligands have been shown to synergize with IFN-γ to hyperinduce production of the CXCR3 ligands, CXCL9/Mig and CXCL11/ITAC (IFN-inducible T cell α-chemo-attractant) (45). We previously demonstrated increased IFN-γ mRNA expression in these same axillary LN tissues (14), and therefore synergy between IFN-γ and viral and/or cellular (apoptotic body) dsRNA.
TLR3 ligands could be occurring in these tissues, thereby contributing to the up-regulation of CXCL9/Mig (14) and CXCL10/IP-10 (Fig. 3). Taken together, our data indicate that production of proinflammatory mediators in lymphoid tissues is likely due in part to increased expression and signaling of TLR3 during SIV infection. Persistent TLR3 expression and signaling could be injurious to the local tissues due to its contribution to chronic inflammation and its outcomes, including increased recruitment of lytic cells and cells susceptible to HIV/SIV infection, and the action of metalloproteinasises expressed by infiltrating cells leading to tissue damage and remodeling.

As touched on by Bafica et al. (28), contrasting results have been obtained with regards to the effects of TLR signaling on stimulation of viral transcription relative to studies of the effects of TLR ligands on overall viral replication. Given that TLR ligands activate transcription of the viral LTR, we examined the effects of TLR3 and TLR9 ligands on SIV replication. To model virus/cell interactions within lymphoid tissues in vivo, we exposed primary cells obtained from macaque LN and spleen to infectious SIV and measured the extent of virus replication. Poly(I:C) and CpG ODNs both inhibited viral replication in all cell populations examined (Fig. 8). Schlafpeier et al. (39) have recently used a similar approach to demonstrate the antiviral effects of CpG ODNs on HIV-1 replication in tonsillar cell populations. Studies of the effects of mismatched dsRNA (poly(I)-poly C12U; Refs. 46, 47), which is a variant of poly(I:C), and CpG (39) have been shown to inhibit HIV-1 replication in multiple cell types in vitro. Poly(I):C12U was actually shown in 1987 to improve CD4+ cell counts and immunological status and to inhibit viral replication in HIV-1-infected patients (46, 47). Our data suggest that the benefits of poly(I):C12U could have been mediated through TLR3 and its ability to simultaneously stimulate the viral LTR—and thereby stimulate viral protein expression and presentation of antigenic peptides to virus-specific T cells—and inhibit ongoing viral replication. These data raise the possibility that TLR ligands might be useful as therapeutical strategies to stimulate viral gene expression and flush out the long-lived, latent viral reservoir of cells that remains despite sustained suppression of viral replication during antiretroviral therapy (48), while simultaneously inhibiting further viral propagation. Our findings reveal a number of quandaries regarding the effectiveness of the host response in lymphoid tissues with respect to controlling viral replication. We have shown in this study that TLR3 and SIV RNA levels are significantly positively correlated. Similarly, Abel et al. (15) have also shown in the SIV/macaque model that IFN-α and SIV RNAs are significantly positively correlated. Therefore, despite increased TLR3 and IFN-α expression, which have positive effects on each other’s expression, local SIV replication levels are not proportionately contained. We propose that this is due to the inflammatory milieu present at the same time in these tissues, mediated in part through increased expression and signaling through TLR3. We have previously proposed that a chronic inflammatory loop is sustained in lymphoid tissues during SIV infection (14), partly due to IFN-γ induction of CXCLs 9–11 and their subsequent recruitment of CXCR3+ Th1/Th1c1 cells, which then produce more IFN-γ. Increased TLR3 expression and signaling would feed the local inflammation due to the action of dsRNA ligands formed from cellular apoptotic bodies (42) or from viral RNA, leading to the induction of IFN-α, IFN-γ, and ultimately inflammatory chemokines. One of the most deleterious consequences of this chronic inflammation would be the continual recruitment of new cellular substrates for ongoing viral propagation and the loss of peripheral immune function due to ectopic homing to lymphoid tissues. Increased homing of cells to LNs during HIV-1 infection and its amelioration following antiretroviral therapy have recently been demonstrated (39). Heggehdal et al. (31) have recently proposed a similar model for the effects of TLR signaling on ongoing viral propagation.

There will be a formidable challenge in attempting to use this information to benefit HIV-1-infected individuals. Increased TLR3 expression in lymphoid tissues during SIV infection, and the resulting effects on inflammation and viral gene expression, suggest that approaches that block or redirect TLR3 signaling could help to reduce inflammation in lymphoid tissues. In contrast, it has been shown in vitro and in vivo that TLR3 ligands are effective at inhibiting viral replication. Therefore, any immunomodulatory strategy targeted toward TLR3 must balance the need to maintain the appropriate strength and nature of adaptive immune responses that will act on virus-infected cells, with the combined goal of reducing the inflammatory consequences of TLR3 action and maintaining the antiviral consequences of TLR3 signaling. TLR3, therefore, appears to lie at a precarious interface between benefit and harm for the host.

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Disclosures

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References


