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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[1]

Ozlem Equils,[2]* Marco L. Schito,[3†] Hiase Karahashi,* Zeynep Madak,[4]* Ayse Yarali,[5]* Kathrin S. Michelsen,* Alan Sher,[†] and Moshe Arditi*

Opportunistic infections are common in HIV-infected patients; they activate HIV replication and contribute to disease progression. In the present study we examined the role of Toll-like receptor 2 (TLR2) and TLR9 in HIV-long terminal repeat (HIV-LTR) *trans*-activation and assessed whether TLR4 synergized with TLR2 or TLR9 to induce HIV replication. Soluble *Mycobacterium tuberculosis* factor (STF) and phenol-soluble modulin from *Staphylococcus epidermidis* induced HIV-LTR *trans*-activation in human microvessel endothelial cells cotransfected with TLR2 cDNA. Stimulation of ex vivo spleen cells from HIV-1 transgenic mice with TLR4, TLR2, and TLR9 ligands (LPS, STF, and CpG DNA, respectively) induced p24 Ag production in a dose-dependent manner. Costimulation of HIV-1 transgenic mice spleen cells with LPS and STF or CpG DNA induced TNF-α and IFN-γ production in a synergistic manner and p24 production in an additive fashion. In the THP-1 human monocytic cell line stably expressing the HIV-LTR-luciferase construct, LPS and STF also induced HIV-LTR *trans*-activation in an additive manner. This is the first time that TLR2 and TLR9 and costimulation of TLRs have been shown to induce HIV replication. Together these results underscore the importance of TLRs in bacterial Ag- and CpG DNA-induced HIV-LTR *trans*-activation and HIV replication. These observations may be important in understanding the role of the innate immune system and the molecular mechanisms involved in the increased HIV replication and HIV disease progression associated with multiple opportunistic infections. ***The Journal of Immunology*, 2003, 170: 5159–5164.**

Human immunodeficiency virus infection is one of the leading causes of mortality in the world. The Joint United Nations Program on HIV/AIDS and World Health Organization estimate that there are >40 million people in the world today living with HIV, the majority of whom cannot get adequate antiretroviral therapy and eventually develop AIDS. The development of AIDS is characterized by a dramatic drop in $CD4^+$ T cell counts and the occurrence of multiple opportunistic infections. Clinical observations in HIV-infected patients show increased plasma HIV loads during opportunistic infections and sexually transmitted diseases, suggesting active HIV replication in response to infection (1, 2). At the transcriptional level, HIV-1 replication is controlled by cellular transcription factors and viral *trans*-activator Tat, acting through the HIV-long terminal repeat (HIV-LTR)[6] (3–6). Infections with pathogenic and nonpathogenic organisms activate the cellular transcription factor, NF-κB, which then binds to consensus binding sites in the HIV-LTR to initiate HIV transcription.

Toll-like receptors (TLRs) are innate immune system receptors expressed on cells of the innate immune system that mediate NF-κB activation by a variety of bacterial, mycobacterial, spirochetal, and viral pathogen-associated molecular patterns (PAMP) (reviewed in Ref. 7). Currently, 10 TLRs have been cloned and described, but only seven have known ligands. TLR4 is the primary signaling receptor for enteric Gram-negative bacterial LPS and chlamydial heat shock protein 60 (8), whereas TLR2 is the signaling receptor for Gram-positive bacterial cell wall components; bacterial, mycobacterial, and spirochetal lipoproteins; and fungi (9–11). TLR3 transduces the response to dsRNA (12), TLR5 is the receptor for bacterial flagellin (13), and TLR9 is the receptor for bacterial DNA, which contains short unmethylated CpG dinucleotides (14, 15). TLR7 has recently been shown to mediate

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[6] Abbreviations used in this paper: LTR, long terminal repeat; EC, endothelial cell; HMEC, human dermal microvessel endothelial cell; Luc, luciferase; MyD88, myeloid differentiation protein; PAMP, pathogen-associated molecular pattern; PSM, phenol-soluble modulin; Tg, transgenic; TLR, Toll-like receptor; STF, soluble tuberculosis factor.

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response to imidazoquinoline compounds, which have antiviral and antitumor activities (16). We have recently shown that TLR4 mediates LPS activation of NF-κB and HIV-LTR *trans*-activation using an in vitro endothelial cell system (17).

HIV-infected patients are frequently coinfected with multiple organisms that can induce HIV replication synergistically or additively. In in vitro and in vivo systems, costimulation with multiple PAMP is known to induce NF-κB activation and inflammatory cytokine production synergistically (18, 19). Costimulation of HIV-infected monocytic cell lines with TNF-α and IL-6 induces HIV expression in a synergistic manner (20–24). It is currently unknown whether costimulation with various TLR ligands, such as TLR4 and TLR2 or TLR9, leads to a synergistic or additive increase in HIV transcription and HIV replication.

In the present study we demonstrate that in addition to LPS, TLR2 ligands mediate HIV-LTR *trans*-activation and HIV replication. We also show for the first time that the TLR9 ligand, CpG DNA, leads to HIV replication in ex vivo HIV-1 transgenic (Tg) mouse spleen cells. Furthermore, our findings indicate that costimulation with TLR4 and TLR2 or TLR9 ligands leads to synergistic TNF-α and IFN-γ production and additive HIV-LTR activation and p24 Ag production. These observations may be important in understanding HIV pathogenesis and the molecular mechanisms involved in the progression of HIV infection during multiple opportunistic infections.

Materials and Methods

Cells and reagents

Immortalized human dermal endothelial cells (HMEC) were obtained from Centers for Disease Control and Prevention (Atlanta, GA) (25). HMEC were cultured in MCDB-131 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 μg/ml penicillin and streptomycin in 24-well plates. The cells were routinely used between passages 10 and 14 as described previously (35). The THP-1 monocytic cell line stably transfected with HIV-LTR-luciferase construct (THP-LTR-Luc) was obtained from S. Klebanoff (University of Washington, Seattle, WA). THP-LTR-Luc cells were used up to a maximum passage number of five to seven. Since THP-1 cells do not express CD14, they were differentiated by 36-h stimulation with vitamin D_3 (0.1 μM) before LPS treatment. Phenol-soluble modulin (PSM), a complex of three small secreted polypeptides purified by phenol extraction of supernatants of stationary *Staphylococcus epidermidis* (26), was obtained from S. Klebanoff (University of Washington). A protein-free, heat-stable, *Mycobacterium tuberculosis*-conditioned culture supernatant, soluble tuberculosis factor (STF), was obtained from M. J. Fenton (Boston University, Boston, MA). Highly purified, phenol-water extracted, and protein-free (<0.0008% protein) *Escherichia coli* LPS, which was prepared according to the method described by McIntire et al. (27), was obtained from S. N. Vogel (Uniformed Services University, Bethesda, MD). The purity of this LPS preparation has been previously demonstrated (28, 29), and this preparation of LPS is active on TLR4-transfected HEK 293 cells and not on TLR2 transfectants (S. N. Vogel, unpublished observation). Bacterial CpG DNA was obtained from A.-K. Yi (University of Tennessee Health Science Center, Memphis, TN). All reagents were verified to be LPS free by the *Limulus* amebocyte lysate assay (Pyrotell, Association of Cape Cod, MA; <0.03 endotoxin units/ml).

HIV-1 Tg mice

Experiments using heterozygous HIV-1 Tg mice complied with all relevant federal guidelines and institutional policies. The HIV-1 Tg line 166 was derived by transfecting the full-length wild-type NL4-3 viral clone into FVB/N mice as previously described (30, 31). The animals contain ~20–60 copies of the transgene at a single integration site and transmit them in a stable Mendelian fashion. Tg mice were maintained under specific pathogen-free conditions in an escape-proof room within animal care facilities of the National Institute of Allergy and Infectious Diseases (Bethesda, MD).

Assay for HIV-1 expression in vitro

Spleens from two to five Tg mice were disrupted through a nylon mesh to obtain single-cell suspensions that were pooled and stimulated with various concentrations of LPS and/or STF or CpG DNA. The cultures (5×10^6 cells/ml) were incubated at 37°C with 5% CO_2 in RPMI 1640 (Life Technologies, Grand Island, NY) medium supplemented with 10% FBS (HyClone, Logan, UT), 10 mM HEPES (Life Technologies), 2 mM glutamine (National Institutes of Health stock), 100 U/ml penicillin, 100 μg/ml streptomycin (National Institutes of Health stock), and 5.5×10^{-5} M 2-ME (Life Technologies). Supernatants were removed daily over a 4-day culture period and were stored frozen at −20°C.

Quantitation of viral protein p24 production

The HIV-1 p24 nucleocapsid Ag was quantified by ELISA using a commercial kit (Beckman-Coulter, Miami, FL) according to the manufacturer's instructions. Supernatants from ex vivo spleen cell cultures were diluted in complete medium, and p24 levels were determined in duplicate. The mean and SD of p24 concentrations on day 4 of culture are reported in *Results*.

TLR2, TLR4, and TLR9 expression in HIV-1 Tg mouse splenocytes

Relative levels of TLR2, TLR4, and TLR9 mRNA were determined by RT-PCR. Total RNA was prepared from 3×10^7 spleen cells using the RNeasy Protect minikit following the manufacturer's instructions (Qiagen, Valencia, CA). Recovered RNA was resuspended in diethyl pyrocarbonate-treated, distilled, deionized water, and cDNA was synthesized using 1 μg of total RNA. PCR reactions were performed for 30 cycles using 10 μl of cDNA in a final volume of 50 μl, and a sample (10 μl) of each PCR reaction was electrophoresed through a 2.0% agarose gel and visualized with ethidium bromide. Gels were photographed with an Epi Chem II Still Video System (UVP, Upland, CA). Primer sequences used were as follows: GAPDH sense, ACA TCA TCC CTG CAT CCA CT; GAPDH antisense, GTC CTC AGT GTA GCC CAA G; TLR2 sense, 5′-GAG TCT GCT GTG CCC TTC TC-3′; TLR2 antisense, 5′-CAA TGG GAA TCC TGC TCA CT-3′ (GenBank accession no. AF185284); TLR4 sense, 5′-CAG CAA AGT CCC TGA TGA CA-3′; TLR4 antisense, 5′-AGA GGT GGT GTA AGC CAT GC-3′ (GenBank accession no. NM021297); TLR9 sense, 5′-GCT TTG GCC TTT CAC TCT TG-3′; and TLR9 antisense, 5′-AAC TGC GCT CTG TGC CTT AT-3′ (GenBank accession no. AF314224).

TNF-α and IFN-γ analysis

After stimulation with various TLR ligands, 24-h supernatants from ex vivo Tg mouse spleen cell cultures were analyzed for TNF-α (R&D Systems, Minneapolis, MN), and 72-h supernatants were analyzed for IFN-γ production by ELISA (BD PharMingen, San Diego, CA) according to the manufacturer's instructions. All data for TNF-α and IFN-γ represent the average of triplicate samples ± SD. Each experiment was repeated at least three times.

Expression vectors

Wild-type human TLR2 cDNA was a gift from Ruslan Medzhitov (Yale University, New Haven, CT). The HIV-LTRwt-Luc vector has been previously described (32). Briefly, it carries U2+R regions of the HIV-LTR (LAI strain) from nucleoside −644 (χhol) to +78 (*Hin*dIII) (33). ELAM-NF-κB-Luc and pCMV-β-galactosidase vectors were used as previously described (34).

Transfection experiments

HMEC were plated at a concentration of 50,000 cells/well in 24-well plates and cultured in MCDB-131 with 10% serum overnight as described previously (35). Cells were cotransfected the following day with FuGene 6 Transfection Reagent (Roche, Indianapolis, IN) following the manufacturer's instructions. The reporter genes pCMV-β-galactosidase (0.1 μg) and HIV-LTRwt-Luc (0.1 μg) expression vector (0.1 μg) were transfected into HMEC with or without human TLR2 cDNA (0.3 μg) overnight as previously described (17). The total amount of cDNA transfected to each well was kept equal with empty vector. HMEC and THP-HIV-LTR-Luc cells were stimulated for 5 h with various concentrations of LPS and/or STF or CpG DNA suspended in growth medium. Cells were then lysed, and Luc activity was measured with a Promega kit (Madison, WI) and a luminometer. In transient transfection experiments β-galactosidase activity was determined to normalize the data for transfection efficiency using colorimetric method as described previously (34).

Statistics

For transfection experiments, data shown are the mean ± SD of three or more independent experiments and are reported as the fold increase in HIV-LTR-Luc over empty vector background. Student's two-tailed t test was used to compare supernatant p24 Ag, TNF-α, and IFN-γ levels. A value of $p < 0.05$ was considered significant.

Results

TLR2 ligands induce HIV-LTR trans*-activation*

We have previously shown that TLR4 mediates enteric Gram-negative bacterial LPS-induced, NF-κB-dependent HIV-LRT *trans*-activation. In this study we determined whether Gram-positive bacterial and mycobacterial cell wall components (i.e., TLR2 ligands) can also lead to HIV-LTR *trans*-activation and HIV replication. We have previously observed that HMEC do not express TLR2 and do not respond to TLR2 ligands as measured by NF-κB activation or cytokine production (35). This system permits us to differentiate between TLR2-dependent and -independent effects of specific microbial ligands such as STF and PSM. We used an endothelial cell system with and without TLR2 transfection to test the role of TLR2 in STF and PSM induction of HIV-LTR *trans*-activation. As expected, in contrast to LPS, TLR2 ligands, i.e., STF and PSM, did not induce HIV-LTR activation in native HMEC transiently transfected with HIV-LTR-Luc construct (Fig. 1). Transfection of TLR2 cDNA restored the response to STF and PSM in HMEC. These results suggest that TLR2 plays a key role in staphylococcal PSM- and STF-induced HIV-1-LTR-*trans*-activation, and that in addition to Gram-negative bacterial LPS (via TLR4), Gram-positive bacterial and mycobacterial cell wall components can induce HIV replication (via TLR2).

Costimulation with LPS and STF induces additive HIV-LTR trans*-activation in THP-LTR-Luc cells*

Clinical observations in HIV-1-infected individuals strongly suggest that opportunistic infections lead to active HIV replication, increased plasma HIV load, and HIV disease progression. To test whether simultaneous stimulation with different TLR ligands would synergistically activate HIV-LTR *trans*-activation we used a human THP-1 monocytic cell line that is stably transfected with HIV-LTR-Luc construct. These cells were costimulated with various concentrations of LPS and STF alone or in combination, including suboptimal doses of each ligand. THP-HIV-LTR cells costimulated with TLR4 and TLR2 ligands showed an additive increase in HIV-LTR *trans*-activation, as measured by luciferase activity (Fig. 2). These results suggest that TLR4 and TLR2 costimulation of THP-HIV-LTR cells leads to additive HIV-1 LTR *trans*-activation.

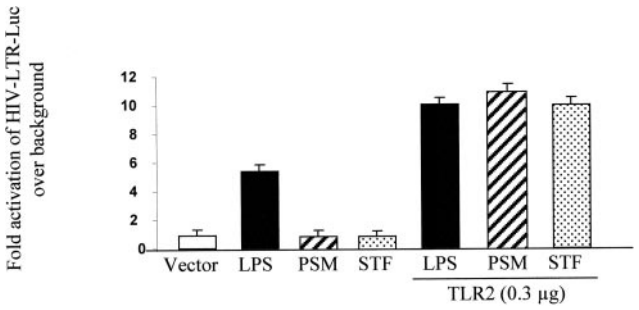

FIGURE 1. TLR2 is required for STF- and PSM-induced HIV-LTR *trans*-activation in the endothelial cell system. HMEC were transfected overnight with HIV-LTR-Luc construct (0.1 μg) and β-galactosidase (0.1 μg) with or without human TLR2 cDNA (0.3 μg). Cells were then stimulated with LPS (50 ng/ml), STF (5 μl/ml), or PSM (100 ng/ml) for 5 h, and Luc activity was determined to assess HIV-LTR activation. A β-galactosidase colorimetric assay was performed to normalize for transfection efficiency. Results are shown as the mean and SD of three or more independent experiments. Ligand-induced HIV-LTR *trans*-activation was reported as the fold increase above that observed with the empty vector control.

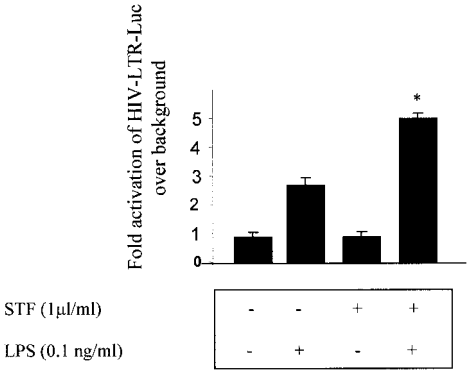

FIGURE 2. Costimulation with LPS and STF induces HIV transcription in an additive manner in THP-LTR-Luc cells. THP-LTR-Luc cells were stimulated for 5 h with various concentrations of LPS and STF (1 μl/ml) alone or together. HIV-LTR activation was assessed by measuring Luc activity using a Promega Luc assay kit and was counted on a 6-detector 1450 Microbeta liquid scintillation counter (PerkinElmer, Gaithersburg, MD). The results are expressed as the fold increase in HIV-LTR-Luc activity above the empty vector control value of three or more independent experiments and are reported as the mean ± SD.

TLR4, TLR2, and TLR9 ligands induce HIV-1 replication in HIV-1 Tg mouse spleen cells ex vivo

In vitro and in vivo *Mycobacterium avium* infection has been shown to induce HIV expression in HIV-1 Tg mice (36), which can be reduced by chemotherapeutic intervention (37). We first confirmed the mRNA expression of TLR2, TLR4, and TLR9 in HIV-1 Tg mouse splenocytes (Fig. 3), then we assessed whether TLR2 and TLR9 ligands induce HIV replication in the HIV-1 Tg mouse system. The tissues of HIV-1 Tg mice contain preintegrated provirus, including HIV-LTR (30, 31). Upon stimulation they produce infectious virus that is recoverable by coculture with human T cells (38); however, due to the lack of appropriate receptors and coreceptors, HIV spread cannot occur. Therefore, these animals are excellent models to examine the factors that regulate latent viral expression (39). To investigate the effects of TLR4, TLR2, and TLR9 ligands on the regulation of latent HIV expression, we stimulated ex vivo spleen cells obtained from HIV-1 Tg mice with LPS, STF, and CpG DNA and assessed HIV replication by measuring supernatant p24 Ag levels. LPS, STF, and CpG DNA stimulation of Tg mice splenocytes induced HIV p24 Ag production in the supernatants in a dose-dependent manner (Fig. 4). These results confirm that spleen cells from HIV-1 Tg mice respond to various TLR ligands with activation of HIV replication, and underscore the role of the innate immune system in up-regulating latent HIV expression through the activation of various TLRs.

TLR4 signaling combined with TLR2 or TLR9 activation results in synergistic Th1 cytokine expression and increased HIV-1 replication in HIV-1 Tg mouse spleen cells ex vivo

HIV-1-infected individuals show increased plasma HIV loads in response to multiple opportunistic infections, which frequently occur simultaneously. Therefore, we hypothesized that simultaneous

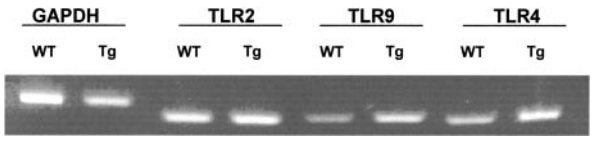

FIGURE 3. HIV-1 Tg mouse splenocytes express TLR2, TLR4, and TLR9. We isolated RNA from HIV-1 Tg mouse splenocytes and performed RT-PCR analysis as described in *Materials and Methods*. The results were compared with those obtained from FVB/N mice.

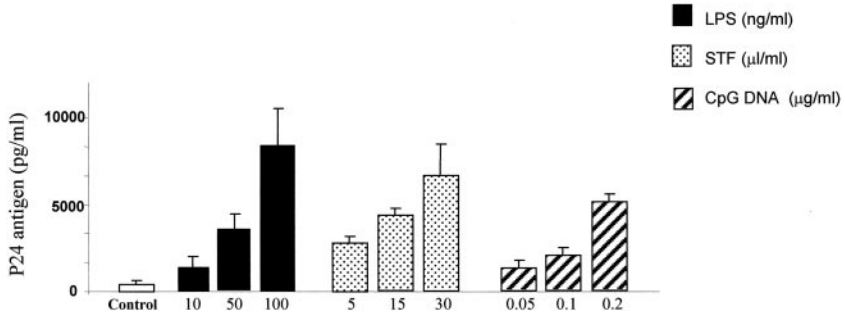

FIGURE 4. LPS, STF, or CpG DNA stimulation of spleen cells from Tg mice induces p24 Ag production in a dose-dependent manner. Ex vivo spleen cells from HIV-1 Tg mice were isolated as described in *Materials and Methods*. Cells were stimulated with various doses of LPS, STF, and CpG DNA for 4 days. Supernatants were collected daily and frozen until p24 Ag levels were determined, using the 96 h samples in batch, by ELISA. The data shown are the mean ± SD of three or more independent experiments.

activation of several TLRs by various microbial ligands may lead to enhanced HIV-1 replication and influence Th1 cytokine production. To test this hypothesis, we used ex vivo spleen cells isolated from HIV-1 Tg mice and examined the effects of costimulation with LPS and either STF or CpG DNA (TLR4, TLR2, and TLR9 ligands, respectively) on the release of Th1 cytokines (i.e., IFN-γ and TNF-α production) and HIV replication. Costimulation of HIV-1 Tg mouse spleen cells ex vivo with LPS and CpG DNA induced IFN-γ production in a synergistic manner (Fig. 5). LPS and STF or CpG DNA costimulation also led to a synergistic increase in TNF-α production (Fig. 6, *A* and *B*).

To investigate the effects of costimulation with LPS and STF or CpG DNA on HIV replication, we used ex vivo spleen cells from Tg mice, stimulated with LPS and STF or CpG DNA, and measured supernatant p24 Ag production by ELISA. LPS and STF or CpG DNA costimulation led to additive p24 Ag production in spleen cells ex vivo (Fig. 7, *A* and *B*). These findings suggest that costimulation of TLR4 and TLR2 or TLR9 induces Th1 cytokine release in a synergistic fashion and HIV translation and replication in an additive manner.

Discussion

HIV infection is characterized by progressive immune dysfunction, leading to AIDS and opportunistic infections by a wide variety of microorganisms. Chronic or recurrent infections contribute to sustained high levels of viremia and thereby accelerate HIV disease progression and immune deterioration. Whalen et al. (39) demonstrated that active *M. tuberculosis* infection is associated with decreased survival among HIV-1-infected individuals after controlling for $CD4^+$ cell count, antiretroviral therapy, and previous opportunistic infections. Alcabes et al. (40) reported an accelerated rate of $CD4^+$ cell decline in HIV-infected individuals during bacterial infections. The development of an opportunistic infection was shown to be an independent risk factor for death in HIV-infected population (41). The innate immune activation-driven HIV replication may in part explain the aggressive course of HIV infection in individuals coinfected with *M. tuberculosis* and may provide a possible mechanism for the more rapid course of immunologic decline among HIV-infected patients in sub-Saharan Africa, where coinfections are common and often continuous (42–44). Currently the molecular mechanisms involved in enhanced HIV replication and the progression of HIV infection following multiple opportunistic infections, such as mycobacterial infections, are not well understood. Therefore, delineating the molecular mechanisms that regulate activation of latent HIV during infections with opportunistic and pathogenic microorganisms is of great clinical significance.

We have recently shown that TLR4 mediates LPS induction of HIV-LTR *trans*-activation through IL-1R signaling molecules and NF-κB activation (17). Here we extend these earlier findings to show that TLR2 plays a central role in PSM- and STF-induced HIV-LTR *trans*-activation, and that bacterial CpG DNA, a TLR9 ligand, leads to enhanced HIV-LTR *trans*-activation and HIV-1

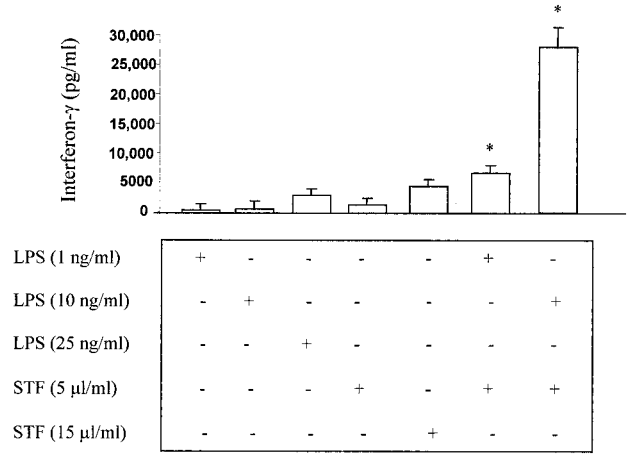

FIGURE 5. Spleen cells from HIV-1 Tg mice costimulated with LPS and STF induce IFN-γ production in a synergistic manner. Ex vivo spleen cells from HIV-1 Tg mice were isolated as described in *Materials and Methods*. Cells were stimulated with suboptimal and optimal concentrations of LPS and STF alone or simultaneously for 4 days. Supernatants were frozen until IFN-γ levels were determined, using the 72 h samples in batch, by ELISA. The data shown are the mean ± SD of three or more independent experiments. *, $p < 0.05$.

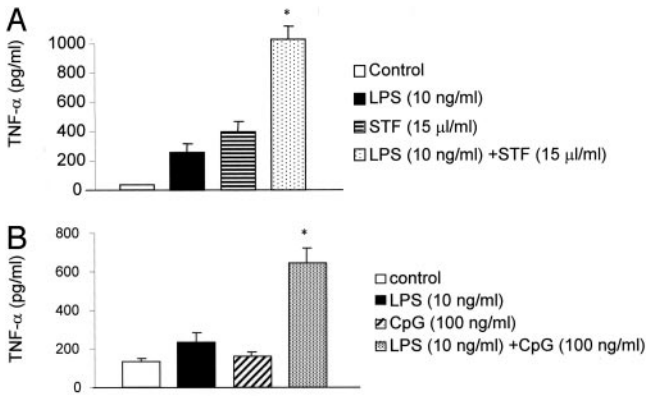

FIGURE 6. Costimulation with LPS and STF or with LPS and CpG DNA induces synergistic TNF-α production in HIV-1 Tg mouse spleen cells. Ex vivo spleen cells from HIV-1 Tg mice were isolated as described. Cells were stimulated with LPS and STF alone or in combination for 4 days (*A*). In separate experiments spleen cells were stimulated with LPS and CpG DNA alone or simultaneously for 4 days (*B*). Supernatants were collected, and TNF-α concentrations were determined, using the 24 h samples in batch, by ELISA. The data shown are the mean ± SD of three or more independent experiments. *, $p < 0.05$.

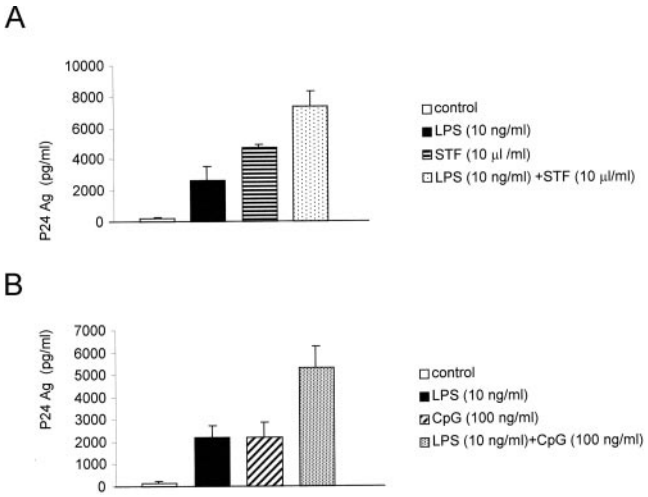

FIGURE 7. Costimulation of HIV-1 Tg mouse spleen cells with LPS and STF or LPS and CpG DNA induces p24 Ag production in an additive manner. Ex vivo spleen cells from HIV-1 Tg mice were isolated as described. Cells were stimulated with LPS and STF alone or simultaneously for 4 days (*A*). In separate experiments spleen cells were stimulated with LPS and CpG DNA alone or in combination for 4 days (*B*). Supernatants were collected, and p24 Ag levels were determined, using the 96 h samples in batch, by ELISA. The data shown are the mean ± SD of three or more independent experiments.

replication, as measured by p24 Ag release from HIV-1 Tg mice spleen cells. Different TLRs are known to induce distinct cellular and systemic responses to infection. Engagement of TLR4, TLR2, and TLR9 induces similar, but distinct, proinflammatory responses (45–47). Indeed, recent studies have suggested that TLR-dependent responses, while similar, are not identical (45–47). TLR2 or TLR9 signaling results in both qualitatively and quantitatively different inflammatory responses compared with TLR4 signaling (45–47). Our results emphasize the role of TLRs, including TLR4, TLR2, and TLR,9 in mediating bacterial and mycobacterial Ag-induced HIV replication and suggest a cooperative up-regulation of HIV *trans*-activation.

CpG DNA conjugate vaccines have been shown to induce strong innate immune responses and clinical trials of vaccines that include CpG DNA are in progress (48). In addition, HIV gp120: CpG DNA conjugate vaccines have been developed and tried in animal models and were shown to induce strong gp120-specific immune responses (49). CpG DNA-based immunization was suggested to hold promise for the development of an effective preventive and therapeutic HIV vaccine. HIV-infected patients are frequently coinfected with multiple pathogens, and their immune system cells are frequently exposed to multiple microbial Ags concurrently. Currently there are no data on the effects of costimulation with CpG DNA and bacterial and mycobacterial Ags on HIV replication. Our data suggest that CpG DNA-induced TLR9 activation also leads to HIV-LTR *trans*-activation and HIV-1 replication. These observations may be of importance for future studies in which DNA-based HIV or non-HIV vaccines will be investigated in HIV-1-infected individuals. Furthermore, understanding the role of the innate immune responses and TLRs by which opportunistic infections lead to progression of HIV-1 infection may have important implications and may lead to novel therapeutic strategies targeting these receptors or their signaling intermediates.

Simultaneous exposure to different antigenic components of bacteria, such as peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* (50), lipoprotein and LPS (51), or CpG DNA and LPS from enteric Gram-negative bacteria (52), has long been recognized to act synergistically to induce cytokine production and lethal shock in in vitro and in vivo animal models. Recently, simultaneous interaction of different TLRs with their respective ligands has been shown to result in synergistic induction of cytokine release. Sato et al. (53) have reported that costimulation of mouse peritoneal macrophages with TLR2 ligand (mycoplasmal lipopeptide (MALP-2) and TLR4 ligand (LPS) resulted in synergistic increase in TNF-α production. Gao and colleagues (54) reported that the TLR9 ligand, CpG-DNA, synergized with TLR4 ligand LPS to enhance TNF-α secretion from mouse macrophages at a post-transcriptional level.

HIV-1 replication is regulated at a transcriptional level by cellular transcription factors, such as NF-κB, NF-AT, AP-1, and Sp1, and at a post-transcriptional level by the interplay of viral proteins (55, 56). Since NF-κB activation plays a key role in bacterial Ag-induced HIV-LTR *trans*-activation and HIV replication (17), and costimulation with various TLR ligands lead to additive, not synergistic, activation of NF-κB (data not shown), it is not surprising that we observed an additive, but not synergistic, effect on HIV replication. We do not think that apoptosis and cell death induced by costimulation of cells with LPS and STF or CpG DNA contributed to the absence of synergy for p24 Ag production, since costimulation with the same PAMPs led to synergistic increases in TNF-α and IFN-γ production.

Here we show that costimulation with TLR4 and TLR2 or TLR9 induces synergistic release of Th1 cytokines, IFN-γ and TNF-α, and additive HIV-LTR *trans*-activation and HIV replication, as measured by p24 Ag release from HIV-1 Tg mouse spleen cells. Th1 cytokines have been shown to play an important role in HIV replication and HIV pathogenesis (57). Our studies do not rule out a contributory synergistic effect of cytokines, i.e., TNF-α and IFN-γ, on CpG DNA-, LPS-, or STF-induced HIV p24 Ag production in ex vivo Tg mouse spleen cells. However, transient transfection experiments in THP-LTR-Luc cells showed that CpG DNA stimulation induces HIV-LTR *trans*-activation at 5 h, which suggests that CpG-DNA can directly induce HIV-LTR *trans*-activation in the absence of Th1 cytokines (Fig. 2). TLRs have recently been shown to mediate the generation of adaptive immune responses (58). Interruption of TLR-mediated signaling has been shown to lead to a profound defect in the activation of Ag-specific Th1 immune responses (59). Therefore, understanding the roles of innate immune responses and TLRs in the generation of Th1 and Th2 responses and in the induction of latent HIV-1 replication may have important clinical significance and may lead to the development of novel therapeutic approaches to control HIV disease progression during opportunistic infections.

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