The mechanism of LPS-induced HIV type I activation in transgenic mouse macrophages

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Abstract

In the course of the development of acquired immunodeficiency syndrome (AIDS), bacterial infection causes deleterious effects on the progression of the disease; bacterial LPS in the circulation activate immune cells, resulting in the acceleration of HIV replication. However, the precise HIV activation mechanisms in infected hosts remain largely unknown. Previously, we generated transgenic (Tg) mice carrying the HIV type I (HIV-1) genome and showed that LPS induces the activation of HIV-1 in splenocytes through the induction of tumor necrosis factor (TNF) and IL-1, although similarly induced IFN-γ and IL-6 are not involved. In this study, we analyzed the mechanisms of HIV-1 activation in macrophages using these HIV-1 Tg mice, because macrophages are one of the major reservoirs of HIV-1. In contrast to splenocytes, direct Toll-like receptor (TLR) 4 signaling rather than TLR-induced pro-inflammatory cytokines was responsible for the LPS-induced activation of HIV-1 in macrophages, because the time course of HIV-1 activation was earlier than that observed in splenocytes and TNF neutralization did not inhibit the activation. p38 mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) activation, but neither extracellular signal-regulated kinase nor c-Jun N-terminal kinase activation, were required for the activation, because only inhibitors for p38 MAPK and NF-κB suppressed activation of HIV-1. Furthermore, we showed that myeloid differentiation primary response gene (MyD) 88, rather than Toll/IL-1R domain-containing adaptor inducing IFN-beta (TRIF), was required as an adaptor molecule for this activation using Myd88⁻/⁻ mice and Dynasore, a specific inhibitor for TRIF, and small interfering RNAs specific for Myd88 and Trif. These observations suggest that suppression of these molecules, which are involved in the TLR4–MyD88 pathway and the downstream p38 MAPK and NF-κB pathways, should be beneficial to prevent development of AIDS in HIV-1-infected people.

Keywords: HIV-1, latency, LPS, macrophages

Introduction

Highly active anti-retroviral therapy (HAART) has dramatically reduced the morbidity and mortality of acquired immunodeficiency syndrome (AIDS) especially in developed countries (1–3). However, due to its expensive cost, side effects and difficulties in adherence, many HIV-infected people, particularly in developing countries, cannot fully receive the benefits of HAART (4–6). Although an effective vaccine for HIV infection has been long awaited to overcome these difficulties, one has yet to be developed (7, 8).

High HIV type I (HIV-1) load associated with opportunistic infection is a characteristic of AIDS at the end phase (9). In sub-Saharan Africa, the frequency of bacteremia such as non-typhoidal Salmonella serotypes and Streptococcus pneumoniae increases dramatically in HIV-1-infected people (10). Over 90% of HIV-1-seropositive people are co-infected with these bacteria and the mortality is about one-third among these bacteremic patients. Systemic microbial translocation easily occurs because of the destruction of the gut.
mucosal barrier in the chronic phase of HIV-1 infection (11), and bacterial LPS in the circulation causes chronic hyper-activation of both innate and adaptive immunity (11, 12).

Interestingly, simian immunodeficiency virus (SIV) infection of natural hosts, such as sooty mangabeys or African green monkeys, is not lethal, despite robust mucosal CD4 T cell depletion (13–15). In these hosts, microbial translocation and chronic immune activation does not occur (14). When macaques, which are not the natural host of SIV, are infected with SIV, these monkeys develop AIDS (11). Because antibiotic treatment of these monkeys delays the development of AIDS, it was suggested that systemic dissemination of LPS is one of the reasons for the development of AIDS (16). In this context, it was reported that LPS potently stimulates HIV-1 long terminal repeat (LTR)-chloramphenicol acetyltransferase constructs transfected into monocyte/macrophage-like cell lines (17). Toll-like receptor (TLR) 4 can mediate LPS-induced NF-kB and HIV-1 activation in human dermal microvesSEL endothelial cells upon co-transfection with HIV-LTR-luciferase and TLR4 cDNA (18).

Signaling through TLR2, TLR4 and TLR9 also activates HIV-1 replication in latently infected mast cells (19). These observations indicate that immune activation caused by LPS or other bacterial cell components plays a crucial role in the development of AIDS (15, 16). However, so far, the LPS-induced HIV-1 activation mechanisms in infected hosts have not been elucidated completely.

We previously generated transgenic (Tg) mice carrying the HIV-1 genome (HIV-Tg), in which the reverse transcriptase gene has a defective mutation (20). These mice are useful as a model for healthy HIV-1 carriers; the expression levels of HIV-1 in lymphocytes from the spleen or lymph nodes are low under physiological conditions, whereas they are strongly enhanced when mice are treated with LPS. We found that HIV-1 activation in splenocytes was greatly reduced in tumor necrosis factor (TNF)- and IFN-γ-deficient mice, but not in IL-6- or IL6R-γ-deficient mice, suggesting that LPS-induced TNF and IL-1 are involved in the HIV-1 activation in lymphocytes (21). Furthermore, we showed that cell cycle-dependent demethylation of CREB/ATF sites in the LTR is required for the activation (22). However, since TNF and IFN-γ deficiency did not completely suppress HIV-1 expression in splenocytes, we speculated that TLR-mediated direct signaling might also be involved in the HIV-1 activation (21).

It is well known that macrophages and B cells, but not T cells, in splenocytes express TLRs, and macrophages are one of major reservoirs of HIV-1 especially during HAART (23, 24). Thus, in this report, we examined the mechanisms of HIV-1 activation in macrophages from HIV-Tg mice upon treatment with LPS. We found that, in contrast to lymphocytes, cytokines are not involved in the HIV-1 reactivation in macrophages after LPS stimulation, and TLR-mediated direct signaling is important for the activation. p38 Mitogen-activated protein kinase (MAPK) and NF-kB pathways, but neither extracellular signal-regulated kinase (ERK) nor c-Jun N-terminal kinase (JNK) pathways, are used as downstream signal transduction pathways. Furthermore, we showed that myeloid differentiation primary response gene (MyD) 88, rather than Toll/IL-1R domain-containing adaptor inducing IFN-beta (TRIF), is used as an adaptor molecule in the TLR-mediated HIV-1 activation.

**Materials and methods**

**Reagents**

LPS from Escherichia coli O55:B5, polymyxin B sulfate, p38 MAPK inhibitor SB202190 (25), NF-kB inhibitor pyrrolidinedithiocarbamate (PDTC) (26) and dynamin inhibitor Dynasore (27) were purchased from Sigma (St Louis, MO, USA). ERK inhibitor U-0126 (28) and JNK inhibitor SP600125 (29) were obtained from Calbiochem (La Jolla, CA, USA). *Escherichia coli*-type synthetic lipid A (compound 506) was purchased from the PEPTIDE Institute (Osaka, Japan). mAb for mouse TNF (Clone MP6-XT3) and isotype control were obtained from eBiosciences (San Diego, CA, USA).

**Mice**

The HIV-Tg mice of the T1607 line, which carry the polymerase gene-defective HIV-1 genome (NL-4–3–2), on the C3H/HeN or C57BL6/J background were used throughout this study (20). The C3H background was mainly used except when Myd88-deficient HIV-Tg mice were included. For the production of Myd88-deficient HIV-Tg mice, Myd88-deficient mice (30) backcrossed to C57BL6/J mice over eight generations (kindly provided by Shizuho Akira, Osaka, Japan) were crossed to HIV-Tg mice that were backcrossed over eight generations to C57BL6/J. The F1 mice carrying both the Myd88 mutation and the HIV-1 transgene were intercrossed to obtain homozygous Myd88-deficient mice carrying the HIV-1 genome. Sex- and age (6–12 weeks of age)-matched mice were used throughout the study. All mice were housed under specific pathogen-free conditions in an environmentally controlled clean room (24 °C, 40–60% moisture, 8:00–20:00 lighting cycle) at the Center for Experimental Medicine and Systems Biology, Institute of Medical Science, University of Tokyo. The experiments were conducted according to the institutional guidelines for animal experiments and the safety guidelines for gene manipulation experiments.

**Preparation of macrophages**

Peritoneal macrophages were prepared according to Saijo et al. (31). Briefly, mice were injected intraperitoneally with 2 ml of 4% thioglycollate (TGC) (Difco Laboratories; Detroit, MI, USA), and peritoneal exudate cells were isolated from the peritoneal cavity 4 days after injection. Cells collected from two to four mice with the same genotype were pooled and incubated in RPMI1640 medium supplemented with 10% fetal bovine serum, 50 U ml−1 penicillin, 50 μg ml−1 streptomycin and 50 mM 2-mercaptoethanol for 16–20 h at a concentration of 2 × 10^6 cells per milliliter. After incubation, cells were washed three times with PBS and harvested by scraping. Harvested cells were adjusted at a concentration of 2 × 10^6 cells per milliliter and replate into a 24-well plate at 500 microliters per well. We used the adherent cells as peritoneal macrophages for the experiments. Over 90% of adherent cells expressed both CD11b and F4/80, detected by flow cytometry.

**Reverse transcription and real-time quantitative PCR**

Five hundred nanograms of total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the
Adherent macrophages were transfected with 25 nM of RNA interference (RNAi) using ECL plus Western Blotting Detection Reagents (GE Biomedical, Osaka, Japan), HIV-1 Gag or Nef protein was subjected to melting curve analysis using the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA); cDNA from 2.5 ng of total RNA was used for each reaction. The PCR reaction was performed by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA, USA) with 0.4 μM of each primer at a final volume of 20 μl. For all analyses, cDNAs were amplified with 45 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Primers for detecting the HIV-1 tat-rev transcript, hypoxanthine phosphoribosyl transferase 1 (Hprt), Cxcl10 (IP-10), IL6 and Trif mRNA were as follows: gagcagaagctcatacagacagt (tat-f) and gattgctccataagtgtcagaag (tat-r), agctactgtaaatgctatcaacctgc (hprt-f) and aagaggctcttttacacagca (hprt-r), tggctggtgtgttcttacaggg (IP-10-f), gctcaatctcctttatcggc (IP-10-r), gaggtacacctcacaacagcc (IL-6-f) and aagtgcatacatgtgtcataca (IL-6-r), and gcctcccctctcagctct (TNF-f) and cacggtgtggtgtgctaca (TNF-r), respectively. Product sequences were subjected to melt curve analysis using the iCycler iQ System to exclude the amplification of non-specific products.

Detection of HIV-1 proteins
An ELISA Kit (BioAcademia, Osaka, Japan) was used to detect the HIV-1 p24 Gag protein in culture supernatant. For detection of intracellular HIV-1 proteins, splenocytes or macrophages were collected from each well and lysed with 100 microliters per well of SDS-PAGE sample buffer (50 mM Tris–Cl, pH 6.8, 2% SDS, 6% glycerol and 1% 2-mercaptoethanol) and then samples were boiled for 5 min. Cell lysates were electrophoresed on a 12% polyacrylamide gel and transferred electrophoretically onto PVDF membrane (Immobilon-P; Millipore, Billerica, MA, USA) using Trans-Blot SD Semi-dry Transfer Cell (Bio-Rad Laboratories). After blocking with BlockAce (DS Pharma Biomedical, Osaka, Japan), HIV-1 Gag or Nef protein was detected by polyclonal antibody for each antigen (BioAcademia) using ECL plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).

RNA interference
Adherent macrophages were transfected with 25 nM of Silencer Select small interfering RNAs (siRNAs) for Myd88, Trif, or Negative Control siRNA (Applied Biosystems) using INTERFERin siRNA Transfection Reagent (Polyplus-transfection SA, Illkirch, France) as described previously (32). Sequences of each siRNA were 5′-CUAUAGGGCAUACAUACAAATT-3′ and 5′-UUGGUAUAGCGCAUAUAGTG-3′ for Myd88 and 5′-CAGCCUAUGUGCCAUAGCTAT-3′ and 5′-UA-GCUAUGGGCAUAGCGCUGT-3′ for Trif. Although sequence of Negative Control siRNA was a non-disclosure by the manufacturer’s policy, we confirmed that transfection of this siRNA did not affect expression of genes of our interest. Forty-two hours after transfection, macrophages were stimulated with LPS and HIV expression was measured 6 h later.

Results
HIV-1 genes are induced in macrophages from HIV-Tg mice by LPS in a different time course to lymphocytes
To examine whether LPS can directly activate HIV-1 expression in macrophages, we prepared TGC-elicted peritoneal macrophages (TGC-Me) from HIV-Tg mice and treated them with various concentrations of LPS for 48 h, when the HIV-1 reactivation reached the peak in splenocytes (21, 22). The HIV-1 tat-rev transcript was observed in both macrophages and splenocytes in an LPS dose-dependent manner (Fig. 1A). The highest expression was observed at 6 h after LPS stimulation in macrophages (Fig. 1B, left panel), while the expression in splenocytes showed a bimodal peak at 12 and 48 h (Fig. 1B, right panel). Other HIV-1 transcripts corresponding to vif and gag exhibited similar activation time course although activation of these mRNAs have another peak at 1 h after stimulation, which would represent premature RNAs that had not been spliced yet (Supplementary Figure 1A, available at International Immunology Online). Then, we measured HIV-1 p24 levels in the culture supernatant of LPS-stimulated HIV-Tg splenocytes or macrophages by ELISA (Fig. 1C) or in cell lysates by western blotting (Fig. 1D). High levels of p24 were detected in splenocyte cultures after 48–72 h of LPS stimulation by both ELISA and western blot techniques. However, only low levels of p24 were detected in macrophage cultures by ELISA, and we could not detect the p24 protein by western blotting, although we could detect the Nef protein induction by western blotting in macrophages. These results clearly show that HIV-1 in macrophages can be activated by LPS and that HIV mRNAs are translated into proteins in macrophages, as in splenocytes (21, 22). However, the mechanisms of activation may be different between macrophages and splenocytes, because the time course of HIV-1 gene activation was different.

HIV-1 reactivation in macrophages by LPS is independent of TNF and IL-1 production
We previously showed that LPS-induced HIV-1 activation in the spleen of HIV-Tg mice depends on TNF and IL-1 expression, but neither IL-6 nor IFN-γ expression (21). We then examined whether this is also true in macrophages. We analyzed HIV-1 expression at 6 h and LPS concentrations at 100 ng ml⁻¹ or less unless otherwise described, because HIV-1 expression was the highest at that time (Fig. 1B) and sufficient at these concentrations (Supplementary Figure 2A, available at International Immunology Online). Treatment with a neutralizing antibody to TNF did not block the LPS-induced HIV-1 activation (Fig. 2A). Under these conditions, this antibody strongly suppressed TNF-induced HIV-1 activation, and a much lower TNF concentration (<2 ng ml⁻¹) was detected in this culture supernatant than that used in recombinant TNF stimulation (20 ng ml⁻¹). Thus, these results indicate that TNF is not involved in the HIV-1 activation in macrophages (Fig. 2A). Moreover, IL-1β production was below the limit of detection by ELISA (<10 pg ml⁻¹). In addition, TNF antibody treatment significantly suppressed LPS-induced HIV-1 reactivation in splenocytes (Fig. 2B), which is consistent with our previous in vivo results. Anti-TNF treatment did not affect even when macrophages were stimulated with lower (1 ng ml⁻¹) concentration of LPS (Supplementary Figure 2B, available at International Immunology Online). Thus, these results indicate that, in contrast to TNF- and IL-1-dependent activation of HIV-1 in splenocytes, those cytokines are dispensable for the HIV-1 activation in macrophages.
Fig. 1. Activation of HIV-1 gene expression in macrophages from HIV-Tg mice by LPS. (A) LPS dose dependency of HIV-1 expression in macrophages (solid lines) or splenocytes (dashed line). TGC-elicited peritoneal macrophages (TGC-Mø) or splenocytes from HIV-Tg mice were stimulated in vitro at the indicated concentration of LPS. Total RNA was isolated 48 h after stimulation and the HIV-1 tat-rev-specific transcript was quantified by real-time PCR. Expression levels in macrophages at 48 h are indicated with open squares and those of splenocytes are shown by open circles, respectively. Relative expression levels to that of unstimulated splenocytes are indicated. Values are means ± SDs from triplicated wells. These data are representative of three independent experiments within a range of stimulation from 8 to 50 in macrophages and from 8 to 15 in splenocytes at 1000 ng ml⁻¹ at 48 h. (B) Time course of HIV-1 expression after LPS stimulation. TGC-Mø and splenocytes isolated from HIV-Tg were stimulated with 5 ng ml⁻¹ of LPS. Total RNA was isolated at indicated times and HIV-1 expression was quantified as described in (A). Expression levels relative to 0 h are indicated for each cell. Values are means ± SDs from triplicated wells. These data are representative of three independent experiments for each cell within a range of stimulation from 6 to 21 in macrophages and from 9 to 30 in splenocytes at 48 h. (C and D) HIV-1 proteins in LPS-stimulated macrophages and splenocytes. TGC-Mø and splenocytes from HIV-Tg mice were stimulated with 5 µg ml⁻¹ of LPS. Total RNA was isolated at indicated times and HIV-1 expression was quantified as described in (A). Expression levels relative to 0 h are indicated for each cell. Values are means ± SDs from triplicated wells. Data of (C) and (D) are representative of two independent experiments.
macrophages, suggesting that direct TLR signaling may be more important.

**p38 MAPK and NF-κB, but neither ERK nor JNK, are essential for LPS-induced HIV-1 activation in macrophages**

Upon stimulation with LPS, TLR4 activates various downstream signaling molecules. Those include MAPKs such as p38, ERK and JNK, and NF-κB [reviewed in (33)]. Then, we examined the activation of these signaling pathways in macrophages stimulated with LPS. First, we blocked each one of these signaling molecules by specific chemical inhibitors. SB202190, U-0126 and SP600125 are the specific inhibitors for p38 MAPK, ERK and JNK, respectively. We stimulated macrophages at the concentration of 1 ng ml\(^{-1}\) of LPS, because higher concentrations of inhibitors were required at higher LPS concentrations that caused non-specific inhibitory effects. As shown in Fig. 3(A), the p38 MAPK inhibitor suppressed LPS-induced HIV-1 activation by ~70% at a concentration of 0.6 μM. We did not detect any inhibition of p38 phosphorylation by SB202190; this is because this inhibitor only interferes with the p38 kinase activity but not p38 phosphorylation. Consistent with this, its downstream target hsp27 was less phosphorylated by the SB202190 treatment, indicating that p38 activity was inhibited under these experimental conditions (Fig. 3A). We did not observe dose dependency of the inhibition, suggesting that 0.6 μM is already sufficient enough to suppress the HIV-1 activation. Under these conditions, phosphorylation of ERK, JNK and c-Jun was not affected, indicating that SB202190 specifically inhibited p38 activity. On the other hand, ERK and JNK inhibitors had no effect on the HIV-1 expression (Fig. 3B and C). Under these conditions, ERK and JNK phosphorylation were severely suppressed, as examined by western blotting (Fig. 3A–C, middle panels).

We next examined the contribution of NF-κB by inhibiting with PDTC. Treatment with >62.5 μM of PDTC inhibited >80% of LPS-induced HIV-1 activation (Fig. 3D). Under these conditions, the phosphorylation of IκBα was almost completely abolished. Similar tendency was observed for other HIV-1 transcripts *vif* and *gag* (Supplementary Figure 1B, available at *International Immunology* Online). Taken together, these observations indicate that, in macrophages, the p38 MAPK and NF-κB pathways are mainly involved in the LPS-induced HIV-1 activation in the downstream of TLR4, and the ERK and JNK pathways are dispensable for this activation.

**MyD88, rather than TRIF, is involved in the LPS-induced HIV-1 activation in macrophages**

Among TLRs, TLR4 is unique because it utilizes two adaptor molecules, MyD88 and TRIF, to transduce the signal. LPS-inducible genes are subdivided into several groups according to MyD88/TRIF dependency (34). For example, *Tnf* expression is induced in a MyD88-dependent manner, while *Cxc10* (IP-10) or other IFN-responsive genes are induced in a TRIF-dependent manner. Both MyD88 and TRIF are required for the expression of *Il6*, while either one of these molecules is enough for the expression of C-type lectin and TNFR family genes. NF-κB is activated either by MyD88 or TRIF (35, 36). Then, we examined the MyD88 dependency of LPS-induced HIV-1 gene activation. HIV-1 gene expression in macrophages from *Myd88*-deficient mice was only a half of that in wild-type macrophages at 6 h after LPS stimulation, indicating that this molecule is involved in the LPS-induced HIV-1 gene activation (Fig. 4A). This reduced expression of HIV-1 genes was still observed at 12 h after LPS stimulation (Fig. 4B), indicating that the MyD88-mediated pathway is functional not only at the beginning of stimulation but also continues to be functional until the late phase. These
observations indicated that the observed HIV-1 suppression is not due to the delay of NF-κB activation as reported previously in Myd88-deficient macrophages (35).

Next, we tried to exclude the possibility that HIV-1 genes were activated through a TLR4-independent pathway by contaminated molecules in the LPS preparation. When LPS was pre-treated with polymyxin B, an LPS inhibitor, HIV-1 gene expression was almost completely abolished in macrophages (Fig. 4C). Furthermore, HIV-1 gene expression was also suppressed in macrophages from Myd88-deficient

Fig. 3. p38 MAPK and NF-κB, but neither ERK nor JNK, are involved in LPS-induced HIV-1 activation in macrophages. TGC-Mø were stimulated with LPS (1 ng ml⁻¹) in the presence of MAPK inhibitors as indicated. The upper panels of (A–C) and the left panel of (D): after 6 h of LPS treatment, HIV-1 expression was determined by real-time PCR. SB202190 (A), U-0126 (B), SP600125 (C) and PDTC (D) are the inhibitors of p38 MAPK, ERK, JNK and NF-κB, respectively. Values are indicated as relative expression levels to those in the absence of inhibitors. Means ± SDs from triplicated wells are indicated. *P < 0.01 determined by Student's t-test. The middle panels of (A–C) and the right panel of (D): western blot analysis was performed in the presence or absence of each inhibitor. Similar results were obtained in at least two independent experiments for each inhibitor.
HIV-Tg mice when cells were treated with chemically synthesized TLR4 ligand, lipid A compound 506 (Fig. 4D and Supplementary Figure 1C, available at International Immunology Online). Thus, it was clearly shown that observed activation of HIV-1 is caused by LPS itself and not by contaminated materials.

The observation that Myd88 deficiency did not completely abolish the HIV-1 activation suggested a possibility that the
residual expression of HIV-1 in Myd88-deficient macrophages might result from TRIF pathway-dependent NF-κB activation. To verify this possibility, we examined the effect of Dynasore, a chemical inhibitor that inhibits the TRIF-dependent pathway (37). We stimulated macrophages at the concentration of 1 ng ml⁻¹ of LPS, because Dynasore could not inhibit TRIF-dependent genes at higher LPS concentrations. As shown in Fig. 4(E) (left panel) and Supplementary Figure 1D (available at International Immunology Online), Dynasore treatment did not affect LPS-induced HIV-1 activation. Under these conditions, the expression of TRIF-dependent genes such as Cxcl10 and Il6 was greatly suppressed (Fig. 4E, middle panel), but that of Myd88-dependent genes such as Tnf was not affected (Fig. 4E, right panel), indicating that the TRIF pathway was efficiently inhibited by this treatment. We obtained similar results using RNAi techniques. As shown in Fig. 4(F), knockdown of Myd88, but not Trif, suppressed HIV-1 reactivation by LPS. Similar tendency was observed when another siRNA for Myd88 RNAi techniques. As shown in Fig. 4(E) (left panel) and Supplementary Figure 1D (available at International Immunology Online), Dynasore treatment did not affect LPS-induced HIV-1 activation. Under these conditions, the expression of TRIF-dependent genes such as Cxcl10 and Il6 was greatly suppressed (Fig. 4E, middle panel) but that of Myd88-dependent genes such as Tnf was not affected (Fig. 4E, right panel), indicating that the TRIF pathway was efficiently inhibited by this treatment. We obtained similar results using RNAi techniques. As shown in Fig. 4(F), knockdown of Myd88, but not Trif, suppressed HIV-1 reactivation by LPS. Similar tendency was observed when another siRNA for Myd88 and Trif was used (Supplementary Figure 3A, available at International Immunology Online). From these results, we concluded that the MyD88 pathway is primarily important in activating HIV-1 in HIV-Tg macrophages and the TRIF pathway may have a compensatory role under Myd88-deficient conditions.

Discussion

Previously, we showed that the activation of HIV-1 genes in the spleen cells upon stimulation with LPS occurs in a TNF-and IL-1-dependent manner using Tg mice carrying the HIV-1 genome (21). In this report, we investigated the activation mechanisms of HIV-1 in macrophages of the same mice. In contrast to splenic HIV-1 activation (21, 22), HIV-1 activation in macrophages occurred much earlier; the highest stimulation in the spleen was observed at 12 and 48 h after stimulation with LPS while it took only 6 h in macrophages. Furthermore, cytokine production such as Tnf was not required for the activation of HIV-1 in macrophages and HIV-1 activation in macrophages was not inhibited by the treatment with the anti-TNF antibody, making a clear contrast to the case of splenocytes. These observations suggest that the HIV-1 activation mechanism is different between splenocytes and macrophages and that LPS-induced TLR4 signaling, but not LPS-induced cytokines, mainly activates HIV-1 expression in macrophages.

We have demonstrated that MyD88 is important for the activation of latent HIV-1 in macrophages downstream of TLR4. The involvement of MyD88 in HIV-1 activation was also suggested in human microvessel endothelial cells transfected with HIV-LTR followed by luciferase gene (18). The observation that Toll-IL-1R domain-containing adaptor protein (TIRAP) is involved in the HIV-1-LTR activation through the TLR1, TLR2, TLR4 and TLR6 signaling is also consistent with the idea that MyD88 is used as the adaptor molecule, because TIRAP only interacts with MyD88 but not TRIF (38). Although the HIV-1 tat-rev induction was significantly reduced in Myd88⁻/⁻ mice, the induction was not completely abolished. Thus, it was suggested that other pathways such as the TRIF-mediated signaling pathway might compensate for the signaling. However, the inhibition of the TRIF pathway by a specific inhibitor, Dynasore, or siRNAs specific for Trif did not significantly suppress the induction of HIV-1 expression, although TRIF-dependent Cxcl10 and Il6 expression was significantly suppressed under these conditions (Fig. 4E and F). These results may suggest that the TRIF-dependent pathway may be only functional when the Myd88 pathway is unavailable. However, treatment with both Myd88 and TRIF siRNA did not further suppress the HIV-1 and other LPS-inducible gene induction (Supplementary Figure 3B, available at International Immunology Online). It is possible that small amount of TRIF expression that we could not inhibit completely may be sufficient for HIV-1 activation in our experimental conditions.

We showed that MAPKs such as p38 MAPK, ERK and JNK, and NF-κB were activated by the stimulation with LPS in the downstream of Myd88. Inhibition of p38 MAPK, but not ERK or JNK, significantly suppressed HIV-1 activation in macrophages, indicating that p38 MAPK, but neither ERK nor JNK, activation is important for the activation of HIV-1. Consistent with our observation, Equils et al. (18) reported that LPS-induced HIV-1-LTR-luciferase activation in human microvessel endothelial cells is partially suppressed by the p38 MAPK inhibitor SB203580. On the other hand, dominant-negative p38 MAPK did not inhibit LPS-induced activation of HIV-1-LTR-luciferase in HEK293 human embryonic kidney cell line (39), suggesting that the activation mechanisms may be different among different cell types and/or different HIV-1 gene constructs. We have also shown that the activation of NF-κB is necessary for the activation of HIV-1. There are two NF-κB-responsive elements in viral LTR. Pomerantz et al. (17) showed that LPS-dependent activation of HIV-1-LTR-CAT was not observed when the construct with mutations in the NF-κB-binding sites was transfected into THP-1 and U937 monocytic cell lines. Furthermore, a dominant-negative form of IκKB inhibits LPS-induced LTR-luciferase activation in HEK293 cells (39). PDTC also prevents activation of NF-κB by inhibiting IκB phosphorylation and release from NF-κB, the reaction which is induced by IκKB. Our observation that inhibition of NF-κB activation by PDTC leads to almost complete suppression of HIV-1 activation in HIV-Tg macrophages is consistent with these reports. Taken together, our observations suggest that HIV-1 in macrophages is activated mainly through the MyD88–p38 MAPK and MyD88–NF-κB pathways upon stimulation with LPS, and the TRIF pathway is not functional in the presence of MyD88.

Brenchley et al. (11) have shown that plasma LPS levels are elevated in chronically HIV-1-infected patients and SIV-infected macaques. Our results suggest that this elevated LPS would reactivate latently infected HIV-1 in macrophages through the MyD88–p38 MAPK and MyD88–NF-κB pathways. At the same time, LPS may also activate macrophages to produce pro-inflammatory cytokines that reactivate latent HIV-1 in lymphocytes (21). Furthermore, HIV-1 infection induces TLR4 expression in human peripheral lymphocytes (40) and LPS-induced pro-inflammatory cytokine production is enhanced by HIV-1 infection in human monocyte cell lines and primary macrophages (41–43), suggesting the presence of an auto-stimulating loop between HIV-1 infection and LPS activation. Thus, the activation of macrophages by LPS may be critically important for the reactivation of HIV-1 in latent HIV-1 carriers.
These observations suggest that targeted inhibition of the MyD88–p38 MAPK and MyD88–NF-κB pathways would be effective to prevent HIV-1 activation in infected hosts by inhibiting both LPS-induced cytokine production and direct HIV-1 activation in macrophages. Our Tg mice should be useful to further analyze the relationship between HIV-1 activation and immune activation as a small animal model.

**Supplementary data**

Supplementary data are available at *International Immunology* Online.

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**References**

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