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Lipid-associated membrane proteins of *Mycoplasma fermentans* and *M. penetrans* activate human immunodeficiency virus long-terminal repeats through Toll-like receptors

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SUMMARY

Mycoplasmas are known to enhance human immunodeficiency virus (HIV) replication, and mycoplasma-derived lipid extracts have been reported to activate nuclear factor-κB (NF-κB) through Toll-like receptors (TLRs). In this study, we examined the involvement of TLRs in the activation of HIV long-terminal repeats (LTR) by mycoplasma and their active components responsible for the TLR activation. Lipid-associated membrane proteins (LAMPs) from two species of mycoplasma (*Mycoplasma fermentans* and *M. penetrans*) that are associated with acquired immune-deficiency syndrome (AIDS), were found to activate HIV LTRs in a human monocytic cell line, THP-1. NF-κB deletion from the LTR resulted in inhibition of the activation. The LTR activation by *M. fermentans* LAMPs was inhibited by a dominant negative (DN) construct of TLR1 and TLR6, whereas HIV LTR activation by *M. penetrans* LAMPs was inhibited by DN TLR1, but not by DN TLR6. These results indicate that the activation of HIV LTRs by *M. fermentans* and *M. penetrans* LAMPs is dependent on NF-κB, and that the activation of HIV LTR by *M. fermentans* LAMPs is mediated through TLR1, TLR2 and TLR6. In contrast, the LTR activation by *M. penetrans* LAMPs is carried out through TLR1 and TLR2, but not TLR6. Subsequently, the active component of *M. penetrans* and *M. fermentans* LAMPs was purified by reverse-phase high-performance liquid chromatography (HPLC). Interestingly, the purified lipoprotein of *M. penetrans* LAMPs (LPMp) was able to activate NF-κB through TLR1 and TLR2. On the other hand, the activation of NF-κB by purified lipoprotein of *M. fermentans* LAMPs (LPMf) was mediated through TLR2 and TLR6, but not TLR1.

Keywords HIV; lipoprotein; mycoplasma; Toll-like receptor

INTRODUCTION

Human immunodeficiency virus (HIV) is recognized as the aetiological agent of acquired immune-deficiency syndrome (AIDS). However, the progression of AIDS is highly variable in different individuals, and several factors, such as viral strains or host factors, have been attributed as the possible cause of such variations. Infectious agents, including various viruses, parasites and bacteria, are considered to be cofactors in the progression of AIDS.[1] Mycoplasmas are wall-less parasitic Gram-positive bacteria, and the smallest organisms capable of self-replication.[2] *Mycoplasma fermentans* and *M. penetrans* have been isolated from the tissues and urine of patients with AIDS[3–5] and were shown to enhance the cytopathic effect of HIV-1 infection.[6,7] In addition, mycoplasmas and acholeplasmas have been reported to enhance HIV-1 replication *in vitro*.[8,9] Thus, mycoplasmas might be reasonable cofactor candidates in the progression of AIDS.

A nuclear transcription factor – nuclear factor-κB (NF-κB) – is thought to play a major role in the regulation of HIV-1 gene expression.[10] Although the HIV long-terminal repeat (LTR) alone can serve as its own promoter, early mRNA transcription appears to rely primarily on the

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binding of cellular transcription factors, including NF-κB, to the LTR.[11] The activation of cytoplasmic NF-κB by various cytokines, including interleukin (IL)-2, IL-6 and tumour necrosis factor-α (TNF-α), or after infection with other viruses, induces HIV replication.[12–17] These findings indicate increased rates of HIV replication, probably through NF-κB-mediated regulation of the HIV LTR. TNF-α induced by mycoplasma had been thought to induce NF-κB and HIV replication; however, anti-TNF-α immunoglobulin failed to inhibit the enhancement of HIV replication by mycoplasma.[18] Although activation of NF-κB has been implicated in the mycoplasma-induced enhancement of HIV replication, the receptor(s) and the pathways of signal transduction via NF-κB have not been clearly defined.

Recently, it has been reported that Toll-like receptors (TLRs) are pattern-recognition receptors in the innate immune system and play important roles in early innate recognition and inflammatory responses by the host to microbial challenges.[19] Among nine TLR family members reported, TLR2, 4, 5 and 9 have been implicated in the recognition of different bacterial components. Pepidoglycan, lipoarabinomannan, zymosan and lipoproteins from various micro-organisms are recognized by TLR2.[20–28] On the other hand, lipopolysaccharide (LPS), bacterial flagellin and bacterial DNA are recognized by TLR4, TLR5 and TLR9, respectively.[29–33] These TLR family members have been shown to activate NF-κB via IL-1R-associated signal molecules, including myeloid differentiation protein (MyD88), IL-1R-activated kinase (IRAK), TNFR-associated factor 6 (TRAF6), and NF-κB-inducing kinase (NIK).[34] However, the precise mechanisms by which mycoplasma activate HIV LTR have not been fully clarified.

In this study, we examined the involvement of TLRs in the activation of HIV LTRs by mycoplasmas and their active components responsible for the TLR activation. We observed that lipid-associated membrane proteins (LAMPs) from the AIDS-associated mycoplasmas, *M. penetrans* and *M. fermentans*, activated the HIV LTR in a human monocytic cell line (THP-1) through NF-κB. Activation of the HIV LTR by LAMPs from *M. fermentans* (referred to as *M. fermentans* LAMPs hereafter) was apparently dependent on TLR1, TLR2 and TLR6. In contrast, activation of the HIV LTR by LAMPs from *M. penetrans* (referred to as *M. penetrans* LAMPs hereafter) was dependent on TLR1 and TLR2, but not on TLR6. Furthermore, the active components of *M. penetrans* and *M. fermentans* LAMPs were purified by reverse-phase HPLC. The activity of purified lipoprotein from *M. penetrans* LAMPs (LPMp) to induce NF-κB was dependent on TLR1 and TLR2. On the other hand, the activity of purified lipoprotein from *M. fermentans* LAMPs (LPMf) was dependent on TLR2 and TLR6, but not on TLR1.

MATERIALS AND METHODS

Cells

Cells of a human monocytic cell line, THP-1, were cultured in RPMI-1640 containing 10% fetal calf serum (FCS; Mitsubishi Chemical, Tokyo, Japan), 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin. Cells of a human kidney cell line, 293T, were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin G and 100 μg/ml streptomycin.

Antibodies

The mouse anti-human TLR2 monoclonal antibodies (mAbs) ABM-8320 and IMG-416 were obtained from Cascade Bioscience (Winchester, MA) and Imgenex (San Diego, CA).[35] Normal mouse immunoglobulin G (IgG)2a was purchased from PharMingen (San Diego, CA).

Pathogen-associated molecular patterns (PAMPs)

(S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH.3HCl (Pam3CSK4) was purchased from Calbiochem (Darmstadt, Germany). *M. fermentans* macrophage-activating lipopeptide 2 (MALP-2) was kindly provided by Dr M. Matsumoto (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan).[36,37]

Preparation of LAMPs from M. fermentans *and* M. penetrans

M. fermentans and *M. penetrans* were cultured in PPLO medium and SP-4 medium, respectively, to the start of stationary phase, and then pelleted by centrifugation for 10 min at 12 000 ***g***. Preparation of LAMPs was performed as described previously by Feng *et al.*[38,39] Briefly, a mycoplasma pellet was suspended in Tris-buffered saline (TBS) (50 mM Tris, 0·15 M NaCl, pH 8·0) containing 1 mM EDTA (TBSE), solubilized by adding TX-114 to a final concentration of 2% and incubated at 4° for 1 hr. The lysate was incubated at 37° for 10 min prior to phase separation. After centrifugation at 10 000 ***g*** for 20 min, the upper aqueous phase was removed and replaced with the same volume of TBSE. The procedure of phase separation was repeated twice. The final TX-114 phase was resuspended in TBSE to the original volume, 2·5 volumes of ethanol were added to precipitate membrane components and the phase was incubated at −20° overnight. After centrifugation, the pellet was suspended in phosphate-buffered saline (PBS) followed by sonication for 30 seconds at output 5 (Sonifier cell disruptor 200; Branson, Danbury, CT). The protein concentration of the suspension was measured by using the Coomassie Protein Assay Regent (Pierce, Rockford, IL).

Expression vectors

To prepare TLR1, TLR2 and TLR6 expression vectors (pFLAG-TLR1, pFLAG-TLR2, and pFLAG-TLR6, respectively), the coding regions of TLR1, TLR2 and TLR6, minus the respective N-terminal signal sequences, were amplified by polymerase chain reaction (PCR) from a cDNA of THP-1 and cloned into the expression vector pFLAG-CMV1 (Sigma, St Louis, MO), in which a preprotrypsin leader precedes an N-terminal FLAG epitope. Dominant negative (DN) TLR1 and TLR6 expression vectors were constructed by subcloning TIR (Toll and interleukin 1 receptor) homology domain-deleted TLR1 and TLR6 fragments into pFLAG-CMV1 (pFLAG-

dTLR1 and pFLAG-dTLR6). pHIV-LTR-luc, a mutant lacking the NF-κB-binding site (pHIV-LTRΔκB-luc), a mutant lacking the SP-1-binding site (pHIV-LTRΔSP1-luc), and a mutant lacking both NF-κB- and SP-1-binding sites (pHIV-LTRΔκBSP1-luc) were gifts from Dr Y. Koyanagi (Tohoku University Graduate School of Medicine, Sendai, Japan).[40] The NF-κB Cis-Reporting System, containing pNF-kB-luc, a plasmid in which the luciferase reporter gene is fused to the NF-κB enhancer, was purchased from Stratagene (La Jolla, CA).

Transfection and luciferase assay

Transient transfection was performed by using FuGENE6 (Roche, Basel, Switzerland), according to the manufacturer's instructions. A total of 4×10^5 THP-1 cells, or 1×10^5 293 T cells, were transfected with 0·1 μg of pFLAG-TLR2, 0·01 μg of pHIV-LTR-luc, 0·01 μg of the pRL-TK internal control plasmid (Promega, Madison, WI), and DN TLRs expressing plasmid, in 24-well plates. After 20 hr, transfected cells were stimulated with 1·0 μg/ml *M. fermentans* LAMPs or 0·5 μg/ml *M. penetrans* LAMPs. After a further 24 hr of incubation, cells were lysed and assayed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega). Both firefly and Renilla luciferase activity were monitored using a Lumat LB9507 luminometer (Berthold, Wildbad, Germany). Normalized reporter activity is expressed as the firefly luciferase value divided by the Renilla luciferase value. Relative fold induction is calculated as the normalized reporter activity of the test samples divided by the unstimulated samples.

Reverse-phase high-performance liquid chromatography (HPLC)

LAMPs were dissolved in 6 M guanidine hydrochloride, and 100 μg of LAMPs were applied on μBondasphere C18 300A (Waters, Milford, MA). Elution was carried out using a 0–100% linear water/2-propanol gradient. The flow rate was 1·0 ml/min. Each fraction was dried *in vacuo* at room temperature and dissolved in 25 mM n-octyl-β-gulucopyranoside. Protein concentration was measured by using the Coomassie Protein Assay Regent (Pierce).

Lipoprotein lipase treatment

Approxmately 100 ng/ml LPMf and LPMp, separated from *M. fermentans* and *M. penetrans* LAMPs, respectively, were treated with 100 μg/ml lipoprotein lipase (Sigma) at 37° for 2 hr. 293T cells transfected with 0·02 μg/ml pNF-kB-luc and 0·2 μg/ml pFLAG-TLR2 were stimulated with 10 ng/ml of the lipoprotein lipase-treated LPMf and LPMp. Luciferase activity was measured as described above.

RESULTS

Activation of HIV LTR by LAMPs

LAMPs of *M. penetrans* have been reported to stimulate macrophages.[38,39] We therefore initially examined whether LAMPs from *M. fermentans* and *M. penetrans* can enhance HIV replication in macrophages. To determine the enhancement of HIV replication, THP-1 cells were first transfected with a plasmid in which the luciferase reporter gene was fused to HIV LTR (pHIV-LTR-luc) and then were stimulated with *M. fermentans* and *M. penetrans* LAMPs. The level of luciferase expression was enhanced by *M. fermentans* or *M. penetrans* LAMPs in a dose-dependent manner (Fig. 1a). When 0·5 μg/ml *M. penetrans* LAMPs was added, the luciferase expression was maximal and ≈12-fold higher than that of the unstimulated control. In contrast, the expression of THP-1 cells was maximal when stimulated with 1·0 μg/ml *M. fermentans* LAMPs,

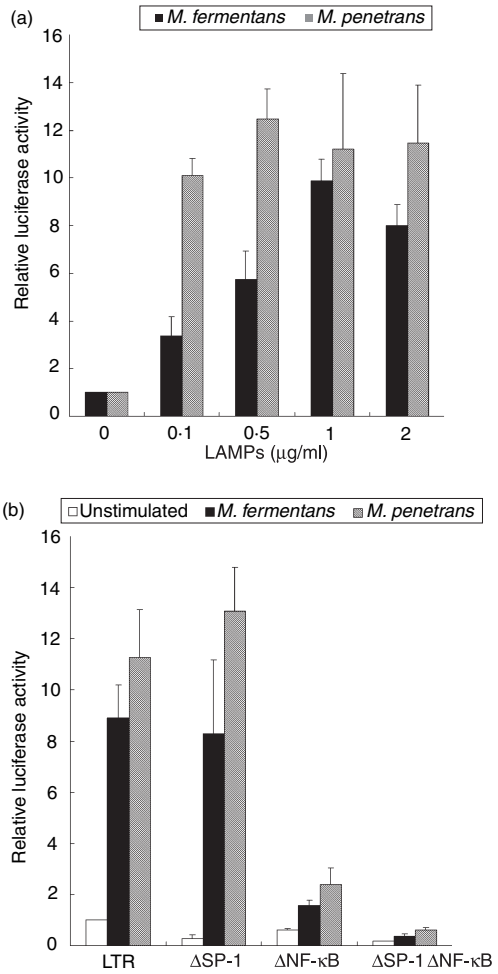

Figure 1. Enhancement of long-terminal repeat (LTR) activation by lipid-associated membrane proteins (LAMPs) through nuclear factor-kappa B (NF-κB). (a) THP-1 cells were transfected with 0·1 μg/ml pHIV-LTR-luc and 0·01 μg/ml pRL-TK. The cells were stimulated with the indicated concentrations of LAMPs. All values represent the mean and standard deviation (SD) of three assays. (b) THP-1 cells were transfected with 0·1 μg/ml pHIV-LTR-luc (LTR), pHIV-LTRΔSP1-luc (ΔSP-1), pHIV-LTRΔκB-luc (ΔNFκB) or pHIV-LTRΔκBSP1-luc (ΔSP-1ΔNF-κB) in combination with 0·01 μg/ml pRL-TK. The cells were stimulated with 0·5 μg/ml *Mycoplasma penetrans* LAMPs and 1·0 μg/ml *M. fermentans* LAMPs. All values represent the mean and SD of three assays.

being ≈10-fold higher compared with the control. These results indicate that LAMPs from *M. fermentans* and *M. penetrans* can enhance HIV replication.

Activation of HIV LTR through NF-κB

HIV LTR contains various binding sites of cellular transcription factors, including NF-κB, SP-1, AP2, TCF-1, USF-1 and Ets; in particular, NF-κB and SP-1 are thought to be major transcription factors.[41] To examine the roles of NF-κB and SP-1 in the activation of HIV LTR, pHIV-LTRΔκB-luc and pHIV-LTRΔSP1-luc (in which the NF-κB- and SP-1-binding sites, respectively, have been deleted from pHIV-LTR-luc) were prepared (Fig. 1b). When pHIV-LTRΔκB-luc-transfected THP-1 cells were stimulated with *M. fermentans* and *M. penetrans* LAMPs, the level of luciferase expression was lower than that of the control. In contrast, the level of luciferase expression was almost constant when pHIV-LTRΔSP1-luc was transfected. Moreover, the deletion of both NF-κB- and SP-1-binding sites (pHIV-LTRΔκBSP1-luc) resulted in a decrease of the expression level down to the level of the unstimulated control. These results indicate that NF-κB may be a major transcription factor induced by LAMPs.

Inhibition of LTR activation by anti-TLR2 mAb

It was reported that a lipopeptide of *M. fermentans* – MALP-2 – can activate NF-κB through TLR2.[28] We therefore examined whether the enhancement of HIV LTR activation with LAMPs is mediated through TLR2. Anti-human TLR2 mAb (ABM-8320)-pretreated THP-1 cells were transfected with pHIV-LTR-luc, followed by stimulation with *M. fermentans* and *M. penetrans* LAMPs. Pretreatment with anti-TLR2 mAb decreased the expression level of luciferase, and control antibody (mouse IgG2a) had no effect on luciferase (Fig. 2a). These results indicate that the activation of HIV LTR by LAMPs is TLR2-mediated.

Activation of HIV LTR through TLR2

To confirm whether the activation of NF-κB by LAMPs is mediated through TLR2, we constructed a TLR2 expression vector (pFLAG-TLR2). 293T cells were transfected with both pFLAG-TLR2 and pHIV-LTRΔSP1-luc. In this experiment, we used pHIV-LTRΔSP1-luc instead of pHIV-LTR-luc, because the binding site for SP-1 on the LTR resulted in a high level of luciferase activity (data not shown). When 293T cells were transfected with a high dose of pFLAG-TLR2 and then stimulated with *M. fermentans* and *M. penetrans* LAMPs, the levels of luciferase expression were augmented in a dose-dependent manner (Fig. 2b). In contrast, the level of luciferase expression was the same as that of the unstimulated control when 293T cells were transfected with the empty vector pFLAG-CMV1. This suggests that *M. fermentans* and *M. penetrans* LAMPs activate HIV LTR through TLR2.

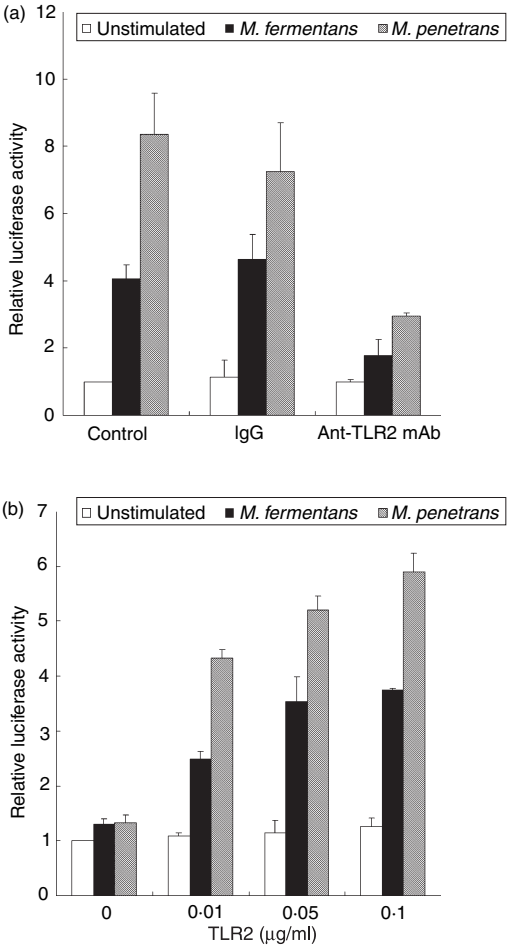

Figure 2. Enhancement of long-terminal repeat (LTR) activation through Toll-like receptor 2 (TLR2). (a) THP-1 cells were transfected with 0·1 μg/ml pHIV-LTR-luc and 0·01 μg/ml pRL-TK. The cells were treated with anti-TLR2 monoclonal antibody (mAb) followed by stimulation with 0·5 μg/ml *Mycoplasma penetrans* lipid-associated membrane proteins (LAMPs) and 1·0 μg/ml *M. fermentans* LAMPs. All values represent the mean and standard deviation (SD) of three assays. (b) 293T cells were transfected with the indicated concentrations of pFLAG-TLR2, 0·01 μg/ml pHIV-LTRΔSP1-luc, and 0·01 μg/ml pRL-TK. The cells were stimulated with 0·5 μg/ml *M. penetrans* LAMPs and 1·0 μg/ml *M. fermentans* LAMPs. All values represent the mean and SD of three assays.

Co-operation of TLR6 and TLR2 for LTR activation

Mouse TLR6 has been reported to recognize diacylated lipopeptides, such as MALP-2, co-operatively with TLR2.[42] To investigate whether *M. fermentans* and *M. penetrans* LAMPs are also recognized by both TLR2 and TLR6 for the activation of HIV LTR, we constructed a plasmid encoding DN TLR6 (pFLAG-dTLR6). 293T cells were transfected with pFLAG-TLR2, pHIV-LTRΔSP1-luc and various concentrations of pFLAG-dTLR6. Initially, the effect of DN TLR6 on the expression of TLR2 was analysed by flow cytometry. The level of TLR2 expression was

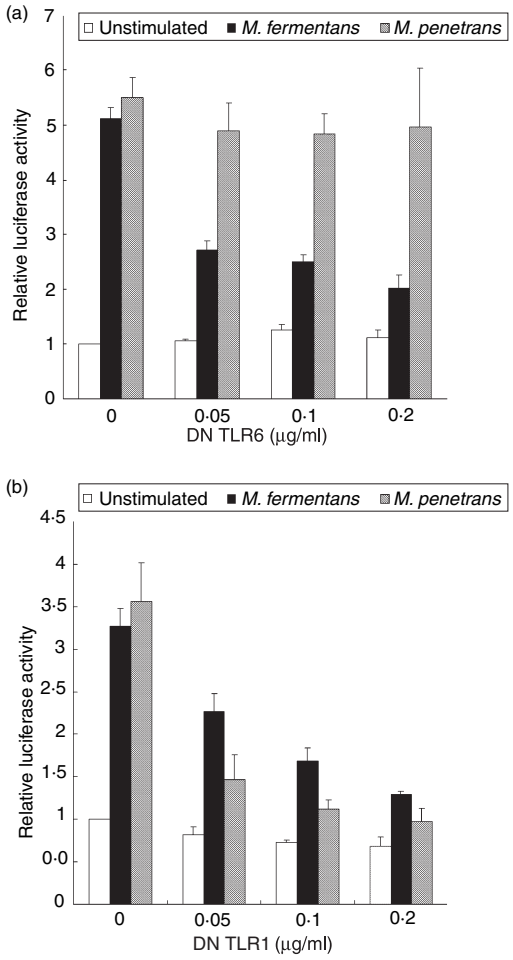

Figure 3. Cooperation of Toll-like receptor (TLR)1, TLR6 and TLR2 for long-terminal repeat (LTR) activation by lipid-associated membrane proteins (LAMPs). 293T cells were transfected with the indicated concentrations of pFLAG-dTLR6 (a) or pFLAG-dTLR1 (b), 0·1 μg/ml pFLAG-TLR2, 0·01 μg/ml pHIV-LTRΔSP1-luc and 0·01 μg/ml pRL-TK. The cells were stimulated with 0·5 μg/ml *Mycoplasma penetrans* LAMPs or 1·0 μg/ml *M. fermentans* LAMPs. All values represent the mean and standard deviation (SD) of three assays. DN, dominant negative.

almost constant, irrespective of the expression of DN TLR6 or of DN TLR1 (data not shown). When the transfected cells were stimulated with *M. fermentans* LAMPs, the level of luciferase expression decreased in a dose-dependent manner (Fig. 3a). Upon transfection with 0·2 μg/ml pFLAG-dTLR6, the expression level decreased to a level similar to that of the unstimulated control. In contrast, the level of luciferase expression was almost constant when the transfected 293T cells were stimulated with *M. penetrans* LAMPs. These results suggest that the LTR activation by *M. fermentans* LAMPs is dependent on TLR2 and TLR6, but the activation by *M. penetrans* LAMPs is not dependent on TLR6. To further examine whether TLR6 alone can mediate the activation of LTR by LAMPs, 293T cells were transfected with a TLR6 expression vector (pFLAG-TLR6). Although the transfected cells were stimulated with *M. fermentans* and *M. penetrans* LAMPs, the level of luciferase expression was not augmented (data not shown). These results indicate that both TLR2 and TLR6 co-operatively mediate the LTR activation by *M. fermentans* LAMPs, but not by *M. penetrans* LAMPs.

Co-operation of TLR1 and TLR2 for LTR activation

Triacylated bacterial lipopeptides, such as Pam3CSK4, were reported to be recognized by murine TLR1, in association with TLR2.[43] The above results (Fig. 3a) suggest that *M. penetrans* LAMPs might contain different active components from *M. fermentans* LAMPs and, like the triacylated lipopeptides, *M. penetrans* LAMPs might be recognized by TLR1 and TLR2. We therefore next determined whether *M. fermentans* and *M. penetrans* LAMPs are recognized by both TLR1 and TLR2 for the activation of HIV LTR. To achieve this, we transfected a plasmid encoding DN TLR1 (pFLAG-dTLR1) into 293T cells containing both pFLAG-TLR2 and pHIV-LTRΔSP1-luc. When the transfected cells were stimulated with *M. penetrans* LAMPs, the level of luciferase expression was decreased in a dose-dependent manner (Fig. 3b). Unexpectedly, the level of luciferase expression of the cells stimulated with *M. fermentans* LAMPs was also decreased. Upon transfection with 0·2 μg/ml pFLAG-dTLR1, the level of expression in both cells decreased down to almost control levels. These results suggest that the LTR activation by *M. fermentans* and *M. penetrans* LAMPs is dependent on both TLR1 and TLR2. To further examine whether TLR1 alone can mediate the activation of LTR by LAMPs, 293T cells were transfected with a TLR1 expression vector (pFLAG-TLR1). Like the TLR6 expression in 293T cells, as mentioned previously, the level of luciferase expression of the transfected cells was not augmented by stimulation with *M. fermentans* and *M. penetrans* LAMPs (data not shown). These results indicate that the co-operation of TLR1 and TLR2 is required for the LTR activation with *M. fermentans* and *M. penetrans* LAMPs.

Purification of active components of LAMPs

To purify the active components of LAMPs, *M. penetrans* and *M. fermentans* LAMPs were fractionated using reverse-phase HPLC with a linear gradient of isopropanol. To measure the activity of fractions to induce NF-κB, each fraction was added to 293T cells transfected with pFLAG-TLR2 and pNF-κB-luc. As shown in Fig. 4(a), the active component of *M. penetrans* LAMPs (LPMp) was eluted by ≈97% isopropanol, whereas the active component of *M. fermentans* LAMPs (LPMf) was eluted by ≈80% isopropanol (Fig. 4b).

Analysis of active components of LAMPs

We next examined whether LPMp and LPMf separated from LAMPs are recognized by TLR2 and TLR6, or TLR1 and TLR2. 293T cells transfected with pFLAG-TLR2,

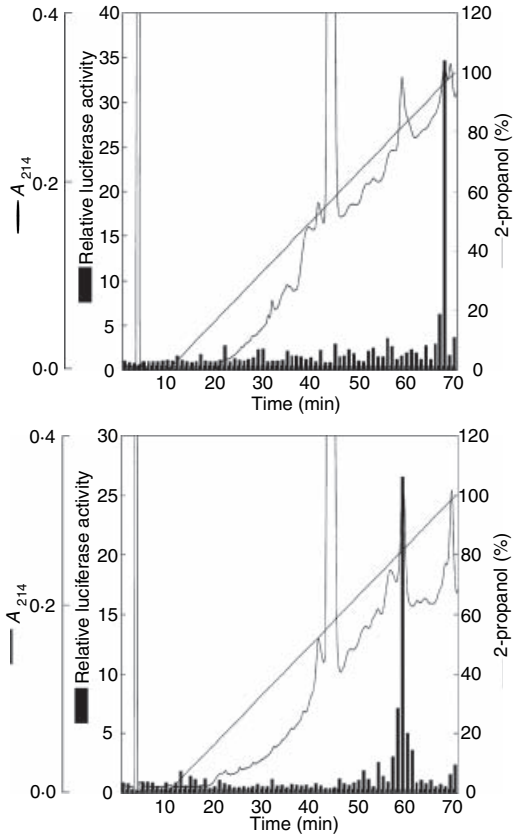

Figure 4. Isolation of active components of lipid-associated membrane proteins (LAMPs). *Mycoplasma penetrans* (a) and *M. fermentans* (b) LAMPS were dissolved in 6 M guanidine hydrochloride, and 100 μg of LAMPs was separated by reverse-phase high-performance liquid chromatography (HPLC). Elution was performed using a 0–100% linear gradient of water/2-propanol. Each fraction was added to THP-1 cells transfected with 0·1 μg/ml pFLAG-TLR2 and 0·1 μg/ml pNF-κB-luc. These results are representative examples of the three independent experiments.

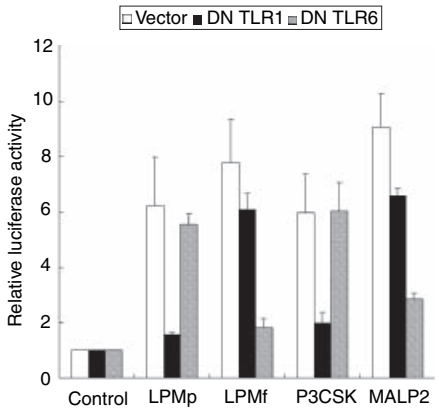

Figure 5. Toll-like receptor (TLR) usage of *Mycoplasma penetrans* lipid-associated membrane proteins (LPMp) and *M. fermentans* lipid-associated membrane proteins (LPMf). 293T cells transfected with 0·01 μg/ml pFLAG-TLR2, 0·01 μg/ml pNF-κB-luc, and 0·2 μg/ml pFLAG-dTLR1 or pFLAG-dTLR6, were stimulated with 10 ng/ml LPMp, 10 ng/ml LPMf, 1 nM MALP-2, and 1 μg of Pam3CSK4 (P3CSK). All values represent the mean and standard deviation (SD) of three assays. DN, dominant negative.

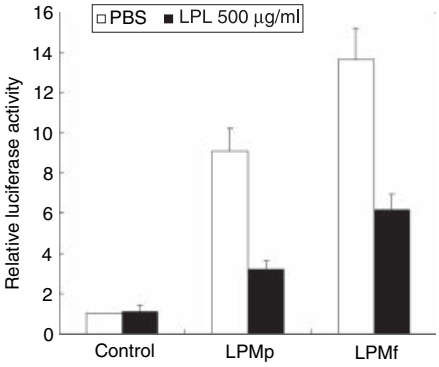

Figure 6. Lipoprotein lipase (LPL) treatment of *Mycoplasma penetrans* lipid-associated membrane proteins (LPMp) and *M. fermentans* lipid-associated membrane proteins (LPMf). One microgram of LPMp and LPMf was treated with 100 μg/ml LPL at 37° for 2 hr. 293T cells transfected with 0·01 μg/ml pNF-κB-luc and 0·01 μg/ml pFLAG-TLR2 were stimulated with 10 ng/ml LPL-treated LPMp and LPMf. All values represent the mean and standard deviation (SD) of three assays. PBS, phosphate-buffered saline.

pNF-κB-luc and pFLAG-dTLR1 or pFLAG-dTLR6 were stimulated with LPMp, LPMf, Pam3CSK4 or MALP-2. As reported previously by Takeuchi *et al.*,[42,43] both DN TLR1 and DN TLR6 suppressed the activity of Pam3CSK4 and MALP-2, respectively, to induce NF-κB (Fig. 5). When the cells were stimulated with LPMp, the relative luciferase activity was reduced by the expression of DN TLR1 (Fig. 6), which is consistent with the results obtained using *M. penetrans* LAMPs (Fig. 3b). In contrast, the 293T cells stimulated with LPMf showed a relatively suppressed level of the luciferase activity when DN TLR6, but not DN TLR1, was expressed, inconsistent with the results that *M. fermentans* LAMPs was recognized by TLR1, TLR2 and TLR6 (Figs 3a and 5).

To analyse the chemical components of LPMf and LPMp, they were treated with lipoprotein lipase and proteinase K. Treatment with proteinase K failed to decrease the activity of LPMf and LPMp (data not shown), while lipoprotein lipase treatment decreased the ability to induce NF-κB (Fig. 6). These results suggest that lipid moiety, but not protein moiety, is required for activity.

DISCUSSION

In this study, we demonstrated that LAMPs from *M. fermentans* and *M. penetrans* activated the HIV LTR through NF-κB. Activation of the LTR by *M. fermentans* LAMPs was TLR1-, TLR2- and TLR6 dependent, while the activation by *M. penetrans* LAMPs was TLR1- and TLR2 dependent. The active components of *M. fermentans* and

M. penetrans LAMPs were purified by reverse-phase HPLC. The purified lipoprotein from *M. penetrans* LAMPs (LPMp) was recognized by TLR1 and TLR2. Although the recognition of *M. fermentans* LAMPs involved TLR1, TLR2 and TLR6 (Fig. 3), the purified lipoprotein from *M. fermentans* LAMPs (LPMf) was recognized only by TLR2 and TLR6. These results indicate that *M. fermentans* LAMPs may contain several active components, in which one component is recognized by TLR2 and TLR6, and other components might be recognized by TLR1 and TLR2. Both LPMf and LPMp showed resistance to proteinase K treatment, in agreement with the previous report by Feng *et al.*[39] In addition, the ability of LPMf and LPMp to induce NF-κB was reduced by treatment with lipoprotein lipase. These results suggest that the active components are attributable to lipid moieties. MALP-2 constitutes a lipopeptide, isolated from *M. fermentans*, which has been well documented as an activator of NF-κB.[44,45] Subsequently, a 44-kDa membrane-bound lipoprotein of *M. salivarium* has been reported to induce TNF-α production in THP-1.[46] To our knowledge, there still seems to be little information on the activity in innate immune responses of lipoproteins derived from mycoplasmas such as *M. fermentans*, *M. penetrans* and *M. salivarium*, although a variety of mycoplasma strains have been shown to exhibit diverse bioactivities in interaction with eukaryotic cells.[47, 48]

In TLR-deficient mice, bacterial lipopeptides containing three acyl chains were reported to be recognized by TLR1 and TLR2,[43] whereas MALP-2, containing two acyl chains, were recognized by TLR2 and TLR6.[42] These findings, and our results, suggest that LPMp might be similar to lipoprotein(s) containing three acyl chains. In contrast, LPMf separated from *M. fermentans* in this study may possess components comparable to MALP-2, as MALP-2 is a lipopeptide derived from *M. fermentans*.

In mycoplasmas, acylated proteins are abundant cell-surface antigens, and many putative lipoprotein-encoding genes have been identified in the sequenced mycoplasma genomes.[49,50] It is, at present, controversial as to whether or not mycoplasmas have triacylated lipoprotein. Chemically identified lipoproteins from *M. fermentans*,[44] *M. hyorhinis*,[51] *M. salivarium*[46] and *M. gallisepticum*[52] are not N-acylated, nor has an *N*-acyltransferase gene been found in *M. pneumoniae*,[53] *M. genitalium*[54] or *M. penetrans*[55] genomes. To date, the presence of proteins with *N*-acyltransferase activity has not been clearly established. However, the study on the ratio of *N*-amide and *O*-ester bonds in *M. gallisepticum* and *M. mycoides* may indicate the presence of diacylated and triacylated lipoproteins.[56] The resistance to Edoman degradation of proteins from *M. mycoides* also indicates the presence of N-acylation.[50] In this study, we found that the lipoprotein separated from *M. penetrans* induced NF-κB through TLR1 and TLR2. Triacylated lipoproteins, such as Pam3-CSK4, have been reported to be recognized by TLR1 and TLR2,[43] whereas diacylated lipoproteins, such as MALP-2, have been shown to be recognized by TLR2 and TLR6.[42] Interestingly, synthetically triacylated MALP-2, N-palamitoyl-MALP-2, was not recognized by TLR6.[57] These findings may indicate the existence of triacylated lipoproteins in mycoplasma species.

Our results indicate that the lipoproteins from *M. fermentans* and *M. penetrans* can activate NF-κB in HIV LTR, leading to the enhancement of HIV replication. The activation of NF-κB was also observed following stimulation with bacterial components, including LPS[30] and peptidoglycan.[24] We have previously reported that glycolipids from *Acholeplasma laidlawii*, binding to both HIV and macrophages, enhance HIV replication.[58,59] In addition to the ability of lipoproteins to induce NF-κB, glycolipids from mycoplasma might, in concert, enhance the replication of HIV. We assume that lipoproteins and glycolipids residing in the mycoplasma membrane can efficiently attach to the surface of HIV-infected cells, as mycoplasmas are completely wall-less bacteria.[2] Moreover, mycoplasmas contain various surface proteins that tend to show high-frequency variation, suggesting that mycoplasmas can escape from immune surveillance and establish a persistent infection.[60] These findings suggest that mycoplasma, rather than bacteria with cell walls, might play an important role in the progression of HIV infection.

We previously reported that a variety of mycoplasma strains can induce TNF-α production in mouse macrophages[61] and THP-1 cells.[62,63] *M. penetrans* LAMPs have been reported to induce TNF-α production in mouse thioglycolate exudate peritoneal macrophage cells.[39] Moreover, TNF-α has been shown to activate HIV LTR through NF-κB.[64] These findings suggest that TNF-α produced by macrophages may activate NF-κB. However, we observed that anti-TNF-α mAb failed to inhibit the activation of LTR by LAMPs (data not shown). It is therefore unlikely that TNF-α produced by LAMPs-stimulated THP-1 cells may directly contribute to the activation of NF-κB.

In summary, we demonstrated that lipoproteins from the AIDS-associated mycoplasmas, *M. fermentans* and *M. penetrans*, can enhance HIV LTR activity in THP-1 cells through NF-κB, and that this enhancement is dependent on TLRs. The enhancement of the HIV LTR activation induced by *M. fermentans* LAMPs was dependent on TLR1, TLR2 and TLR6. Interestingly, LPMf separated from *M. fermentans* LAMPs activated NF-κB through TLR2 and TLR6, but not TLR1. In contrast, the enhancement of the NF-κB activation induced by *M. penetrans* LAMPs, as well as LPMp separated from *M. penetrans* LAMPs, was dependent on TLR1 and TLR2, but not TLR6. Clarifying the mechanisms by which various bacteria, including mycoplasma, enhance HIV replication may have therapeutic values in preventing the progression of AIDS during opportunistic infection.

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