Suppression of human immunodeficiency virus type 1 replication in macrophages by commensal bacteria preferentially stimulating Toll-like receptor 4

Nursarat Ahmed, Takaya Hayashi, Atsuhiko Hasegawa, Hiroyuki Furukawa, Noboru Okamura, Toshio Chida, Takao Masuda and Mari Kannagi

1Department of Immunotherapeutics, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan
2Laboratory of Microbiology and Immunology, Graduate School of Health Care Sciences, Tokyo Medical and Dental University, Tokyo, Japan

Protection from primary human immunodeficiency virus type 1 (HIV-1) infection has not yet been accomplished by vaccines inducing HIV-1-specific acquired immunity. Nevertheless, it has been reported that a small subgroup of women remain resistant to HIV-1 infection under natural conditions. If similar conditions can be induced in uninfected individuals, it will contribute the first line of protection against HIV-1 infection, and also improve the effects of anti-HIV-1 vaccines. We reasoned that innate immunity may be involved in the resistance to HIV-1 infection, and investigated the effects of various Toll-like receptor (TLR) ligands and commensal bacteria on HIV-1 replication in macrophages, one of the initial targets of HIV-1 infection and also the main mediators of innate immunity. We established the HIV-1 reporter monocytic cell line, THP-1/NL4-3Luc, which could be differentiated into macrophage-like cells in vitro. In these cells, stimulation of TLR3 and TLR4 by their ligands suppressed HIV-1 expression partly through type I interferon (IFN). Among the commensal bacteria tested, Escherichia coli, Veillonella parvula and Neisseria mucosa suppressed HIV-1 expression, whereas Lactobacillus acidophilus, Prevotella melaninogena, P. bivia and Mycobacterium smegmatis enhanced it. The bacteria with suppressive effects preferentially stimulated TLR4, whereas the ones with enhancing effects stimulated TLR2. Neutralizing antibodies against TLR4 and IFN-α/β receptor abrogated bacterially mediated HIV-1 suppression. Suppressive effects of E. coli, V. parvula and N. mucosa on HIV-1 replication were reproducible in primary monocyte-derived macrophages following acute HIV-1 infection. These findings suggest that certain commensal bacteria preferentially stimulating TLR4 potentially produce local environments resistant to HIV-1 infection.

INTRODUCTION

More than 60 million people worldwide have been infected with human immunodeficiency virus type 1 (HIV-1) and nearly half of these individuals have died in the last 25 years, since HIV-1 was identified as the causative agent of AIDS (Barouch, 2008). The development of a safe and effective HIV-1 vaccine is the best solution for the ultimate control of the worldwide AIDS pandemic. It is believed that induction of acquired immune responses, especially T-cell responses, is required for a successful vaccine. However, a recent HIV-1 vaccine candidate formulated as a trivalent mixture of rAd5 vectors expressing HIV-1 clade B Gag, Pol and Nef failed to protect against HIV-1 infection or to reduce viral loads after HIV-1 infection, despite its strong ability to induce T-cell responses (Barouch, 2008; Ravanfar et al., 2009; Sekaly, 2008). It is unclear whether this simply represents the failure of the vaccine product or the overall T-cell-based vaccine concept.

Although HIV-1 vaccine-development efforts have not yet proven successful, several reports have indicated that a small subgroup of female sex workers in Nairobi (Kenya) have remained uninfected for periods of up to 15 years (Fowke et al., 1996; Plummer et al., 1999). In these individuals, HIV-1-specific IgG and HIV-1 RNA were undetectable in the plasma; however, HIV-1-specific T cells and IgA were sometimes detected in these individuals, especially in the vaginal wash (Kaul et al., 1999, 2000). This suggests that HIV-1 infection might have occurred at a primary site, but failed to establish systemic persistent infection because of limited HIV-1 replication. Further studies revealed that some of these individuals seroconverted...
years later. This indicates that the resistance to HIV-1 infection observed earlier was transient and not genetic in nature (Kaul et al., 2001).

It is ironic that protection from HIV-1 infection can be achieved under certain natural circumstances, but not by sophisticated vaccines. This implies that some mechanisms other than acquired immunity may be primarily involved in producing an environment resistant to HIV-1 infection or limiting its replication; we supposed that innate immunity might be involved. If similar conditions can be induced in uninfected individuals, it would markedly improve the efficiency of protection against HIV-1 infection in conjunction with T-cell-directed HIV-1 vaccines.

Toll-like receptors (TLRs) play a key role in the activation of innate immunity to produce interferons (IFNs) and pro-inflammatory cytokines (Takeda & Akira, 2004). To date, 11 TLRs have been identified, and they recognize various components of microenvironments through pathogen-associated molecular patterns. For example, TLR2 recognizes lipoteichoic acid (LTA), TLR3 recognizes dsRNA, TLR4 recognizes lipopolysaccharide (LPS), TLR5 recognizes flagellin, TLR7/8 recognizes ssRNA and TLR9 recognizes CpG DNA. A recent report indicated that the patterns of cytokine responses against various TLR ligands in the HIV-1-exposed seronegative individuals partly differed from those of controls (Biasin et al., 2010). Some TLR ligands have been shown to suppress HIV-1 replication. HIV-1 ssRNA itself stimulates TLR7/8 and suppresses virus replication in plasmacytoid DCs (Beignon et al., 2005; Gurney et al., 2004; Meier et al., 2007). LPS and poly(I:C) potentially suppress productive HIV-1 infection in primary macrophages in vitro (Equils et al., 2006; Trapp et al., 2009).

Although IFNs and pro-inflammatory cytokines usually exhibit antiviral effects, recent reports indicate that increase of these cytokines may also be a risk factor in accelerating the development of AIDS (Herbeuval & Shearer, 2007; Hosmalin & Lebon, 2006). Therefore, protective effects of TLR-mediated innate immunity on HIV-1 infection, if any, would be limited to the primary site of infection. In addition, even though some TLR ligands possess the potential to suppress HIV-1 replication, there is the problem of how those effects can be maintained in vivo before HIV-1 infection. In order to create HIV-1-resistant conditions before HIV-1 infection, non-invasive TLR ligands must be maintained continuously at the primary site of HIV-1 infection.

Studies in germ-free mice indicated that continuous stimulation of TLRs by commensal bacteria is necessary to maintain physiological levels of innate immunity (Bouskra et al., 2008; Troy & Kasper, 2010). It is conceivable that some kinds of commensal bacteria might be able to produce an HIV-1-resistant local environment in vivo by continuously stimulating innate immunity. However, very little is known about the relationship between commensal bacteria and HIV-1 infection. Since macrophages and DCs are the cellular targets of primary HIV-1 infection and also the major mediator of innate immune responses, the status of these cells at the site of primary infection might be crucial in determining susceptibility to HIV-1 infection (Meltzer et al., 1990). In the present study, we established a macrophage-like reporter system for HIV-1 replication. By using these cells, we sought to examine the effects of various commensal bacteria, especially those found in the genital tracts, on HIV-1 replication, and demonstrated that some, but not all, of the Gram-negative bacteria tested inhibited HIV-1 replication, depending on their abilities to stimulate TLR4 or TLR2.

### RESULTS

#### Suppression of HIV-1 gene expression by TLR ligands through type I IFN response in THP-1/NL4-3luc cells

In order to establish a system to assess the effects of signals through TLRs on HIV-1 gene expression in macrophages, we employed the human monocytic cell line THP-1, which is known to differentiate into adherent macrophage-like cells in vitro following treatment with phorbol 12-myristate 13-acetate (PMA) (Auwerx, 1991; Shattock et al., 1993). We detected strong expression of TLR2 and TLR4 mRNA, and little expression of TLR5 mRNA, in THP-1 cells by RT-PCR (data not shown). TLR3 mRNA was detectable in these cells only after PMA and LPS stimulation. These features resembled those of macrophages (Hijiya et al., 2002; Zarember & Godowski, 2002). We then infected THP-1 cells with pseudotyped HIV-1 NL4-3 expressing luciferase (Planelles et al., 1995) and obtained THP-1/NL4-3luc cells that stably expressed luciferase.

By using THP-1/NL4-3luc-derived macrophage-like cells, we first examined the effects of known TLR ligands on HIV-1 gene expression. As shown in Fig. 1a, poly(I:C) (TLR3 ligand) and LPS (TLR4 ligand), but not LTA (TLR2 ligand) or flagellin (TLR5 ligand), reduced HIV-1 expression significantly in THP-1/NL4-3luc cells ($P<0.01$). This is consistent with previous observations in primary macrophages (Equils et al., 2003, 2006; Heggelund et al., 2004; Trapp et al., 2009). Thus, THP-1/NL4-3luc cells mimic primary macrophages with respect to innate immune responses against TLR ligands and the sequential suppressive effects on HIV-1 replication.

We then evaluated whether type I IFNs were involved in TLR3- and TLR4-mediated suppression of HIV-1 expression in THP-1/NL4-3luc cells. Following stimulation with poly(I:C) or LPS, IFN-β mRNA became detectable by RT-PCR in total RNA samples from THP-1/NL4-3luc cells, peaking 2–4 h after stimulation, then gradually decreasing (Fig. 1b). LTA, which had no role in the suppression of HIV-1 expression, did not induce detectable levels of IFN-β mRNA. We further examined the effects of neutralizing antibodies to the IFN-α/β receptor on TLR-mediated
suppression of HIV-1 replication. As shown in Fig. 1(c), poly(I:C)- or LPS-mediated HIV-1 suppression was abrogated significantly by antibodies directed towards the anti-IFN-α/β receptors in a dose-dependent manner, but not by control antibodies (P<0.05). These results indicated that IFN-β-mediated mechanisms were involved in TLR3- and TLR4-mediated HIV-1 suppression.

**Effects of commensal bacteria on HIV-1 expression**

In order to determine whether any local environment potentially influenced HIV-1 replication, we assessed the effects of commensal bacteria on HIV-1 expression. As *Escherichia coli* is a representative commensal bacterium possessing LPS, we added different amounts of formalin-fixed *E. coli* into THP-1/NL4-3luc cell cultures and evaluated its effect on HIV-1 expression and cell toxicity (Fig. 2a). The highest inoculum of *E. coli* (3 × 10^8 cells ml^-1) inhibited HIV-1 expression almost completely. This could be partly attributed to cell toxicity, as the THP-1/NL4-3luc cell numbers decreased markedly on the addition of this amount of *E. coli*. However, serially diluted *E. coli* in the range of 3 × 10^7 to 3 × 10^4 cells ml^-1 still suppressed HIV-1 expression significantly in a dose-dependent manner without showing apparent cell toxicity (P<0.005).

We further examined whether vaginal commensal organisms have any role in suppression of HIV-1 replication, as the vagina is a primary infection site of sexually transmitted diseases. We fixed a series of bacteria with formalin and added them to THP-1/NL4-3luc cells. The results are shown in Fig. 2(b). Among the bacteria tested, *Neisseria mucosa* and *Veillonella parvula* showed potent suppressive effects on HIV-1 expression. However, *Prevotella melaninogenica* and *Lactobacillus acidophilus* enhanced HIV-1 expression, with *P. bivia* and *Mycobacterium smegmatis* elevating HIV-1 expression to a lesser extent. These bacteria did not markedly affect the viability of the cells under the conditions tested except for *V. parvula* (Fig. 2b). As *V. parvula* seemed to be slightly toxic for the cells, we further diluted *V. parvula* and confirmed that 1:50–1:200 dilutions of this bacterium still suppressed HIV-1 expression without apparent cell toxicity (Fig. 2c).

**Involvement of type I IFNs in bacterially mediated suppression of HIV-1 expression**

We further evaluated the involvement of type I IFNs on bacterially mediated HIV-1 suppression in THP-1/NL4-3luc cells. IFN-β mRNA became detectable by RT-PCR in total RNA samples from THP-1/NL4-3luc cells following stimulation with *E. coli*, *V. parvula* or *N. mucosa*, but not with *L. acidophilus* (Fig. 3a). We also examined the effects of neutralizing antibodies to the IFN-α/β receptor on bacterially mediated suppression of HIV-1. As shown in Fig. 3(b), the suppressive effects on HIV-1 expression by *E. coli*, *N. mucosa* and *V. parvula* were diminished when the signal through IFN-α/β receptor was blocked by the
antibodies. These results indicated that a type I IFN-mediated mechanism was involved in bacterially mediated HIV-1 suppression in THP-1/NL4-3luc cells.

Preference of TLRs stimulated by commensal bacteria

We next investigated the relationship between suppression of HIV-1 expression and the preference for TLRs stimulated by the commensal bacteria tested. As we used whole bacterial cells and not each component, we assessed the net ability of the bacteria to stimulate TLRs 2 and 4. For this purpose, we employed 293/hTLR-2 and 293/hTLR-4/CD14/MD2 cell lines, which stably expressed human TLR2 or TLR4 with CD14 and MD2, respectively. We transfected these cells with a nuclear factor κB (NF-κB)/Luc reporter plasmid together with the pRL-CMV reporter plasmid as an internal control, and then added various formalin-fixed bacteria. The results are shown in Fig. 4. P. melaninogenica, L. acidophilus and M. smegmatis, which did not suppress HIV-1 expression, stimulated TLR2 but not TLR4. In contrast, E. coli, V. parvula and N. mucosa, which suppressed HIV-1 expression, preferentially stimulated TLR4, with minimal stimulation of TLR2. P. bivia had little enhancing effect on HIV-1 expression but stimulated both TLR2 and TLR4. These results suggest that suppress-

![Fig. 2. Effects of various commensal bacteria on HIV-1 expression. (a) Dose-dependent suppressive effects of formalin-fixed E. coli. Two sets of 24-well plates containing THP-1/NL4-3luc-derived macrophage-like cells (approx. 5×10⁵ cells per well) were prepared and cultured in the presence of 100 μl of the serially diluted, formalin-fixed E. coli in a total of 1 ml culture per well. After 24 h incubation, one plate was harvested for luciferase assays (left) and the other plate was harvested for viability assays (right). The starting E. coli sample contained 3×10⁹ cells ml⁻¹ and the final concentrations of E. coli in 1 ml cultures are indicated. (b, c) Various commensal bacteria, as indicated, were treated similarly with formalin and co-cultured with THP-1/NL4-3luc-derived macrophage-like cells for 24 h, followed by evaluation of luciferase activities (left) and viability (right). Cell densities of the original bacterial suspensions were between 10⁹ and 10¹⁰ cells ml⁻¹, and were used at dilutions of 1 : 20 (b) or 1 : 50~200 (c). Results represent the mean±sd of duplicate samples. *P<0.05 compared with PBS controls.](http://vir.sgmjournals.org)
sion of HIV-1 expression was mainly mediated through TLR4, and selective stimulation of TLR4 is required to evoke optimal protective effects against HIV-1.

Suppression of HIV-1 gene expression by commensal bacteria in monocyte-derived macrophages (MDMs)

We next verified the suppressive effects of commensal bacteria on HIV-1 expression in primary human MDMs (Fig. 5). We infected these cells with pseudotyped HIV-1 (VSVG/pNL4-3luc) (Planelles et al., 1995) and then added formalin-fixed bacteria. Similar to the observations in THP-1/NL4-3luc cells, *E. coli*, *V. parvula*, *N. mucosa*, but not *L. acidophilus*, suppressed HIV-1 expression significantly (*P*<0.0005) (Fig. 5a), without apparent toxicities to MDMs in the same experiment (Fig. 5b).

We further examined the effects of neutralizing antibodies to TLR2, TLR4 and IFN-α/β receptor on *E. coli*-mediated suppression of HIV-1 expression in MDMs. As shown in Fig. 5(c), pretreatment with anti-TLR4 and -IFN-α/β receptor antibodies abrogated the *E. coli*-mediated suppressive effect almost completely, whereas anti-TLR2 antibodies showed little effects. These results indicated that the TLR4-dependent signalling pathway was involved in the suppression of HIV-1 gene expression in MDMs, which is mediated through type I IFNs.

**Effects of commensal bacteria on HIV-1 replication in acutely infected MDMs**

Finally, we examined the effects of commensal bacteria on HIV-1 replication in primary MDMs following acute infection with a replication-competent HIV-1 strain, JR-CSF (Koyanagi et al., 1987). As bacterial components induce chemokines as well as IFNs that potently suppress the acute phase of HIV-1 infection, especially in macrophages (Simard et al., 2008; Verani et al., 1997), we employed two different experimental systems. In one experiment, formalin-fixed commensal bacteria were added simultaneously with HIV-1 infection (0 h) and, in the other experiment, bacteria were added following the
HIV-1 infection period (16 h). When the bacteria were added simultaneously with HIV-1 infection, all bacteria tested, including *L. acidophilus*, *E. coli*, *V. parvula* and *N. mucosa*, suppressed HIV-1 p24 production during 4 days incubation (Fig. 6a). In contrast, when the bacteria were added 16 h after the initiation of HIV-1 infection, only *E. coli*, *V. parvula* and *N. mucosa*, but not *L. acidophilus*, suppressed HIV-1 replication significantly (*P*<0.05; Fig. 6b). Cell viabilities were not affected by the addition of formalin-fixed bacteria.

Thus, *E. coli*, *V. parvula* and *N. mucosa* suppressed HIV-1 replication in any cases where the bacteria were added at the early or late phases of acute HIV-1 infection in MDMs. A further time-course study indicated that the suppressive effects on HIV-1 replication by *E. coli* and *V. parvula* were gradually reduced but still significant up to 10 days after infection (Fig. 6c).

### DISCUSSION

In this study, we demonstrated that commensal bacteria such as *E. coli*, *N. mucosa* and *V. parvula*, but not *L. acidophilus*, *M. smegmatis*, *P. melaninogenica* or *P. bivia*, suppressed HIV-1 expression in macrophage-like THP-1 cells. The bacterially mediated HIV-1-inhibitory effects were attributed to signalling through TLR4 and subsequent type I IFN responses.

Suppression of HIV-1 replication by *E. coli*, *N. mucosa* and *V. parvula* was reproduced in primary MDMs acutely infected with HIV-1 JR-CSF, even when the bacteria were added following acute infection, consistent with the results that these bacteria could suppress HIV-1 expression. In contrast, *L. acidophilus*, which did not suppress HIV-1 expression, reduced HIV-1 replication only when added simultaneously with infection, but not when added after infection (Fig. 6). The transient HIV-1 suppression by *L. acidophilus* might be partly explained by induction of chemokines that potentially suppress HIV-1 entry steps (Cocchi et al., 1995). However, for more efficient suppression of HIV-1 replication, the inhibitory effects on the viral gene-expression steps seemed to be critical in macrophages at primary HIV-1 infection.

Among the bacteria used in the present study, *L. acidophilus* and *M. smegmatis* are Gram-positive, whilst *P. melaninogenica*, *P. bivia*, *N. mucosa*, *V. parvula* and *E. coli* are Gram-negative. All bacteria exhibiting inhibitory effects on HIV-1 expression in the present study were Gram-negative. *E. coli* and *V. parvula* are known to possess LPS and *N. mucosa* possesses lipooligosaccharide (LOS) on the outer membrane. *E. coli* LPS has been shown to suppress HIV-1 transcription in macrophages (Equils et al., 2006), while the LOS of *Neisseria gonorrhoeae*, the causative agent of gonorrhoea, suppresses HIV-1 infection in human primary macrophages (Liu et al., 2006). In our study, non-pathogenic *N. mucosa* also demonstrated suppressive effects on HIV-1 replication. Although the effects of *V. parvula* on HIV-1 replication have not been reported before, the LPS of *V. parvula* induces cytokine production in human and murine models as well as p38 MAPK activation in a TLR4-dependent manner (Matera et al., 2009). Thus, HIV-1-inhibitory effects by commensal bacteria are most likely to be contributed by the LPS or LOS that stimulates TLR4.

However, not all LPS-possessing Gram-negative bacteria used suppressed the expression of HIV-1. *P. melaninogenica* and *P. bivia* are Gram-negative bacteria possessing LPS, but failed to suppress HIV-1. Reporter assays revealed that...
P. melaninogenica and M. smegmatis markedly stimulated TLR2 and P. bivia stimulated both TLRs 2 and 4. Others have reported that Prevotella species potentially stimulated persistent HIV-1 infection through TLR2 (Mares et al., 2008; Spear et al., 2007). These observations suggest that the integrity of the outer membrane of bacteria to stimulate TLR2 or TLR4 is critical in rendering macrophages susceptible or resistant to HIV-1 infection.

None of the Gram-positive bacteria tested suppressed HIV-1 expression; instead, it was enhanced. L. acidophilus is normally found in the small intestine and vagina of humans. M. smegmatis is an acid-fast bacterial species found in genito-urinary tracts. L. acidophilus produces H₂O₂, which is virucidal to HIV-1 (Klebanoff & Coombs, 1991). However, in the present study, L. acidophilus preferentially stimulated TLR2 over TLR4 (Fig. 4) and showed an enhancing effect on HIV-1 expression (Fig. 2b). Our results are consistent with a previous report indicating enhanced susceptibility to HIV-1 in Langerhans cells or DCs through TLR2 stimulation by Gram-positive bacteria, including L. acidophilus (Ogawa et al., 2009).

Neutralizing antibodies against IFN-α/β receptors effectively blocked bacterially mediated suppression of HIV-1 expression (Fig. 3b), indicating that HIV-1 suppression was mediated partly by type I IFNs. Activation of TLR3/4 can induce antiviral functions through the adaptor TRIF via the MyD88-independent pathway (Takeda & Akira, 2004). IRF3 in the downstream signalling pathway is critical for evoking early antiviral proteins, including IFN-β (Doyle et al., 2002). A recent report indicated that poly(I:C) also inhibited HIV-1 replication in DCs via type I IFN-mediated activation of APOBEC3G (Trapp et al., 2009). The involvement of APOBEC3G in TLR4-induced HIV-1 suppression remains to be elucidated.

Commensal bacteria in the gastrointestinal tract are involved in the development of the intestinal lymphoid system and maintenance of intestinal homeostasis (Bouskra et al., 2008). Particular strains of Lactobacillus spp. protect intestinal barrier dysfunctions caused by invasive pathogens or pro-inflammatory cytokines (Johnson-Henry et al., 2008; Resta-Lenert & Barrett, 2006). However, very little is known about commensal bacterially induced responses in the gastrointestinal tract.
vagina. Among the commensal bacteria exhibiting anti-HIV-1 effects in the present study, *V. parvula* is found in the genito-urinary tract and is often associated with bacterial vaginosis, suggesting that some commensal bacteria under physiological and subclinical conditions may potentially limit HIV-1 replication.

A previous report indicated that HIV-1 gene expression in THP89GFP, a monocyte cell line latently infected with a fully competent dual-tropic viral strain (i.e. 89.6/R5X4) was induced by LPS, while LPS diminished virus production in primary MDMs (Simard *et al.*, 2008). In the present study, we established an HIV-1 reporter monocytic cell line (THP-1/NL4-3luc), which demonstrated innate immune responses against various TLR ligands in a similar manner to primary macrophages. This cell line therefore provides a useful tool for screening commensal bacteria or other substances that can suppress HIV-1 replication in macrophages.

In conclusion, commensal bacteria that selectively stimulate TLR4, such as *E. coli*, *N. mucosa* and *V. parvula*, inhibited HIV-1 expression in macrophages. This implies that such commensal organisms might contribute to naturally occurring resistance to HIV-1 infection. Application of harmless species of commensal bacteria that selectively stimulate TLR4 might be a potential prophylactic approach in mounting protective innate immune responses at the primary site of HIV-1 infection.

**METHODS**

Cell culture. Human PBMCs were isolated from buffy coats of healthy HIV-1-seronegative donors by Ficoll-Hypaque density-gradient centrifugation (Ficoll-Paque PLUS; Amersham Biosciences). Monocytes were purified from fresh PBMCs by magnetic cell sorting using a monocyte isolation kit (Miltenyi Biotec). Monocytes were cultured in RPMI 1640 medium (Gibco-BRL) supplemented with 5% heat-inactivated human AB blood group serum (Sigma), 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹ (Wako) and 5 µg recombinant human M-CSF ml⁻¹ (R&D Systems), in order to generate MDMs. 293-hTLR4/MD2-CD14 and 293-hTLR2 stable cell lines were purchased from Invivogen. AZT (3'-azido-3'-deoxothymidine; Sigma Aldrich) was used at a concentration of 5 µM in culture.

**TLR ligands.** The TLR ligands LTA (5–20 µg ml⁻¹), poly(I:C) (50–200 µg ml⁻¹), LPS (50–200 ng ml⁻¹) and flagellin (0.5–2.0 µg ml⁻¹) were purchased from Invivogen. AZT (3'-azido-3'-deoxothymidine; Sigma Aldrich) was used at a concentration of 5 µM in culture.

**Antibodies and reagents.** TL2.1, a neutralizing mAb specific for human TLR2, and HTA-125, a neutralizing mAb specific for human TLR4, were purchased from ebioscience and used at 10 µg ml⁻¹ as indicated previously (Romano Carratelli *et al.*, 2009). Anti-human IFN-α/β receptor chain 2 neutralizing mAb clone MMHAR-2 and IgG2a isotype antibody were purchased from PBL Interferon Source.

**Plasmids.** The pNL43lucAenv vector, an envelope-defective pNL4-3 vector containing the luciferase gene inserted at the Nef site, was kindly provided by Dr Irvin S. Y. Chen (University of California, Los Angeles, CA, USA) (Planelles *et al.*, 1995). The pMD.G vector expressing vesicular stomatitis virus (VSV) G envelope protein was also obtained from Dr Chen. A reporter plasmid expressing firefly luciferase driven by the NF-κB promoter (κB-Luc) was provided by Dr Junichi Fujisawa (Kansai Medical University, Osaka, Japan) (Hirai *et al.*, 1994). A control plasmid expressing Renilla luciferase driven by the cytomegalovirus promoter (pRL-CMV; Promega) was used as a control.

**Virus preparation and infection.** Pseudotype viruses were generated by co-transfection of 293T cells (1 × 10⁶ cells) with the pNL43lucAenv vector (3 µg) and pMD.G (1 µg) using Lipofectamine 2000 (Invitrogen) in a 60 mm dish (Ikeda *et al.*, 2004). After 48 h culture, supernatants were harvested, passed through a filter with 0.45 µm pores and stored at −80 °C until used. Virus titres were evaluated by using a p24 HIV-1 ELISA kit (RETRO-TEK; ZeptoMetrix Corp.) and VSV/pNL4-3luc pseudotype viruses containing 100 ng p24 were used to infect one well of THP-1 cells in a 24-well plate.

HIV-1 JR-CSF strain (Koyanagi *et al.*, 1987) was grown in phytohaemagglutinin (PHA; Difco Laboratories)-stimulated PBMCs cultured in RPMI 1640 medium supplemented with 10% FCS and 10 IU recombinant human interleukin-2 ml⁻¹ (Shionogi) for 5–7 days. The supernatants were filtered and stored at −80 °C until use. The culture supernatant that contained HIV-1 JR-CSF (20 ng p24 ml⁻¹ per well) was added to MDMs grown in a 24-well plate and incubated for 16 h at 37 °C.

**RT-PCR.** Total RNA was extracted from cells by using Isogen (Nippon Gene), and cDNA was synthesized from 1.0 µg total RNA using ReverTraAce for RT-PCR with Oligo(dt)₉₀ primers (TOYOBO Ltd). The cDNA was used as a template for PCR in a mixture containing cDNA, dNTPs (TOYOBO) and 10 × PCR buffer (TOYOBO). PCR cycling conditions consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 5 s and extension at 72 °C for 30 s. The primers were 5'-GGCCGAGTTGACCATC-3' and 5'-CACAGTACTGATCCTCC-3' for human IFN-β, and 5'-GTGAAAGTCCGGAATTCAACCGATTG-3' and 5'-TGATTGTGAGGAAATCTGCTCTTGGAAAG-3' for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The predicted sizes of amplicons were 262 and 247 bp for IFN-β and GAPDH, respectively. PCR products were stained with ethidium bromide following electrophoresis on a 2% (w/v) agarose gel.
Cell-viability assay. Cell viability was evaluated by using a Cell Counting kit-8 (Dojindo), which measures formazan colour development in viable cells. Cell Counting kit-8 solution (10 μl) was added to 100 μl aliquots of cell cultures in a 24-well plate. After incubation in a CO₂ incubator for 40 min, culture supernatants were transferred to 96-well microplates and A₅₁₀ was measured. The negative-control value of the well containing medium alone was subtracted from the sample values. After the cell-counting assay, the cells were washed twice with PBS then subjected to the luciferase assay.

Luciferase reporter assay. For the HIV-1 reporter assay, THP-1/NL4-3uc cells (5 × 10⁴ cells per well) were seeded onto 24-well plates, stimulated with PMA (10 ng ml⁻¹) for 24 h, washed, and then cultured for an additional 48 h. The cells were further stimulated at 37 °C with various TLR ligands for 24 h or with fixed bacteria for 24 h prior to harvesting. For the TLR2 or TLR4 reporter assays, 2 × 10⁵ 293-hTLR2 cells or 293-hTLR4/MD2-Cd14 cells, respectively, were co-transfected with κB-Luc (100 ng) and phRL-CMV (10 ng) plasmids using Lipofectamine 2000. Various fixed bacteria were added to the cultures 24 h post-transfection and incubated for another 24 h. For the luciferase assays, all cells in each well were washed twice with PBS and lysed with 100 μl 1 × luciferase cell culture lysis reagent (Promega), and firefly and Renilla luciferase activities in 10 μl lysate were measured by using the luciferase and Renilla luciferase assay systems (both from Promega) on a luminometer (Lumat LB 9507; EG&G Berthold), according to the manufacturer’s instructions.

Statistics. A Student’s t-test was used for evaluating differences between two groups of samples. P-values of <0.05 were considered to be statistically significant.

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REFERENCES


