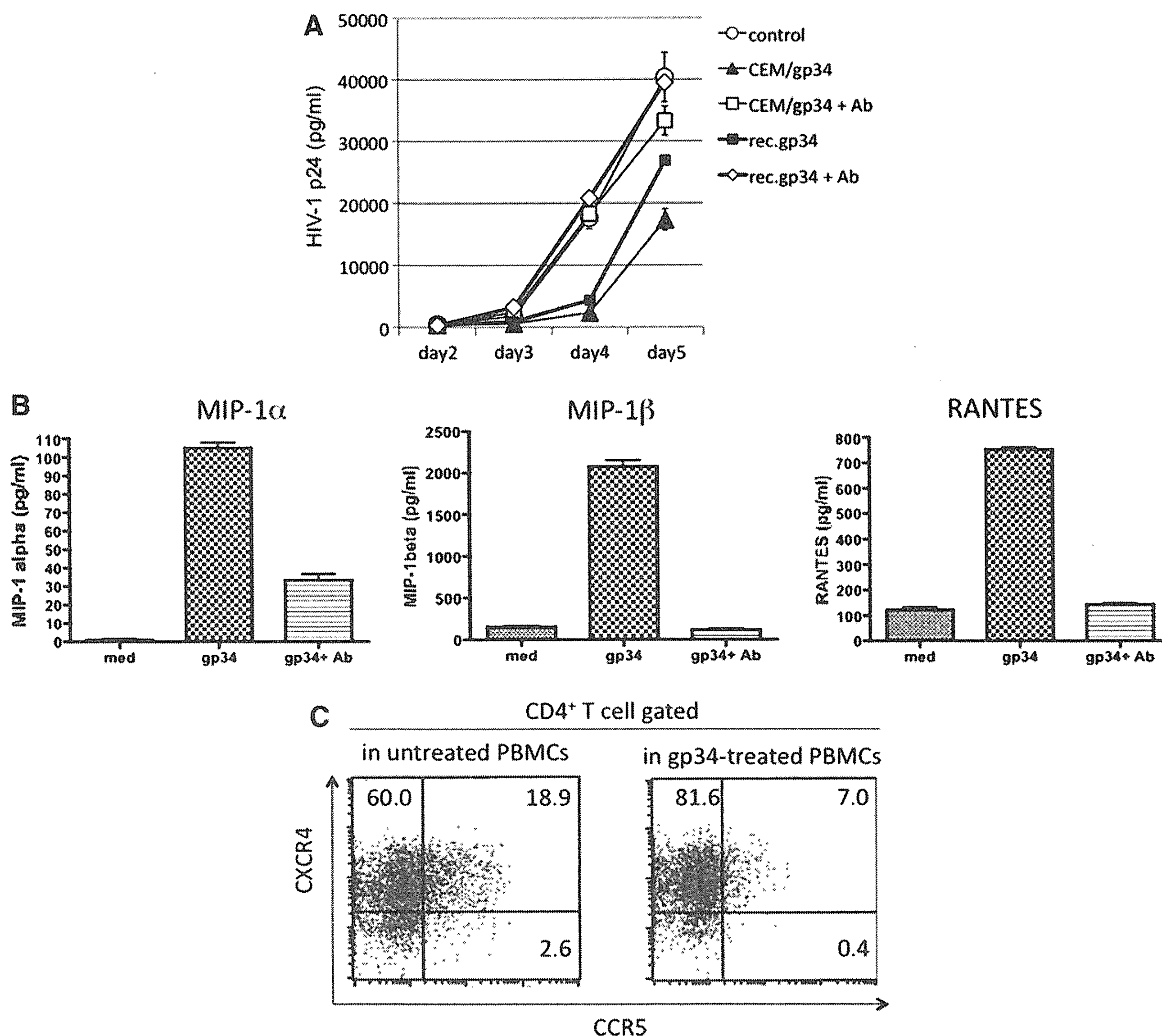

FIG. 4. Enhanced production of CCR5-binding β-chemokines is involved in the R5 HIV-1 inhibition by OX40 stimulation. **(A)** HIV-$1_{JR\text{-}FL}$-infected anti-CD3/CD28-activated PBMCs (5×10^5 cells/ml) prepared as in Fig. 2 were cocultured with 2×10^5 cells/ml of CEM/gp34, 1 μg/ml soluble recombinant OX40L (rec.gp34), or medium alone (control) either in the presence of the mixture of mAbs (Ab) against MIP-1α, MIP-1β, and RANTES at 10 μg/ml each. Production of HIV-1 was monitored by measuring levels of p24 by ELISA. **(B)** Anti-CD3/CD28-activated fresh PBMCs (5×10^5 cells/ml) as in Fig. 1 were cultured in media alone (med) or in media containing 1 μg/ml soluble recombinant OX40 (rec.gp34) either in the presence or absence of 10 μg/ml anti-OX40 neutralizing mAb (clone W4-54) for 24 h. β-Chemokines produced in the culture supernatants were quantitated by ELISA. **(C)** PBMCs prepared as in **(B)** were examined for the expression of CCR5 and CXCR4 on CD4-gated cells by multicolor FCM. Data shown are representative data of three independent experiments using three different donors.

vectors that express OX40L or OX40L mRNA-transfected dendritic cells.[40,41] We submit that the use of T cells expressing OX40L that are induced by *ex vivo* activation under DNA-damaging conditions for autologous transfusion-based therapy may provide yet another therapeutic option.[32] Further studies exploring the direct *in vivo* effects of OX40 stimulation on HIV-1 production in the SIV-infected nonhuman primate model and in the hu-PBL-SCID mouse model are in progress.

Similar to our findings on OX40 costimulation, it is well known that a strong CD28 costimulation together with CD3 stimulation of purified $CD4^+$ T cells from HIV-1-negative or HIV-positive humans results in inhibition of R5 HIV-1 infection due to the production of the three CCR5-binding β-chemokines.[42,43] Data from our preliminary experiments show that activated PBMCs stimulated with immobilized anti-CD3 mAb without additional CD28 costimulation were permissive to R5 HIV-1 proliferation *in vitro* irrespective of OX40L stimulation (data not shown), suggesting that the CD28 costimulation is essential for OX40-mediated R5 HIV-1 suppression. As it has been demonstrated that CD28 costimulation augments and sustains OX40 expression on T cells in the murine system,[24,44] we also confirmed it in our human system (data not shown). It might be possible that CD28 ligation is required to enhance OX40 function. On the other hand, it will be interesting to

reveal whether an OX40–OX40L interaction is involved in CD3/CD28 costimulation-mediated R5 HIV-1 inhibition.

Finally, since OX40-stimulation has been shown to include adjuvant effects,[1,2,9] it is reasoned that OX40 activation in addition to its anti-R5 HIV-1 effect would also promote $CD4^+$ and $CD8^+$ T cell immune responses against HIV-1 and enhance $OX40^+$ NKT cell and neutrophil activities.[8,9] Indeed, it has been shown that OX40 ligation of human $CD4^+$ T cells enhanced HIV-1-specific CTL responses *in vitro*.[45] These OX40 activities in concert may have great therapeutic potential in HIV-1-infected individuals.

Acknowledgments

This work was supported by grants from a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and Research on HIV/AIDS and Health Sciences focusing on Drug Innovation from the Ministry of Health, Labor and Welfare of Japan.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Yuetsu Tanaka
Department of Immunology
Graduate School of Medicine
University of the Ryukyus
Uehara 207, Nishihara-cho, Nakagami-gun
Okinawa 903-0215, Japan

E-mail: yuetsu@s4.dion.ne.jp

Journal of General Virology (2010), **91**, 2804–2813 DOI 10.1099/vir.0.022442-0

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

Nursarat Ahmed,[1] Takaya Hayashi,[1] Atsuhiko Hasegawa,[1] Hiroyuki Furukawa,[1] Noboru Okamura,[2] Toshio Chida,[2] Takao Masuda[1] and Mari Kannagi[1]

Correspondence
Mari Kannagi
kann.impt@tmd.ac.jp

[1]Department of Immunotherapeutics, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

[2]Laboratory 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 melaninogenica*, *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.

Received 29 March 2010
Accepted 15 August 2010

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

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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. 1(a), 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

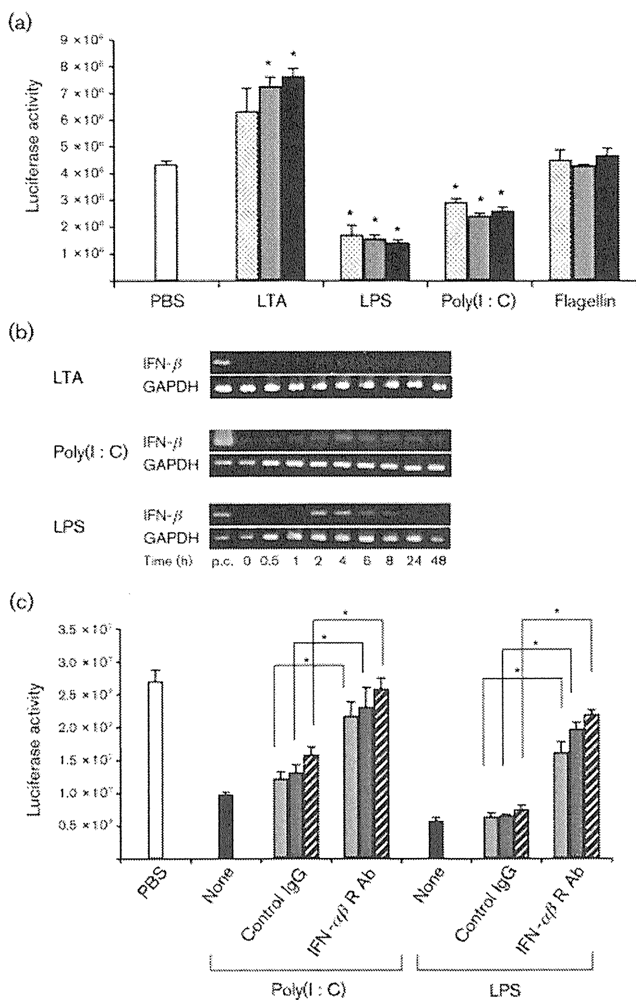

Fig. 1. Effects of various TLR ligands on HIV-1 expression and type I IFN responses in the THP-1/NL4-3luc reporter cell line. (a) THP-1/NL4-3luc cells were differentiated into macrophage-like cells by stimulation with PMA for 24 h and were further cultured with various TLR ligands for 48 h. Then, luciferase activities in the total cell lysates were measured. Light grey, dark grey and black bars respectively indicate low, middle and high doses of each TLR ligand: 5, 10 and 20 μg ml^{-1} for LTA; 50, 100 and 200 μg ml^{-1} for poly (I : C); 50, 100 and 200 ng ml^{-1} for LPS; and 0.5, 1.0 and 2.0 μg ml^{-1} for flagellin, respectively. Results indicate the mean ± SD of duplicate samples. *$P<0.05$ compared with PBS controls. (b) IFN-β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were amplified by RT-PCR from the total RNA of THP-1/NL4-3luc-derived macrophages before (0 h) or after stimulation with LTA (10 μg ml^{-1}), poly(I : C) (100 μg ml^{-1}) or LPS (100 ng ml^{-1}) at the indicated time points. PCR products were visualized by staining with ethidium bromide following agarose gel electrophoresis. (c) THP-1/NL4-3luc-derived macrophage-like cells were untreated (empty bars) or treated with poly(I : C) or LPS, in the absence (filled bars) or presence of anti-IFN-α/β receptor chain 2 neutralizing antibody or control mouse IgG at 0.25 μg ml^{-1} (light grey bars), 1 μg ml^{-1} (dark grey bars) or 4 μg ml^{-1} (hatched bars) as indicated. Luciferase activities in cell lysates were measured after 48 h incubation. The data represent the mean ± SD of duplicate samples. *$P<0.05$.

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* enhancing it 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

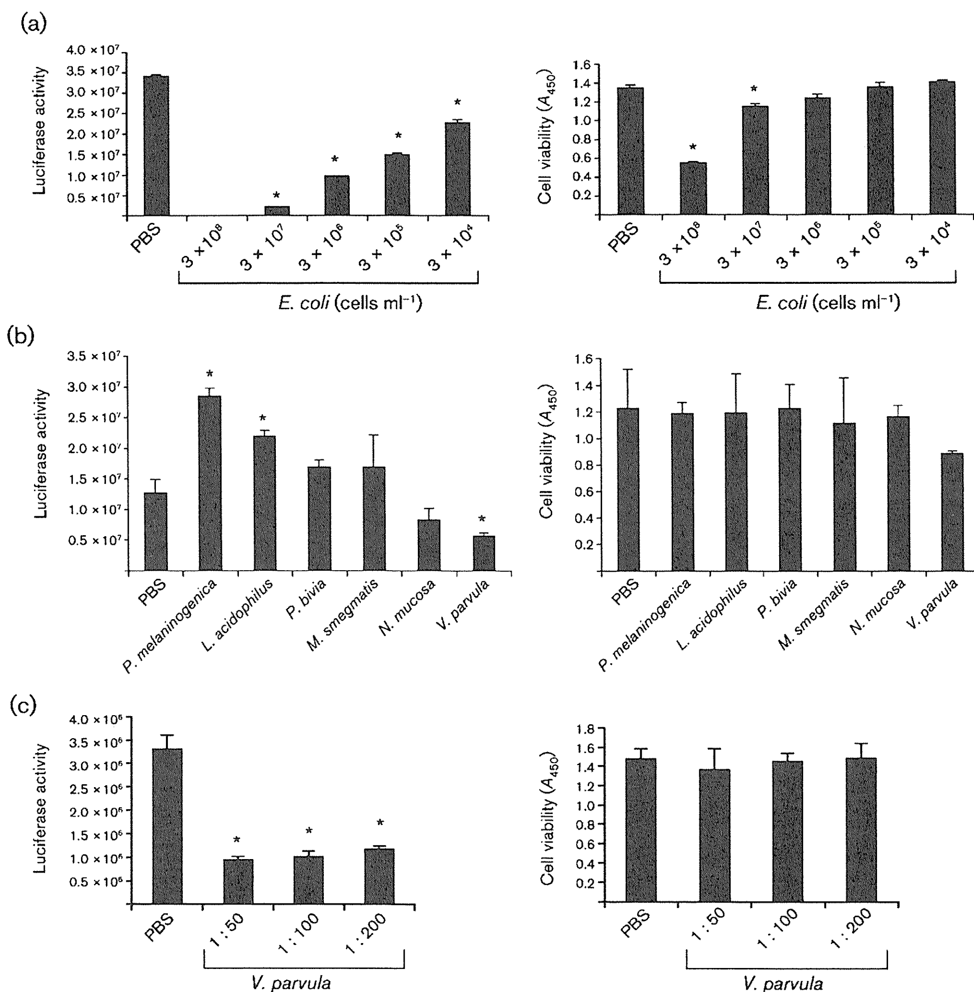

Fig. 2. Effects of various commensal bacteria on HIV-1 expression. (a) Dose-dependent suppressive effects of formalin-fixed *E. coli*. Two sets of 24-well plates containing THP-1/NL4-3luc-derived macrophage-like cells (approx. 5×10^5 cells per well) were prepared and cultured in the presence of 100 μl 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 3×10^9 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 10^9 and 10^{10} 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.

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 suppres-

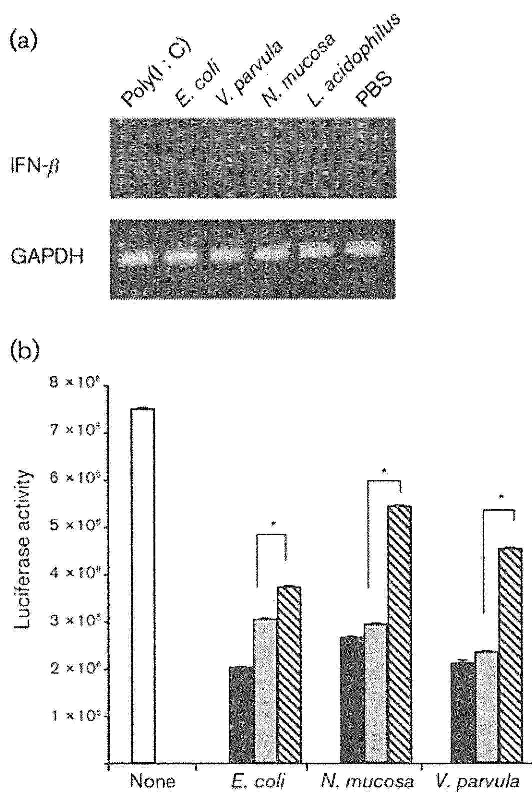

Fig. 3. Involvement of type I IFNs in bacterially mediated suppression of HIV-1 expression. (a) THP-1/NL4-3luc-derived macrophages were stimulated with poly(I : C) (positive control), *E. coli*, *V. parvula*, *N. mucosa* or *L. acidophilus* for 12 h, and total RNA extracted from the cells was subjected to RT-PCR using primers specific for human IFN-β and GAPDH. PCR products were visualized by staining with ethidium bromide following agarose gel electrophoresis. (b) THP-1/NL4-3luc cells were untreated (empty bars) or treated with formalin-fixed bacteria as indicated, in the absence (filled bars) or presence of mouse IgG (grey bars) or anti-IFN-α/β receptor neutralizing antibody (hatched bars), both at 2 μg ml^{-1}. Luciferase activities in cell lysates were measured after 24 h incubation. Data represent the mean ± SD of duplicate samples. *$P<0.05$.

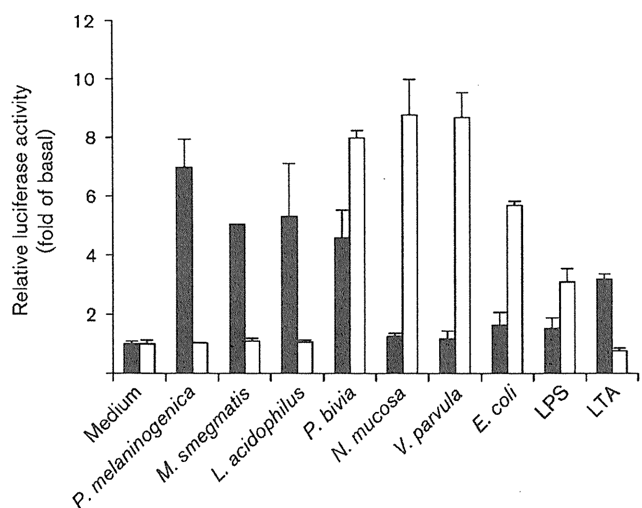

Fig. 4. Preference of various commensal bacteria to stimulate different TLRs. 293-hTLR2 (filled bars) and 293-hTLR4/MD2-CD14 (empty bars) cells were transfected with 0.1 μg kB-Luc and 0.01 μg phRL-CMV plasmids, then cultured in the presence of various formalin-fixed bacteria or positive controls (LPS and LTA), as indicated. Luciferase assays were performed after a 24 h incubation, with firefly luciferase activity normalized to *Renilla* luciferase activity. The data represent fold increases against the control sample incubated with PBS, and show the mean ± SD of duplicate samples.

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 commensal bacteria. Similar to the observations in THP-1/NL4-3luc cells, *E. coli*, *V. parvula* and *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

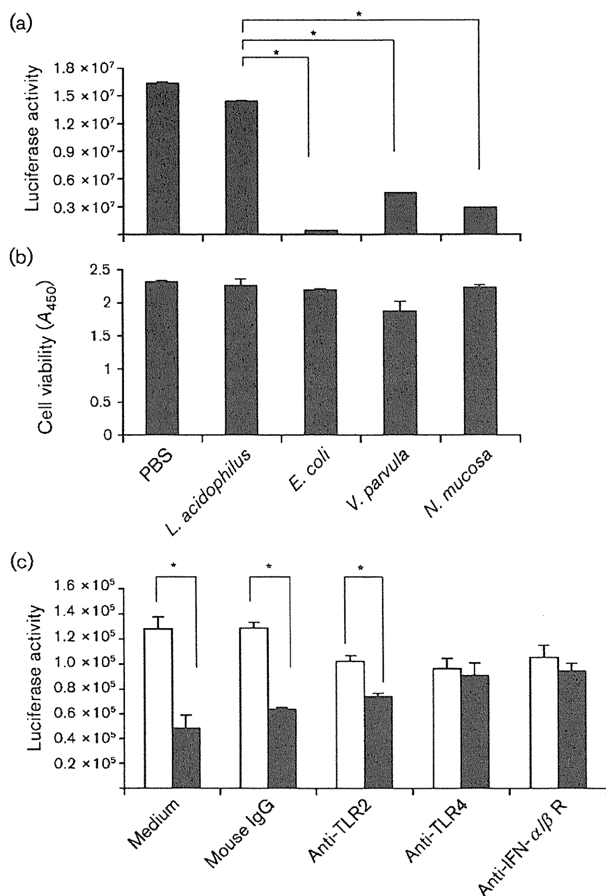

Fig. 5. Suppressive effects of commensal bacteria on HIV-1 expression in MDMs. (a, b) MDMs were prepared from uninfected PBMCs in 7 day cultures as described in Methods, and infected with VSVG/pNL4-3luc pseudotype virus for 16 h. After washing with PBS, infected MDMs were cultured for a further 24 h in the presence or absence of formalin-fixed bacteria that were prepared as described in the legend to Fig. 2, and then luciferase activity (a) and viable cell numbers (A_{450}; b) were evaluated. (c) MDMs were infected with VSVG/pNL4-3luc pseudotype virus for 16 h, and then incubated with anti-TLR2 (10 μg ml^{-1}), anti-TLR4 (10 μg ml^{-1}), anti-IFN-α/β receptor (2 μg ml^{-1}) antibodies or control mouse IgG 2a (2 μg ml^{-1}) for 1 h. Luciferase activities were evaluated after another 24 h culture in the presence of 3×10^6 formalin-fixed *E. coli* ml^{-1} (filled bars) or PBS (empty bars). The data indicate the mean ± SD of duplicate samples. *$P<0.05$.

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 suppresive 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-1cells. 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

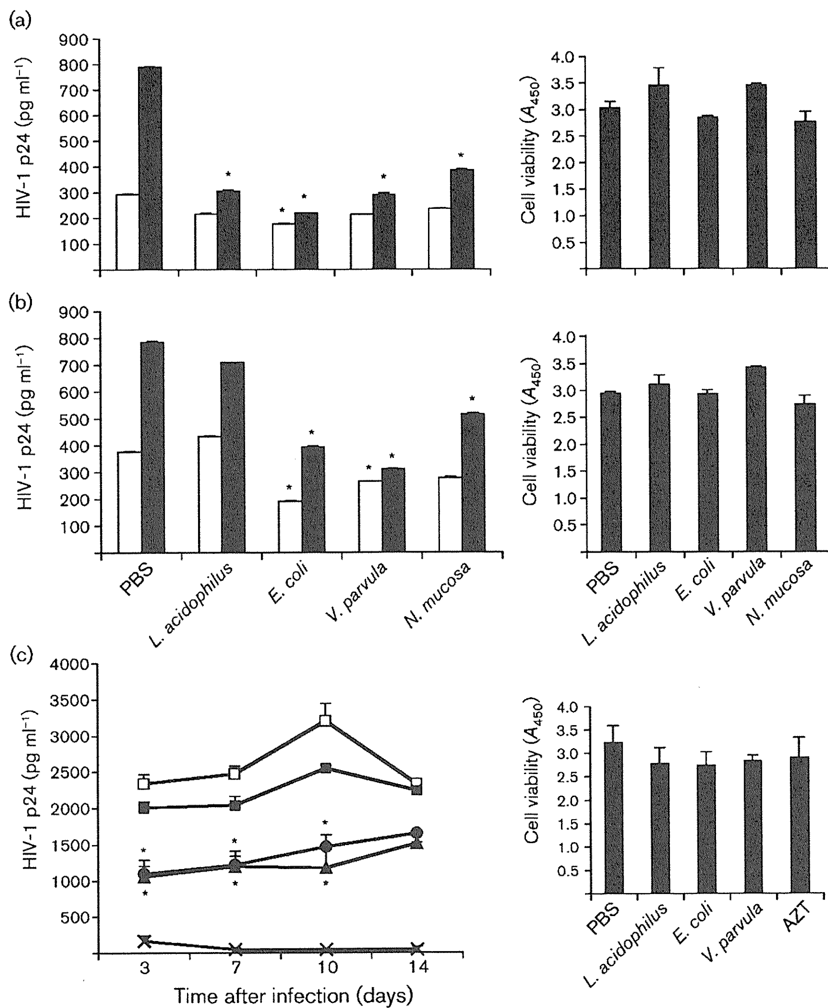

Fig. 6. Effects of commensal organisms on HIV-1 replication in acutely infected MDMs. (a) MDMs were infected with replication-competent HIV-1 (JR-CSF) for 16 h in the presence or absence of formalin-fixed bacteria as indicated. The cells were washed four times and cultured further in the presence of the same levels of the bacteria. The amounts of HIV-1 p24 in the culture supernatants on day 2 (open bars) and day 4 (filled bars) were monitored by ELISA (left panel), and the viable cell numbers on day 4 were evaluated (right panel). (b) MDMs infected with HIV-1 JR-CSF for 16 h were washed four times, and then incubated with formalin-fixed bacteria as indicated. HIV-1 p24 and viable cell numbers were measured as described for (a). Experiments for (a) and (b) were performed at the same time using MDMs from the same donor. (c) MDMs infected with HIV-1 JR-CSF for 16 h were washed and cultured up to 14 days in the absence (□) or presence of *L. acidophilus* (■), *E. coli* (▲) and *V. parvula* (●). MDMs infected and cultured in the presence of 5 μM AZT (×) served as negative controls. Half of the medium in each well was replaced with fresh medium supplemented with the same concentrations of bacteria or AZT every 3–4 days. HIV-1 p24 in the culture supernatants at the time indicated (left panel) and cell viability at day 14 (right panel) were measured as described above. Dilutions of bacteria used were 1 : 40 for *L. acidophilus*, 1 : 100 for *E. coli*, 1 : 200 for *V. parvula* and 1 : 20 for *N. mucosa*. Results represent the mean ± SD of duplicate samples. *$P<0.05$ compared with PBS controls.

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 H_2O_2, 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

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 monocytic 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 ng 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 and cultured in Dulbecco's modified Eagle's medium supplemented with 10 % heat-inactivated FBS (Sigma) and antibiotics (100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}). An HIV-1 reporter monocytic cell line, THP-1/NL4-3luc, was established by *in vitro* infection of THP-1 cells with VSV/pNL4-3luc pseudotype virus followed by limiting dilution in flat-bottom 96-well plates. THP-1/NL4-3luc cells were differentiated into macrophage-like cells by stimulation with PMA (10 ng ml^{-1}; Sigma) for 24 h. THP-1 and THP-1/NL4-3luc cells were cultured in RPMI 1640 medium supplemented with 10 % FBS and antibiotics (100 U penicillin ml^{-1} and 100 μg streptomycin ml^{-1}).

Bacterial culture. The bacterial strains *L. acidophilus* (JCM 2124), *P. bivia* (JCM 6331^T), *P. melaninogenica* (JCM 6321, 6325^T), *V. parvula* (JCM 12972^T), *N. mucosa* (JCM 12992^T), *M. smegmatis* (JCM 5866^T, 20379) and *E. coli* (JCM 5491, 20135) were obtained from the Japan Collection of Microorganisms, RIKEN Bio Resource Center (Ibaraki, Japan). *L. acidophilus* was grown on BL agar (Nissui); *P. bivia*, *P. melaninogenica* and *V. parvula* were grown on EG agar (Nissui); and *N. mucosa* was grown on Columbia blood agar (Oxoid) under anaerobic conditions using an AnaeroPack-Kenki (Mitsubishi Gas Chemical Company). *M. smegmatis* was grown on Middle Brook 7H10 agar (Difco) and *E. coli* in LB broth (Merck) under aerobic conditions. Concentrations of bacteria were evaluated by using the McFarland standard (NCCLS, 1992). Bacteria were fixed with 1 % formalin in PBS for 30 min at room temperature, then washed three times with PBS and stored at −80 °C.

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′-deoxythymidine; 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 pNL43lucΔenv 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 (phRL-CMV; Promega) was used as a control.

Virus preparation and infection. Pseudotype viruses were generated by co-transfection of 293T cells (1×10^6 cells) with the pNL43lucΔenv 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 needed. 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$(dT)_{20}$ 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′-GCCGCATTGACCATC-3′ and 5′-CACAGTGACT-GTACTCCT-3′ for human IFN-β, and 5′-GTGAAGGTCGGAGT-CAACGGATTTG-3′ and 5′-TGATTTTGGAGGGATCTCGCTCCT-GGAAGA-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 CO_2 incubator for 40 min, culture supernatants were transferred to 96-well microplates and A_{450} 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/NL4-3luc cells (5×10^5 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 48 h or with fixed bacteria for 24 h prior to harvesting. For the TLR2 or TLR4 reporter assays, 2×10^5 293-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.

ACKNOWLEDGEMENTS

We thank Mr Yoshio Inagaki (Tokyo Medical and Dental University) for his technical advice regarding bacterial culture. This study was supported by grants from the Ministry of Health, Welfare and Labor of Japan and Japan Science Technology Agency. The authors have no financial conflict of interest to declare.

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Microbes and Infection 12 (2010) 937–947

Microbes and Infection

www.elsevier.com/locate/micinf

Original article

DNA-dependent activator of IFN-regulatory factors enhances the transcription of HIV-1 through NF-κB

Takaya Hayashi [a], Hironori Nishitsuji [a,b], Ayako Takamori [a], Atsuhiko Hasegawa [a], Takao Masuda [a,*], Mari Kannagi [a]

[a] Department of Immunotherapeutics, Graduate School of Medicine and Dentistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

[b] Department of Life and Environmental Science and High Technology Research Center, Chiba Institute of Technology, 2-17-1 Tsudanuma, Narashino-shi, Chiba 275-0016, Japan

Received 26 February 2010; accepted 11 June 2010
Available online 22 June 2010

Abstract

Pattern recognition receptors (PRRs) play a pivotal role in host innate immune responses against microbial infection. Viruses are primarily recognized by PRRs such as Toll-like receptor 3, 7, 8 and 9, and RIG-I-like receptors. Recent studies have demonstrated that DNA-dependent activator of IFN-regulatory factors (DAI) is a cytosolic sensor molecule for dsDNA, and is implicated in antiviral responses to some DNA viruses. Soon after infection, human immunodeficiency virus type-1 (HIV-1) synthesizes viral dsDNA in the cytoplasm by reverse transcriptase. In addition, an immune compromised state due to chronic HIV-1 infection results in opportunistic infection with some microbes that potentially activate DAI. However, it has not been elucidated whether DAI affects HIV-1 replication, or its possible mechanisms. Here, we showed that forced expression of DAI markedly enhanced HIV-1 replication, which was largely impaired by mutations at κB sites in HIV-1 LTR or by suppressing activation of NF-κB. Moreover, intact structure around the D3 region (174–232 aa) and two RIP homotypic interaction motifs (198–214 aa and 256–272 aa) within DAI were critical for its activity. These results suggest that activation of DAI might contribute to augment HIV-1 replication through DAI- NF-κB pathway.
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Keywords: HIV-1; DAI; NF-κB

1. Introduction

Various pattern recognition receptors (PRRs) in host cells act as sensors for microorganisms in infection and mediate initial signals for innate immune responses [1]. The PRRs that are able to sense nucleic acids play critical roles in the initiation of host defense against viral infection. There are two types of PRRs that sense nucleic acids, membrane-bound Toll-like receptors (TLRs) and cytosolic sensors [2]. Among the TLR family, TLR3, TLR7/8 and TLR9 recognize dsRNA, ssRNA and unmethylated CpG DNA, respectively. The retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) such as RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) are cytosolic nucleic acid sensors that have been shown to recognize viral RNA [3]. DNA-dependent activator of IFN-regulatory factors (DAI) (also known as ZBP1 or DLM-1) [4–6] has been recently identified as a cytosolic DNA sensor, and dsDNA-stimulated DAI activates interferon-regulatory factor (IRF) 3 and nuclear factor of κB (NF-κB) leading to the production of type-I interferons (IFNs) and inflammatory cytokines [7].

Some viruses take advantage of innate immune signals for viral replication by selectively inhibiting expression or function of type-I IFNs. In human rhinovirus type-14 infection,

* Corresponding author. Tel.: +81 03 5803 5799; fax: +81 03 5803 0235.
E-mail address: tmasu.impt@tmd.ac.jp (T. Masuda).

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doi:10.1016/j.micinf.2010.06.003

ATF-2/c-Jun and NF-κB, but not IRF3, are activated, which in turn promotes transcription of the viral receptor intercellular adhesion molecule-1 (ICAM-1) without production of type-I IFNs [8]. Bovine respiratory syncytial virus encodes non-structural proteins, non-structural protein (NS) 1 and 2, that inhibit IRF3 activity and may exploit coincidentally induced NF-κB signaling for growth and survival of infected cells [9]. Epstein–Barr virus (EBV)-encoded small RNAs (EBERs) stimulate RIG-I and TLR3 to induce the secretion of type-I IFNs and interleukin (IL)-10 from EBV-infected cells [10,11]. However, a type-I IFN-mediated pro-apoptotic effect is inhibited by the interaction of EBERs with dsRNA-dependent protein kinase (PKR), and IL-10 eventually promotes the growth of EBV-infected cells [12]. Human immunodeficiency virus type-1 (HIV-1) also possesses strategies to inhibit the antiviral effect of type-I IFNs through the HIV-1 accessory proteins, Vif and Vpr, which individually promote proteasome-dependent IRF3 degradation [13,14].

It has been shown that GU-rich ssRNA from HIV-1 stimulates plasmacytoid dendritic cells (pDCs) through mouse TLR7 and human TLR8, and induces the production of IFN-α and inflammatory cytokines [15–17]. Increased cellular cytotoxicity and IFN-γ production from natural killer (NK) cells have been shown upon addition of HIV-1 ssRNA to pDCs or CD14(+) monocyte co-cultures [18]. Treatment of *ex vivo* HIV-1-infected lymphocytes with CpG DNA almost completely inhibited the replication of HIV-1 [19]. Similarly, thymocyte infection with HIV-1 results in the secretion of IFN-α by thymic pDCs, which in turn suppresses HIV-1 replication [20].

During HIV-1 replication cycles, the viral genome exists in both RNA and DNA forms. The viral RNA is transcribed by host RNA polymerase II, thereby modifying the 5′ ends of the viral RNA by the addition of a cap structure. Therefore, it seems unlikely that RLRs recognize HIV-1 genomic RNAs because the 5′-triphosphate, an essential structure for RIG-I recognition, is masked [21,22]. Instead, HIV-1 dsDNA that is synthesized by reverse transcriptase soon after infection might be potentially recognized in the cytoplasm by the dsDNA-recognizing PRRs, including DAI. Additionally, these PRRs can be activated by various co-infecting microorganisms. It has been described that opportunistic infection with herpes simplex virus type-1 (HSV-1) or human cytomegalovirus (HCMV) increased HIV-1 replication and that human herpes virus type-6 (HHV-6) infection induced reactivation of latently infected HIV-1 [23–26]. These DNA viruses potentially activate DAI.

DAI is ubiquitously expressed in various types of cells including lymphocytes and macrophages [4], and its expression level is enhanced by type-I and type-II IFNs [4,7]. DAI recognizes and responds to mammalian, microbial and synthesized dsDNA [7,27], presumably based on structure, and subsequently induces the production of IFN-β. Additionally, knocking down of DAI in mouse fibroblasts reduces IFN-β production and augments susceptibility to HSV-1 and EMCV infection [7]. DAI might also contribute to IFN responses in some bacterial infections [28,29].

In this study we aimed to clarify whether DAI positively or negatively affects HIV-1 replication. Here, we showed that DAI-mediated signals enhanced HIV-1 replication at a transcriptional step through the NF-κB pathway. In addition, the D3 region and two receptor interaction protein (RIP) homotypic interaction motifs (RHIMs) of DAI were involved in this enhancement.

2. Materials and methods

2.1. Construction of plasmids

A series of protein expression vectors were constructed based on the pEF6/myc-His vector. To generate the pEF6/myc-His vector, a pSecTag2 vector (Invitrogen, USA) was digested with NotI and PmaCI, and the resulting 1.7 kb fragment was inserted into the 4.1 kb NotI–PmaCI digested pEF6/V5-His vector. To construct a myc-tagged LacZ-expressing control vector, pEF6/LacZ, the pEF6/V5-His/LacZ vector (Invitrogen, USA) was digested with BamHI and NotI, and the fragment containing the LacZ coding gene was inserted between the BamHI and NotI sites of pEF6/myc-His. Human DAI mRNA was purified from healthy human peripheral blood mononuclear cells by Isogen (Nippon Gene Co., Japan) and amplified by RT-PCR (ReverTra Ace; Toyobo Co., Japan) using the primers dai-fw, 5′-CAC CAT GGC CCA GGC TCC TGC TGA CCC G-3′ and dai-rev, 5′-AAT CCC ACC TCC CCA CCA GCT CCC CTC GTG-3′. Amplified full-length DAI DNA was inserted into the pGEM-T easy vector (Promega, USA) by TA cloning, producing pGEM-DAI. The pEF6/DAI-full plasmid was obtained by ligating the NotI-digested DAI-insert from pGEM-DAI into NotI-digested pEF6/myc-His. All of the truncated DAI-expressing mutant vectors used in this study, pEF6/DAI-ΔZα, pEF6/DAI-ΔZαβ, pEF6/DAI-ΔN199, pEF6/DAI-ΔN234, pEF6/DAI-ΔC115 and pEF6/DAI-Zαβ, were generated as pEF6/DAI-full. Deletion of the D3 region (pEF6/DAI-ΔD3), point mutations of either or both RHIMs in DAI (pEF6/DAI-mRHIM-1, pEF6/DAI-mRHIM-2 and pEF6/DAI-mRHIM-1, 2), and alanine substitutions (pEF6/DAI-179A5, pEF6/DAI-184A5, pEF6/DAI-189A5 and pEF6/DAI-194A5) were introduced by a PCR method in which each primer containing mutated sequences was used.

The reporter plasmid expressing luciferase driven by the HIV-1 5′ long terminal repeat (LTR), pLTR-luc, was constructed as-described below. A fraction consisting of the 5′LTR and a part of *gag* was amplified by PCR using the primers LTR-BamHI-fw, 5′-CAC CGG ATC CTG GAA GGG CTA ATT TGG-3′ and LTR-SA-rev, 5′-TTT TTG TAA TTT GTT TTT GTA ATT CTT TAG TCT TAC TTT TGT TTT GCT CTT CCT CTA TC-3′. The PCR products were purified and used as a template for the next round of PCR. The second round of PCR was performed with the following primer sets, LTR-BamHI-fw described above and LTR-SA-SpeI-rev, 5′-ACT AGT CCT GTA ATA AAC CCG AAA ATT TTG AAT TTT TGT AAT TTG TTT TTG TAA TTC TTT AGT C-3′. A 1.2 kb PCR product was digested with BamHI and SpeI and then inserted into the BamHI and SpeI sites of the pEF6/V5-

His vector from which the promoter region was removed by HindIII digestion. Finally, the vector was digested with XhoI and ligated with a 1.7 kb fragment from the pFB-Luc vector (Stratagene, USA) digested with SalI and XhoI. To generate the *cis*-element mutated reporter plasmids, the point mutations and deletions were introduced into pLTR-luc with the following primers: LTRmκB-fw, 5′-GCT TTC TAC AAT TTA CTT TCC GCT TTT TAC TTT CCA GGG AG-3′ and LTRmκB-rev, 5′-CTC CCT GGA AAG TAA AAA GCG GAA AGT AAA TTG TAG AAA GC-3′ for pLTRmκB-luc; LTRΔISRE-fw, 5′-CAG TGG CGC CCG AAC AGG GAC CAG AGG AGA TCT CTC GAC GC-3′ and LTRΔISRE-rev, 5′-GCG TCG AGA GAT CTC CTC TGG TCC CTG TTC GGG CGC CAC TG-3′ for pLTRΔISRE-luc.

pNL4-3*luc*Δ*env* and pNL4-3*thy1*Δ*env* (kindly provided by Dr. I.S.Y. Chen, University of California, Los Angeles, U.S.A.) [30,31], where the *env* gene has been deleted from pNL4-3 [32] and *nef* gene has been replaced with the *firefly luciferase* and *thy1.2* genes, respectively, and pHCMVG or pJD-1, were used for the production of pseudotyped HIV-1. The Moloney murine leukemia virus (Mo-MuLV)-based retrovirus vector, pFB-luc, was used for the production of pseudotyped MLV. The expression plasmid for the super-repressor form of IκBα (pSR-IκBα) was a kind gift from Dr. Shoji Yamaoka (Tokyo Medical and Dental University, Tokyo, Japan). A reporter plasmid, κB-luc, expressing firefly luciferase driven by five tandem NF-κB binding sites derived from IL-2 receptor α was kindly provided by Dr. Junichi Fujisawa (Kansai Medical University, Osaka, Japan).

2.2. Cells

293T cells and the 293-10A1 retroviral packaging cell line were maintained in Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 10% fetal bovine serum (Sigma, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (Invitrogen, USA).

2.3. Virus preparation

Pseudotyped viruses were generated as previously described [33]. Briefly, 293T cells or 293-10A1 cells (1.0×10^6) were plated on 60 mm dishes and transfected with pNL4-3lucΔenv (2.0 μg) together with vesicular stomatitis virus-G (VSV-G) (pHCMVG; 1.0 μg) or pmpho-MLV (pJD-1; 2.0 μg; kind gifts from Dr. I.S.Y. Chen), or with pFB-Luc (2.0 μg) using Lipofectamine 2000 (Invitrogen, USA). After 6 h, the medium was refreshed and cells were incubated for a further 48 h. Cultured supernatants were harvested and filtered through 0.45 μm pore-size filters. The virus preparation was treated with 20 μg/ml DNase I (Worthington Biochemical Corp., USA) in the presence of 10 mM $MgCl_2$ at 37° C for 40 min to avoid plasmid DNA contamination, aliquoted and stored at −80 °C.

2.4. Transfection and infection

Approximately 2–4 μg of expression vectors, pEF6/LacZ, pEF6/DAI-full or a series of pEF6/DAI-mutants, were transfected into 293T cells (5.0×10^5) seeded on 6-well plates using Lipofectamine 2000 and Opti-MEM (Invitrogen, USA) according to the manufacturer's instructions. After 6 h, the medium was aspirated and 2 ml of fresh medium was added and cells were incubated for a further 24 h. The transfected cells were harvested, reseeded (4.0×10^5) into 24-well plates, and incubated for an additional 24 h. Infection of pseudotyped viruses to 293T cells was performed for 6 h, then cells were washed with PBS, and cultured in fresh medium.

2.5. Single round HIV-1 infection assay

The concentration of HIV-1 p24 antigen in the virus preparation was determined using a p24 ELISA kit (ZeptoMetrix, USA). Forced expression of DAI or LacZ in 293T cells was conducted as described above. For infection of each HIV-1 pseudotype virus, a virus preparation corresponding to 10 ng of p24 was inoculated into cell cultures for 6 h. Cells were washed with PBS, incubated for 18–42 h, and lysed with 0.2 ml 1× passive lysis buffer (Promega, USA). The luciferase activities of each cell lysate were measured and relative values were indicated as the fold changes compared with control.

2.6. FACS analysis

To detect mouse Thy1.2 expression as an HIV-1-reporter antigen in 293T cells infected with HIV-1 pseudotyped with VSV-G (pNL4-3thy1Δenv/VSV-G), the cells were harvested with Cell Dissociation Solution (Sigma, USA), serially washed with DMEM containing 10% FCS and PBS containing 1% FCS, then incubated with or without rat anti-mouse Thy1.2-FITC antibody (Sigma, USA) or rat IgG2b-FITC isotype control antibody (MBL Co., Japan) in the dark at 4 °C for 30 min. Cells were washed with 1% PBS twice, fixed with 1% formalin, and then analyzed on an FACSCalibur (Becton Dickinson, USA) and data analysis was performed using CellQuest software (BD Biosciences, USA).

2.7. Reporter assay

Transfection of LacZ, DAI or mutant DAI-expressing vectors into 293T cells was described above. After 48 h, the cells were again transfected with 0.1 μg of pLTR-luc, pLTRmκB-luc or pLTRΔISRE-luc together with 0.01 μg of the Renilla luciferase expressing pRL-TK vector (Promega, USA) for 16 h, and then lysed with 0.2 ml of 1× passive lysis buffer. Firefly and Renilla luciferase activities of each cell lysate were measured and relative values were determined by dividing the value of Firefly luciferase activity by that of Renilla luciferase activity. Each of the calculated values was shown as the fold activation of luciferase activity relative to the control LacZ group.

2.8. Quantitative PCR (qPCR)

Total DNA was isolated from cells at 1 day post-infection by using the urea lysis method [30]. Briefly, cells were lysed

with 0.3 ml of urea lysis buffer (7 M urea, 2% sodium dodecyl sulfate, 1 mM EDTA, 10 mM Tris–HCl pH 8.0, 350 mM NaCl). Total DNA was purified from the cell lysates by phenol–chloroform extraction followed by ethanol preparation. Quantitative analyses of the rate of viral cDNA synthesis and the amplified products were performed using real-time qPCR (LightCycler; Roche Diagnostics, USA) as described previously [34].

2.9. Western blotting

293T cells were transfected with LacZ, DAI or DAI mutants expressing vector. At 2 days post-transfection, cells were harvested and lysed with cell lysis buffer (2% sodium dodecyl sulfate, 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 50 mM Tris–HCl pH 8.0, 150 mM NaCl), followed by centrifugation at 15,000*g* for 20 min at 4 °C. The supernatants were subjected to SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane and reacted with anti-myc antibody (Cell Signaling Technology, U.S.A.) or anti-human-α-tubulin antibody (Cedarlane laboratories, Canada). Visualization was performed using an LAS-1000 imaging system (Fuji Photo Fm, Japan).

2.10. Statistical analysis

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.

3. Results

3.1. Effect of DAI expression on HIV-1 replication

To investigate the effect of DAI on HIV-1 replication, 293T cells were transfected with DAI- or control LacZ-expressing vectors, and then infected with HIV-1 pseudotyped with VSV-G. Expression of DAI or LacZ was confirmed by Western blot using an anti-myc antibody. The level of HIV-1 gene expression measured by luciferase activity was more than 7-fold higher in cells expressing DAI than that in LacZ-expressing controls (Fig. 1a). In contrast, the replication of Mo-MuLV pseudotyped with 10A1-MLV envelope was comparable between cells expressing DAI and LacZ (Fig. 1b). Thus, this effect of DAI was not always observed among retroviruses as replication of Mo-MuLV was barely affected. Since the VSV-G envelope exploits the endocytotic pathway and the 10A1-MLV envelope mainly utilizes direct fusion for virus entry, we further examined the effects of DAI on replication of HIV-1 pseudotyped with amphotropic MuLV envelope (pJD-1), which uses the same receptor, Pit2, as the 10A1 envelope. As shown in Fig. 1c, replication of HIV-1 pseudotyped with the MuLV envelope was also enhanced under the expression of DAI. These results suggested that DAI increased replication of HIV-1 regardless of the pathway for viral entry.

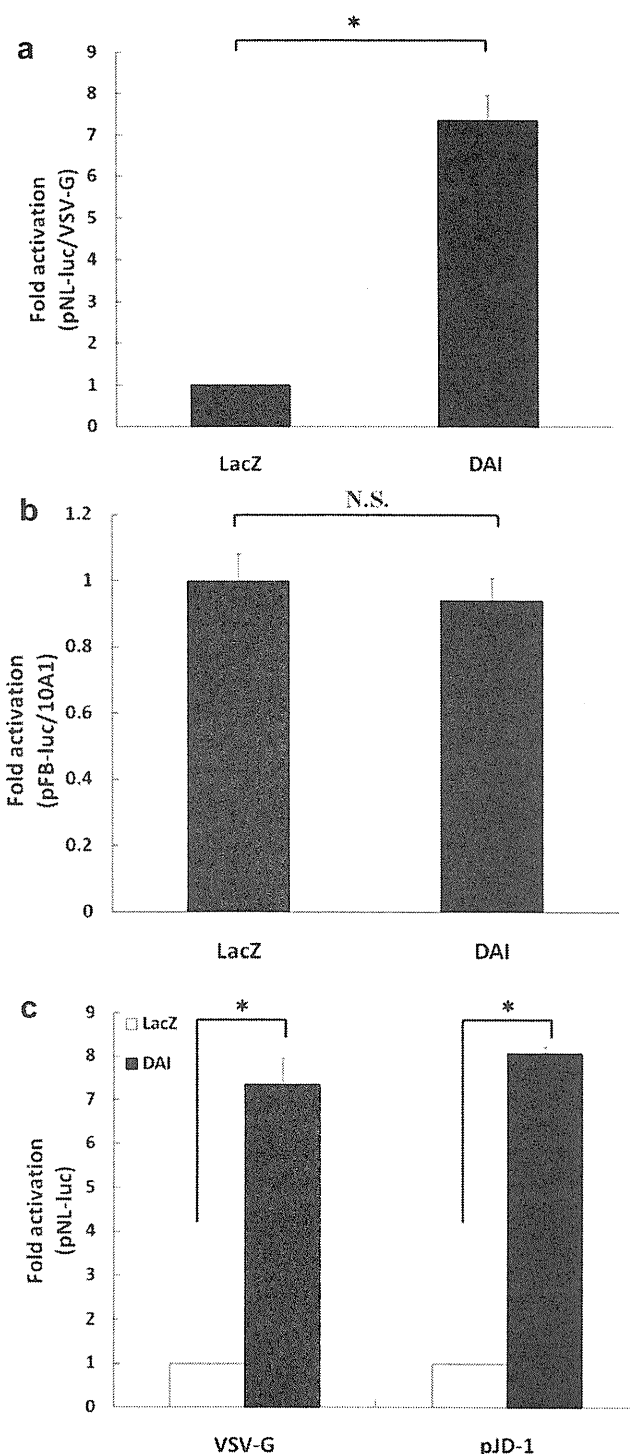

Fig. 1. Effects of DAI expression on HIV-1 replication. 293T cells were transfected with pEF-LacZ or pEF-DAI and two days after transfection, cells were infected with various viruses: (a) HIV-1 pseudotyped with VSV-G (pNL4-3lucΔenv/VSV-G); (b) Mo-MuLV pseudotyped with dual tropic MLV envelope (pFB-luc/10A1); (c) pNL4-3lucΔenv/VSV-G and HIV-1 pseudotyped with amphotropic MLV envelope (pNL4-3lucΔenv/pJD-1) for 6 h. Luciferase activities in infected cells were measured at 24 h post-infection. Data are shown as a fold activation of luciferase activity relative to that in pEF-LacZ-transfected controls, and represent means ±S.D. of duplicate or triplicate samples. N.S. represents not significant. *$P < 0.01$.

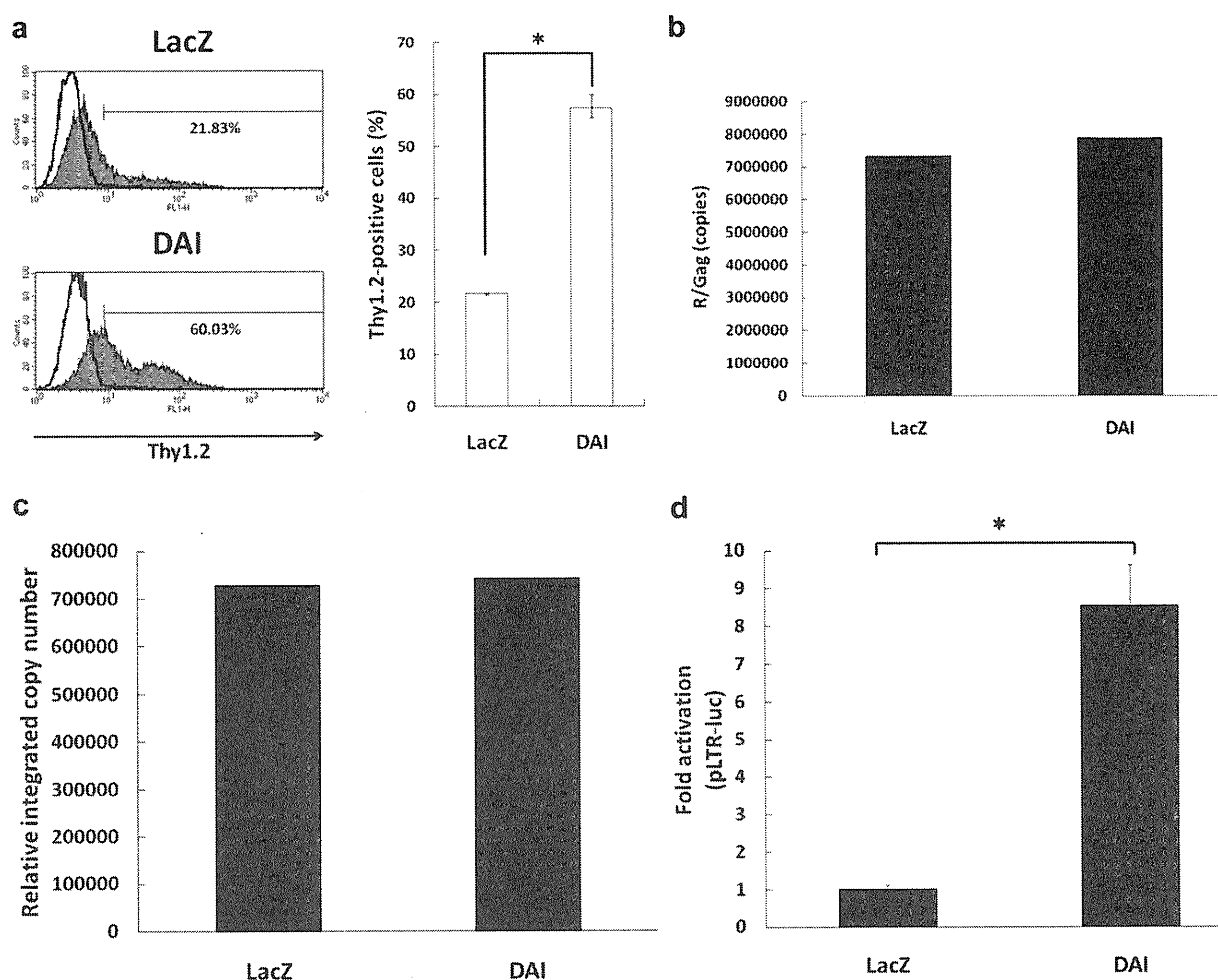

Fig. 2. HIV-1 replication step(s) affected by the expression of DAI. 293T cells were transfected with pEF-LacZ or pEF-DAI. At 2 days post-transfection, cells were infected with HIV-1 pseudotype expressing mouse Thy1.2 (pNL4-3thy1Δenv/VSV-G) and subjected to FACS analysis (a) following staining with FITC-conjugated anti-Thy1.2 (closed histogram) or isotype control antibody (solid line) 24 h post-infection (left). The rate of Thy1.2-positive cells in LacZ- or DAI-transfected cells were shown (right). (b, c) LacZ- or DAI-expressing 293T cells were infected with HIV-1 pseudotyped with VSV-G (pNL4-3lucΔenv/VSV-G), and the level of *de novo* synthesized viral DNA (b) and relative amounts of integrated proviral DNA (c) were measured by PCR using R/Gag or R/U5 primer sets as-described in Methods. (d) 293T cells were transfected with pEF-LacZ or pEF-DAI. At 2 days post-transfection, cells were co-transfected with HIV-1 LTR reporter plasmid (pLTR-luc) and control reporter plasmid (pRL-TK). Luciferase activities in these cells were measured after a 16 h incubation, and the values are shown as a fold activation of luciferase activity relative to that in pEF-LacZ-transfected controls. Data represent means ±S.D. of duplicate or triplicate samples. *$P < 0.01$.

3.2. HIV-1 replication cycle affected by DAI

To determine which steps in the HIV-1 replication cycle were affected by DAI, we used flow cytometry following HIV-1 infection of LacZ- or DAI-expressing 293T cells (Fig. 2a). In this experiment, we used HIV-1-thy1, in which the mouse thy1.2 gene was inserted for expression marker to monitor the levels of viral infection and gene expression in a single cell level [31]. Compared to the control, the percentage of HIV-1-infected cells (Fig. 2a) and mean fluorescence intensity of thy1.2 expression (data not shown) were significantly increased in the culture of DAI-expressing cells. We next investigated whether efficiency of reverse transcription and integration of viral DNA might be affected by DAI expression using qPCR with different primer sets [34]. As shown in Fig. 2b and c, neither the levels of *de novo* synthesized viral DNA by reverse transcription nor integrated forms of viral DNA were enhanced by DAI expression. We then examined the effect of DAI on HIV-1 transcription using a reporter plasmid expressing luciferase driven by HIV-1 LTR (pLTR-Luc). We found that transcription of HIV-1 was markedly increased by expression of DAI (Fig. 2d). Thus, DAI promoted replication of HIV-1 by upregulating viral transcription, but did not affect reverse transcription and integration of viral DNA.

3.3. A cis-element in the HIV-1 LTR was implicated in DAI-mediated enhancement of transcription

Previous studies have indicated that dsDNA-stimulated DAI led to the activation of the transcription factors, IRF3 and NF-κB [7,35,36]. The HIV-1 genome contains binding sites

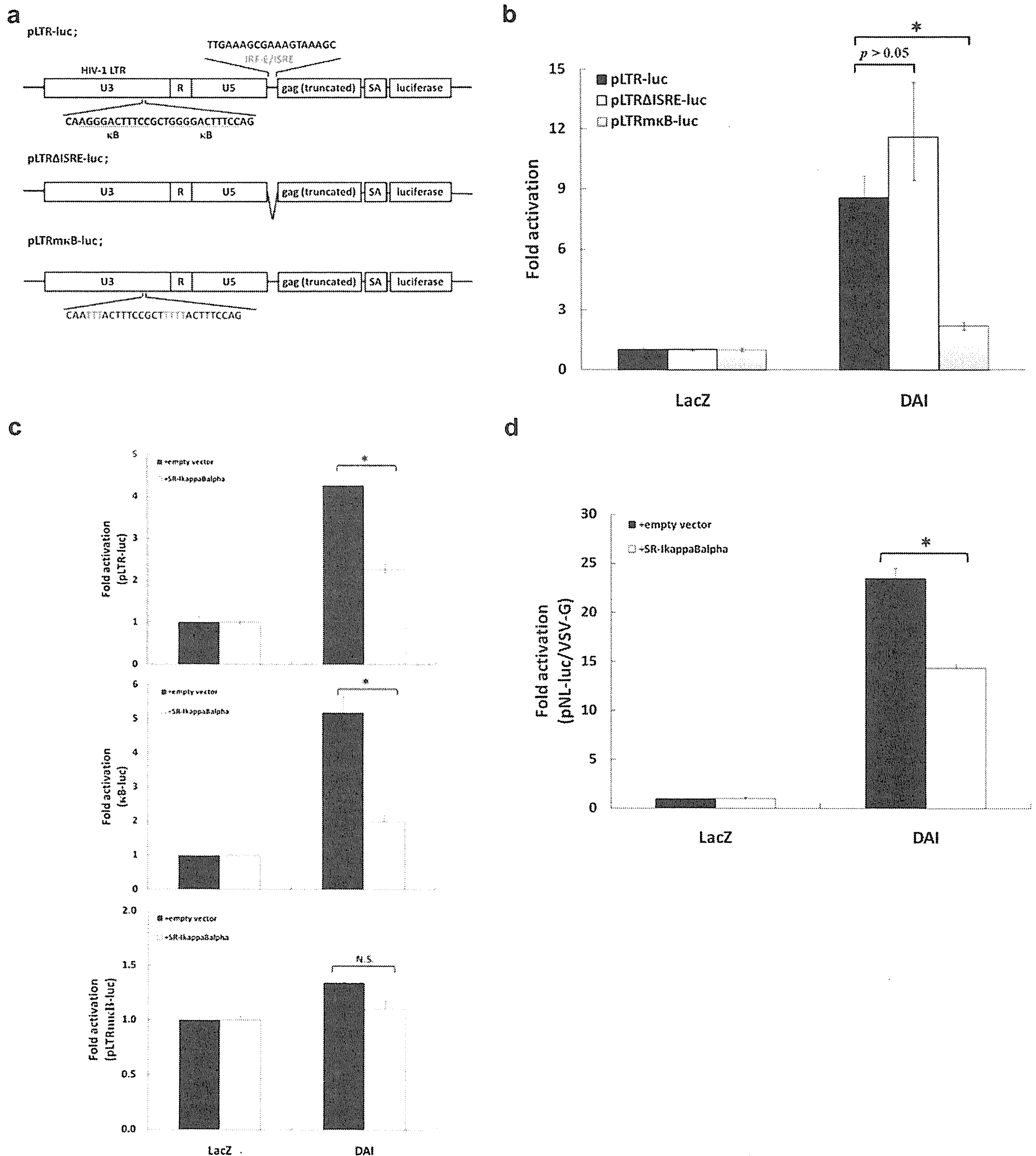

Fig. 3. The *cis*-element in the HIV-1 LTR responsible for DAI-mediated activation. (a) The positions and nucleotide sequences for two κB sites and the IRF-E/ISRE site present in the HIV-1 LTR reporter plasmid (pLTR-luc) are indicated. A mutant reporter plasmid, pLTRΔISRE-luc was constructed by deletion of IRF-E/ISRE. Another mutant reporter plasmid (pLTRmκB-luc) was constructed by substitution of two series of G with T (red) in κB sites (underlined). (b) HIV-1 LTR reporter assays were conducted in 293T cells that were transfected with pEF-LacZ or pEF-DAI. Two days later, cells were transfected with wild type (pLTR-luc) or mutant (pLTRmκB-luc or pLTRΔISRE-luc) reporter plasmids. After a 16 h incubation, luciferase activities were measured. (c) 293T cells were co-transfected with pEF-LacZ or pEF-DAI, and pSR-IκBα (shaded bar) or empty vector (unshaded bar). Reporter assays using pLTR-luc (upper panel), κB-luc (middle panel) or pLTRΔISRE-luc (lower panel) were conducted as-described in (b). (d) 293T cells were co-transfected with pEF-LacZ or pEF-DAI, and pSR-IκBα (shaded bar) or empty vector (unshaded bar). Single round HIV-1 infection assay using pNL-luc/VSV-G was conducted as-described in Fig. 1a. Values are indicated as the fold activation of luciferase activity relative to pEF-LacZ controls. Data represent means ±S.D. of duplicate or triplicate samples. N.S. represents not significant. $^{*}P < 0.01$.

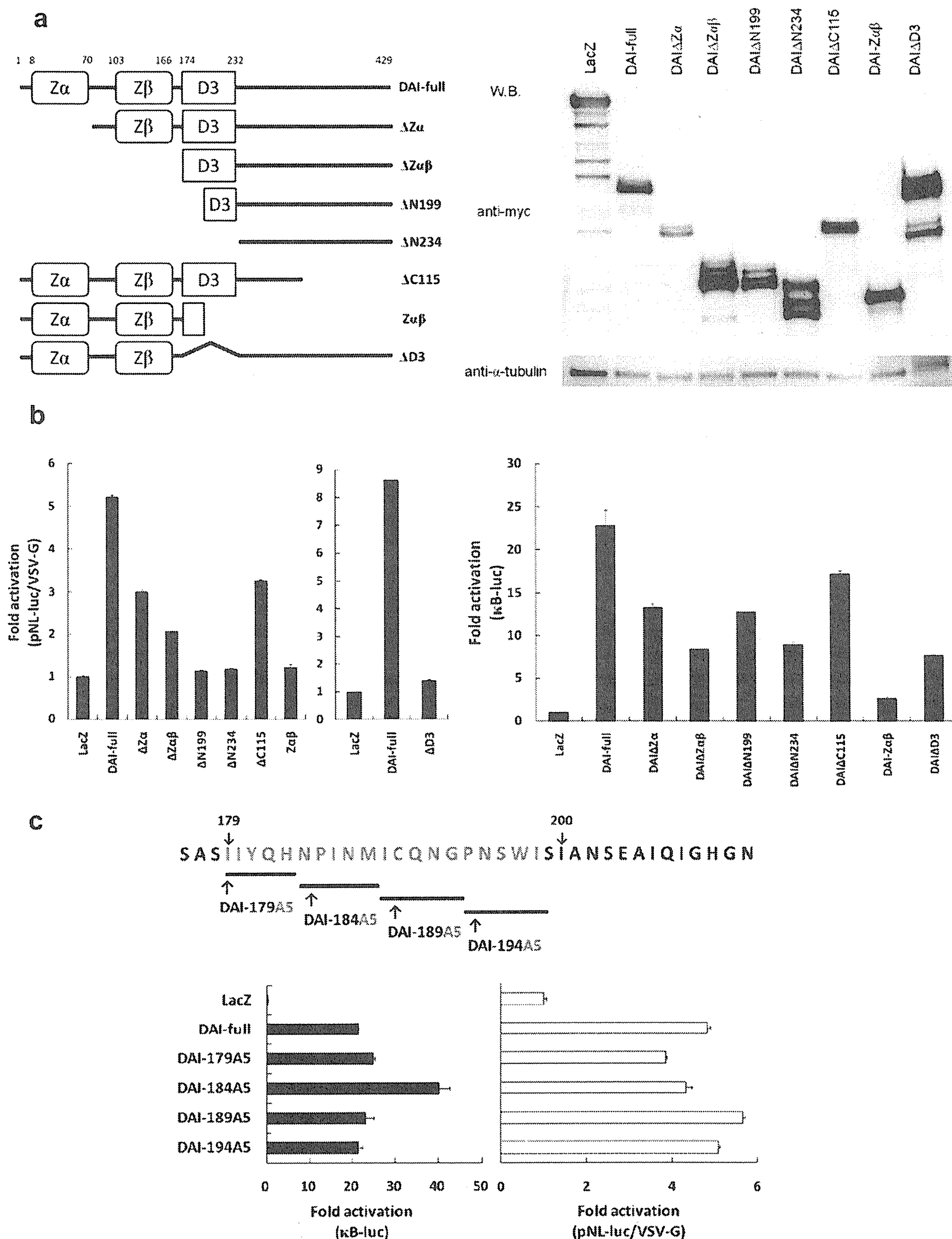

Fig. 4. Responsible region in DAI for the enhancement of HIV-1 transcription. (a) Schematic illustration of wild type DAI and a series of truncated mutant DAI constructs (left). The expression level of LacZ, WT-DAI or each DAI mutant was examined by Western blotting (right). The level of endogenous α-tubulin in each sample was shown as an internal control (lower panel). (b) The wild type and mutant DAI constructs were transfected to 293T cells. Single round infection assay using HIV-1/VSV-G pseudotype virus (left panel and middle), or reporter assay using κB-luc (right panel) were conducted as-described in Fig. 3. (c) Four additional DAI mutants (179A5, 184A5, 189A5, 194A5) were constructed, in which five serial amino acid residues in a part of the D3 region were substituted to alanines. 293T cells were transfected with each expression vector indicated, and luciferase assays were performed at 16 h post-transfection with κB-luc (bottom left) or single round infection assays were conducted at 24 h post-infection (bottom right). The positions of amino acid substitution (red) are indicated in the top panel. The values of fold activation of luciferase activities relative to pEF-LacZ-transfected controls are shown. Data represent means ±S.D. of duplicate samples.