Inhibition of human immunodeficiency virus type 1 replication by Z-100, an immunomodulator extracted from human-type tubercle bacilli, in macrophages

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Z-100 is an arabinomannan extracted from Mycobacterium tuberculosis that has various immunomodulatory activities, such as the induction of interleukin 12, interferon gamma (IFN-γ) and β-chemokines. The effects of Z-100 on human immunodeficiency virus type 1 (HIV-1) replication in human monocyte-derived macrophages (MDMs) are investigated in this paper. In MDMs, Z-100 markedly suppressed the replication of not only macrophage-tropic (M-tropic) HIV-1 strain (HIV-1JR-CSF), but also HIV-1 pseudotypes that possessed amphotropic Moloney murine leukemia virus or vesicular stomatitis virus G envelopes. Z-100 was found to inhibit HIV-1 expression, even when added 24 h after infection. In addition, it substantially inhibited the expression of the pNL43lucΔenv vector (in which the env gene is defective and the nef gene is replaced with the firefly luciferase gene) when this vector was transfected directly into MDMs. These findings suggest that Z-100 inhibits virus replication, mainly at HIV-1 transcription. However, Z-100 also downregulated expression of the cell surface receptors CD4 and CCR5 in MDMs, suggesting some inhibitory effect on HIV-1 entry. Further experiments revealed that Z-100 induced IFN-β production in these cells, resulting in induction of the 16-kDa CCAAT/enhancer binding protein (C/EBP) β transcription factor that represses HIV-1 long terminal repeat transcription. These effects were alleviated by SB 203580, a specific inhibitor of p38 mitogen-activated protein kinases (MAPK), indicating that the p38 MAPK signalling pathway was involved in Z-100-induced repression of HIV-1 replication in MDMs. These findings suggest that Z-100 might be a useful immunomodulator for control of HIV-1 infection.

INTRODUCTION

Monocytes/macrophages are presumed to be the initial target of human immunodeficiency virus type 1 (HIV-1) in mucosa, and these cells might serve as virus reservoirs during all stages of HIV-1 infection (Connor & Ho, 1994; Mann et al., 1990; van’t Wout et al., 1994). HIV-1 entry into monocytes/macrophages is mediated by binding of HIV-1 envelope glycoproteins to the cell-surface receptor CD4 and a coreceptor (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996; Hill et al., 1997), and most primary HIV-1 isolates are macrophage-tropic (M-tropic), utilizing the CCR5 coreceptor. Individuals with the CCR5 Δ32 mutation (Stephens et al., 1998) have partial resistance to HIV-1 infection and this indicates clearly that suppression of virus entry into monocytes/macrophages could be a strategy for limiting primary HIV-1 infection.

HIV-1 replication in monocytes/macrophages is also influenced by their differentiation status. HIV-1 replication can be detected in tissue macrophages present in the lung, skin and mucosa in vivo. HIV-1 replicates well in monocyte-derived macrophages (MDMs) in vitro, but poorly in monocytes (Kalter et al., 1991; Pauza et al., 1988; Smith et al., 1994). However, conversely, in the presence of Mycobacterium tuberculosis, HIV-1 replication is inhibited in macrophages, but enhanced in monocytes (Mancino et al., 1997; Weiden et al., 2000). This observation suggests that permissiveness of monocytes/macrophages for HIV-1 infection depends on their state of activation and differentiation. Zybarth et al. (1999) have demonstrated that lipopolysaccharide (LPS), a potent stimulator of macrophages, inhibits HIV-1 replication in MDMs. Similarly, HIV-1 replication is reduced in dendritic cells where maturation has been driven by CD40 ligand binding.
As LPS also induces dendritic cell maturation (Kaisho et al., 2001), macrophages and dendritic cells might share an LPS-responsive signalling pathway. LPS is known to bind to the cell-surface receptor CD14 and to transmit intracellular signals through Toll-like receptor (TLR) 4 (da Silva Correia et al., 2001). These immunomodulators, in inducing macrophage activation or dendritic-cell maturation, might induce resistance to HIV-1 infection. Interestingly, murabutide, a synthetic derivative of peptidoglycan muramyl dipeptide that is undergoing HIV-1 clinical trials, inhibits HIV-1 replication by activating macrophages (De La Tribonniere et al., 2003; Vidal et al., 2001).

Z-100, a mixture of an arabinomannan and other small-sized components (Kobatake et al., 1981), is used clinically in Japan in patients with leukopenia caused by radiation therapy. This compound is purified from an extract of human-type M. tuberculosis strain Aoyama B. Z-100 consists mainly of three fractions, A, B and C, when separated by high performance gel filtration chromatography. Fraction A contains 67.6 % sugars, mainly arabinomannan; fraction B contains 83.5 % sugars, mainly mannann; and fraction C contains 41.1 % sugars, mainly mannann and glucan, with large amounts of amino-acid-containing peptidoglycan.

The immunomodulatory activities of Z-100 have been described previously. These include anti-tumour (Sasaki et al., 1993; Suzuki et al., 1986) and anti-metastatic (Emori et al., 1996; Kobayashi et al., 1997) activities against several syngeneic tumours in experimental animals, protective effects against opportunistic infections in immunosuppressed hosts (Kawamura et al., 1990; Sasaki et al., 1997) and cytokine-inducing activities, such as interleukin (IL) 12 (Kobayashi et al., 1995) and interferon (IFN)-γ (Hayashi et al., 1981) production in vivo and in vitro. The anti-tumour activities of Z-100 might be due to suppression of tumour-associated Th2-type cytokines, for example IL4 and IL10 (Oka et al., 1999). Our group have demonstrated previously that Z-100 restored the balance of Th1/Th2 cell responses in tumour-bearing mice by upregulating IL12 and downregulating IL10 production by macrophages (Oka et al., 2003).

In addition to its anti-tumour activity, Z-100 decreased the severity of murine AIDS (caused by LP-BM5 murine leukemia virus in C57BL/6 mice) by repressing Th2-type responses (Sasaki et al., 2001). Recently, we found that Z-100 could induce β-chemokines, for example macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES, which are natural ligands for CCR5 in human MDMS. Furthermore, Z-100 induces MIP-1α in a phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophage cell line by activating the p38 mitogen-activated protein kinase (MAPK) signalling pathway (K. Yoshinaga, personal communication). These findings encouraged us to investigate the anti-HIV-1 effects of Z-100.

In this paper, we demonstrate that Z-100 suppresses HIV-1 replication in acutely infected MDMS. Although Z-100 induced β-chemokine production by MDMS, we found that, in the main, Z-100 suppressed late stages of HIV-1 replication, presumably at the level of virus transcription. Further analysis revealed that this suppression required IFN-β induction in MDMS, mediated through activation of the p38 MAPK signalling pathway. These findings suggest that Z-100 might be a useful immunomodulator for control of HIV-1 infection.

**METHODS**

**Cell culture.** Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy seronegative donors by Ficol-Hypaque density-gradient centrifugation (Ficoll-Paque PLUS; Amersham Bioscience). Monocytes were purified from fresh PBMCs by magnetic cell sorting (MACS) using a monoclonal 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 and 5 ng granulocyte/macrophage colony-stimulating factor (GM-CSF) ml⁻¹ (Stemcell Technologies) in order to generate MDMS. THP-1 cells were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated fetal bovine serum (FBS; Sigma) and stimulated to differentiate into macrophages by the addition of PMA (5 nM; Sigma) for 24 h prior to infection. Monkey kidney fibroblast line COS7 or human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10 % FBS.

**Virus preparation and infection.** M-tropic HIV-1JR-CSF strain (Koyanagi et al., 1987) (kindly provided by Y. Koyanagi, Tohoku University) was grown in phytohaemagglutinin (PHA; Difco Laboratories)-stimulated PBMCs cultured in RPMI 1640 medium supplemented with 10 % FBS and 10 IU recombinant IL2 (Shionogi) ml⁻¹ for 5–7 days. The supernatants were filtered and stored at −80 °C until use. To infect MDMS in vitro, culture supernatant that contained HIV-1JR-CSF (1 ng p24 ml⁻¹) was added to each well and incubated for 24–48 h at 37 °C. After this time, virus was removed by washing the cells twice and fresh medium was added. Cultures were incubated for 7 days with fresh medium in the presence or absence of Z-100 at a range of concentrations and at 37 °C in humidified 5 % CO₂. Virus replication was assessed by the level of virus p24 antigen in the supernatants. Pseudotype viruses were generated by co-transfection of the monkey kidney fibroblast line COS7 or human embryonic kidney 293T cells with the pNL43Δenv vector (in which the env gene is replaced with the firefly luciferase gene) and either an M-tropic HIV-1 envelope expression vector (pJR-FL) (kindly provided by Dr Yoshio Koyanagi, Tohoku University), an amphotropic Moloney murine leukemia virus (MuLV) envelope expression vector (pDJ-1) or a vesicular stomatitis virus (VSV)-G envelope protein (pHCMV) (Masuda et al., 1995; Yee et al., 1999) (kindly provided by Dr Irvin S. Y. Chen, University of California), using Lipofectamine (Gibco BRL). Culture supernatants (4–5 ml) from the transfected cells were harvested at 48 h post-transfection, filtered through 0.45-μm-pore filters and used as virus preparations. Viral gene expression from each plasmid vector was confirmed by luciferase activity. The cells were lysed at 48 h post-transfection with 1 ml 1× luciferase lysis buffer (Promega) and 1 μl of each cell lysate was assayed for luciferase. MDMS (2.5×10⁵) were infected with 0.5–1 ml of these viruses in the presence of 10 μg polybrene (Sigma) ml⁻¹ at 37 °C for 6 h. Viruses were then removed and the cells were cultured with fresh medium in the presence or absence of Z-100 at 37 °C in humidified 5 % CO₂. Virus replication in infected...
MDMs was assessed by luciferase activity. In some experiments, Z-100 was added to MDMs either 24 h before infection or simultaneously with infection and then left in the culture medium. MAPK inhibitors were added to the MDM cultures 30 min before Z-100 treatment.

Transfection of viral DNA. MDMs (5 × 10^5) were transfected with the pNL4-3Luc.Aenv vector using JetPEI (PolyPlus transfection). Following 24 h incubation, MDMs were washed, overlaid with fresh medium and incubated at 37 °C in humidified 5% CO_2. Cells were lysed 96 h post-transfection with 100 µl 1 × luciferase lysis buffer and the luciferase activity was assessed.

Reagents and antibodies. Z-100, consisting of a mixture of polysaccharides including arabinox, mannose, glucose and other small-sized components, was prepared from hot-water extracts of M. tuberculosis strain Aoyama B, purified by deproteinization and dialysis, and was supplied by Zeria Pharmaceutical Co. Ltd (Tokyo, Japan). The Z-100 stock solution was of clinical-use grade and confirmed as containing 2 mg D-arabinose ml⁻¹ and being free from endotoxins, and was kept at 4 °C and diluted just before use. The inhibitors SB 203580 and Ro 318220 were purchased from Calbiochem. PD 98059 and PDTC (ammonium pyrrolidine dithiocarbamate) inhibitors were purchased from Sigma. SP 600125 inhibitor was purchased from Tocris Cookson Inc. Anti-CCAAT/ enhancer binding protein (C/EBP) β (C-19) and anti-rabbit horseradish peroxidase (HRP) antibodies were purchased from Santa Cruz Biotechnology. Anti-human IFN-β neutralizing antibody and isotype-matched control antibody (normal goat IgG) were purchased from R&D Systems. Fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and anti-CCR5 monoclonal antibodies (mAbs) and their isotype-matched control immunoglobulins (Igs) were purchased from BD Biosciences.

Flow cytometry analysis. MDMs (5 × 10^5) were incubated for 15 min at room temperature with FITC-conjugated anti-CD4 or FITC-conjugated anti-CCR5 mAbs or with isotype-matched control Igs to assess surface receptor expression. Cells were washed twice, resuspended, fixed in 1% paraformaldehyde and analysed with a FACSCalibur flow cytometer (Becton Dickinson). Live cells were gated according to forward- and side-scatter characteristics and the percentage of positive cells and the mean fluorescence intensity (MFI) were recorded.

ELISA. The level of HIV-1 p24 antigen in the culture supernatants was measured by ELISA using a RETRO-TEK HIV-1 p24 Antigen ELISA kit (Zeptometrix). The level of MIP-1α, MIP-1β and RANTES in MDM culture supernatants was determined using ELISA kits purchased from R&D Systems. An ELISA kit specific for human IFN-β detection (PBL Biomedical Laboratories) was also used.

Luciferase activity. Infected cells were harvested at 4 days post-infection for luciferase analysis. The cell pellet from individual wells was washed twice with PBS and lysed with 150 µl 1 × luciferase lysis buffer. Cell lysate (10 µl) was analysed using a luminometer (Lumat LB 9507; EG&G Berthold). The luciferase activity was indicated as U µl⁻¹.

Preparation of nuclear extracts and Western blot analysis. MDMs (4 × 10^6) or PMA-differentiated THP-1 cells (4 × 10^5) were stimulated with 100 µg Z-100 ml⁻¹ for 96 h at 37 °C. Nuclear extracts were then prepared using a Nuclear Extract kit (Active Motif) according to the manufacturer's instructions. The protein concentration of the extracts was determined by Bradford protein assay (Bio-Rad Laboratories). Nuclear protein (60 µg) was separated by SDS-PAGE on a 10–20% gel (SuperSep; Wako) and transferred onto a nitrocellulose membrane (ATTO). The membrane was incubated with anti-C/EBPβ antibodies followed by anti-rabbit HRP antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (ECL Western blotting detection reagents; Amersham Biosciences) and quantified using a Science Lab 98 Image Gauge (Fuji Photo Film).

Statistical analyses. The paired t-test was used to compare the means of the two groups. For the one-way analysis of variance (ANOVA) followed by multiple comparisons tests, Dunnett’s two-tailed test was used. In both tests, P values <0.05 were considered statistically significant.

RESULTS

Z-100 inhibits wild-type HIV-1 replication in acutely infected MDMs

A range of Z-100 concentrations (0.1–100 µg ml⁻¹) was added to the replication-competent HIV-1JR-CSF-infected MDM culture in order to evaluate its HIV-suppressive activity. Fig. 1(a) shows the level of virus p24 antigen in untreated or Z-100-treated MDM culture supernatant, assayed by ELISA at 7 days post-infection. The addition of Z-100 at 0·1, 1, 10 or 100 µg ml⁻¹ resulted in 31·8, 55·7, 79·0 or 93·5% inhibition of virus replication, respectively; that is, in a dose-dependent manner (P<0·01). Z-100 at a concentration of 100 µg ml⁻¹ inhibited HIV-1 replication markedly; however, Z-100 added at <10 µg ml⁻¹ had varied effects in blood donors. Therefore, a concentration of 100 µg ml⁻¹ was used in further experiments to ensure maximal inhibition.

The effect of adding Z-100 at different time points following HIV-1JR-CSF infection of MDMs was evaluated. Z-100 (100 µg ml⁻¹) was added to MDMs either 24 h before infection (−24 h), simultaneously with infection (0 h) or following the infection period (+48 h) and the results are shown in Fig. 1(b). HIV-1 replication was suppressed markedly (>97·9%) when Z-100 was added either −24 or 0 h from infection. Virus replication was suppressed significantly (78·1%, P<0·01) even when Z-100 was added at +48 h post-infection, indicating that Z-100 suppressed HIV-1 replication even after completion of the initial virus infection.

Effects of Z-100 on β-chemokine production and cell surface HIV-1 receptors

β-Chemokines, natural ligands of CCR5, are known to block M-tropic HIV-1 entry competitively (Cocchi et al., 1995; Verani et al., 1997). High levels of β-chemokines (8·1 ng MIP-1α ml⁻¹, 11·0 ng MIP-1β ml⁻¹ and 2·5 ng RANTES ml⁻¹) were detected in MDM culture supernatants treated with Z-100 at 100 µg ml⁻¹ for 24 h. Therefore, we evaluated whether these β-chemokines are responsible for the HIV-suppressive activity of Z-100 by adding neutralizing antibodies against MIP-1α, MIP-1β and RANTES into Z-100-treated, HIV-1-infected MDM cultures. We found that these neutralizing antibodies had no effect on the Z-100-induced HIV suppression (data not shown).
The influence of Z-100 on CD4 and CCR5 receptor expression in MDMs was examined further by flow cytometry (Fig. 2). Both CD4 and CCR5 receptor expression levels were reduced slightly in MDMs cultured with Z-100 for 24 h (Fig. 2). This result explains partly why Z-100 suppressed HIV-1-replication in MDMs more effectively when added before rather than after HIV-1 infection.

**Z-100 inhibits single-round virus replication of pseudotype HIV-1 in MDMs at late stages**

Single-round infection assay was used to determine the stages of HIV-1 suppression by Z-100. In this assay, we used an *env*-defective HIV-1 clone carrying the luciferase gene (pNLucΔenv) pseudotyped with an envelope from either M-tropic HIV-1JR-FL, amphotropic MuLV or VSV-G. MDMs, pre-treated with Z-100 for 24 h, were exposed to these viruses and maintained for 4 days in the absence or presence of Z-100. Virus expression was evaluated by luciferase activity expressed in MDMs (Fig. 3a). Z-100 suppressed virus expression of M-tropic HIV-1JR-FL (88.1%), amphotropic MuLV (81.9%) and VSV-G (67.1%) significantly. As MuLV and VSV-G pseudotype viruses do not require β-chemokine receptors for infection, this result indicates that Z-100 suppressed HIV-1 replication at the post-entry stages.

The addition of Z-100 to MDM cultures at different time points following amphotropic MuLV or VSV-G pseudotype HIV-1 infection confirmed this result. As shown in Fig. 3(b), pseudotype HIV-1 expression was inhibited significantly in any culture to which Z-100 was added −24 h, or 6 h and 24 h post-infection. It is noteworthy that Z-100 suppressed virus replication even when added 24 h after infection. These findings suggest that Z-100 inhibits virus replication mainly at late stages of virus replication, presumably at the level of virus transcription, because proviral DNA integration must have been completed by 24 h post-infection (Masuda *et al.*, 1995).

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**Fig. 1.** Z-100 inhibits M-tropic HIV-1 replication in acutely infected MDMs. (a) Dose-dependent inhibition of HIV-1 replication by Z-100. MDMs were infected with HIV-1JR-CSF for 24 h, washed and maintained in the absence (○) or presence (●) of Z-100 at 0.1–100 μg ml⁻¹. (b) Effect of Z-100 (100 μg ml⁻¹) on HIV-1 replication in MDMs infected with HIV-1JR-CSF for 48 h when added 24 h before infection (−24 h), simultaneously with infection (0 h) or following infection (+48 h). For the MDM samples pre-treated with Z-100, the same concentration of Z-100 was re-added after washing at 48 h and then left in the culture thereafter. Virus p24 antigen levels were measured in culture supernatants by ELISA 7 days post-infection. Values represent means±SEM of four replicates. ***, *P*<0.01 compared to medium control (Dunnett’s test).

**Fig. 2.** Downregulation by Z-100 of CD4 and CCR5 membrane expression in MDMs. MDMs were cultured in the absence or presence of Z-100 (100 μg ml⁻¹) for 24 h and the expression of CD4 or CCR5 (filled histograms) and isotype-matched control (open histograms) in these cells was analysed using a flow cytometer. The percentage of positive cells and the MFI for CD4 and CCR5 receptors are indicated.
Suppression of virus transcription by Z-100 requires p38 MAPK activation

The p38 MAPK protein is an important mediator of stress- or inflammatory cytokine-induced gene expression (Ono & Han, 2000). In particular, p38 kinase is known to play a key role in LPS-induced signal transduction pathways that lead to cytokine synthesis (Lee & Young, 1996). p38 MAPK activation has been suggested recently as a major mechanism for LPS-mediated HIV-suppressive activity in macrophages (Zybarth et al., 1999). The effect of a specific p38 MAPK inhibitor, SB 203580, on Z-100 inhibition of virus replication was evaluated in order to determine whether Z-100-induced, HIV-1 suppression involves a mechanism similar to that of LPS (Fig. 4a). M-tropic HIV-1\textsubscript{JR-CSF} replication was suppressed by 91.4% in the presence of Z-100 compared to without Z-100. When SB 203580 (1 \mu M) was added to HIV-1-infected MDM cultures 30 min before Z-100 treatment, HIV-1 suppression by Z-100 was reduced significantly, to 27.7%. A similar dose-dependent alleviation of Z-100-mediated HIV-1 suppression was observed when amphotropic MuLV pseudotype HIV-1 was used on MDMs or PMA-differentiated THP-1 cells (Fig. 4b). The addition of SB 203580 alone to infected MDM cultures did not alter virus replication markedly. These results suggest that Z-100 HIV-suppressive activity requires p38 MAPK activation, analogous to that with LPS.

Various inhibitors were used to assess whether other MAPK signalling cascades were involved in Z-100-mediated HIV suppression. These included SP 600125, a selective and reversible inhibitor of c-jun N-terminal kinase (JNK), PD 98059, a selective inhibitor of MAP kinase kinase-1 (MKK1), and PDTC, a specific inhibitor of NF-\kappaB. As shown as Table 1, SB 203580 abolished Z-100-mediated HIV suppression. In addition, PDTC restored virus replication in Z-100-treated MDM cultures (Table 1); however, it was not clear whether there was involvement of NF-\kappaB pathways in Z-100-induced HIV-1 suppression, because PDTC alone markedly enhanced virus replication in MDM cultures without Z-100. We therefore investigated the role of MAPK signalling pathways.

Effects of Z-100 in HIV-1 transcription

The results shown in Fig. 3 suggested that Z-100 inhibits virus replication at post-entry stages of virus replication. Therefore we assessed the inhibitory effect of Z-100 on virus transcription by transfecting MDMs directly with env-defective HIV-1 vector expressing luciferase (pNL43luc\textalpha env). As shown in Fig. 5, luciferase activity in MDMs cultured for 4 days post-transfection with Z-100 was markedly lower than in the control culture without Z-100 (P < 0.01; Fig. 5). Moreover, pre-treatment of MDMs with SB 203580 at 1 \mu M significantly alleviated Z-100 suppression (P < 0.05). These results indicate that Z-100 suppressed virus transcription and that this suppression required p38 MAPK activation.
Z-100 enhances the induction of inhibitory 16-kDa C/EBPβ isoform in macrophages

HIV-1 transcription is controlled by cellular and virus factors that bind the HIV-1 long terminal repeat (LTR) (Gaynor, 1992). Recent reports have demonstrated that the C/EBP family of transcription factors is essential for HIV-1 replication in macrophages, but not in CD4+ T cells (Henderson & Calame, 1997). C/EBPβ (also called NF-IL6) and intact HIV-1 LTR binding sites are required for virus transcription; however, the dominant-negative 16-kDa isoform of C/EBPβ inhibits HIV-1 replication and LTR promoter function (Descombes & Schibler, 1991; Henderson et al., 1995, 1996).

Nuclear protein extracted from PMA-differentiated THP-1 cells incubated with or without Z-100 for 4 days was analysed to evaluate if Z-100 could induce the inhibitory isoform. As shown in Fig. 6, the 16-kDa inhibitory C/EBPβ isoform increased 5-7-fold in the presence of Z-100, whereas expression of the 37-kDa stimulatory C/EBPβ isoform was only increased slightly by Z-100 treatment (Fig. 6a) in these cells. This resulted in an increase in the ratio of inhibitory C/EBPβ isoform to stimulatory isoform of 89% following Z-100 addition. Pre-treatment of THP-1 macrophages with SB 203580 increased the stimulatory isoform and decreased the inhibitory isoform in Z-100-treated THP-1 macrophages (Fig. 6a), resulting in a decrease in the ratio of inhibitory to stimulatory isoforms to 51%. Induction of the inhibitory C/EBPβ isoform by Z-100 and its suppression by SB 203580 was observed in primary macrophages (Fig. 6b). These results are consistent with those in Fig. 4(b) that show Z-100-mediated HIV suppression in MDMs was alleviated greatly by SB 203580 pre-treatment (10 μM).

These findings suggested that Z-100 inhibits HIV-1 transcription by repressing LTR by induction of the inhibitory 16-kDa C/EBPβ isoform, for which p38 MAP kinase activation was required.

Z-100 inhibits HIV-1 replication by induction of the inhibitory C/EBPβ isoform by IFN-β release from MDMs

Honda et al. (1998) have reported that M. tuberculosis infection induces an IFN-β response in macrophages and that IFN-β induces the expression of an inhibitory 16-kDa C/EBPβ transcription factor. Consequently, we examined IFN-β involvement in induction of the inhibitory 16-kDa C/EBPβ isoform in Z-100-treated MDMs. As shown in Fig. 7, the addition of IFN-β-neutralizing antibodies (1 ng ml⁻¹) to Z-100-treated MDMs suppressed the enhanced inhibitory C/EBPβ isoform levels to that of the control (without Z-100). The role of IFN-β in Z-100-induced HIV-1 suppression was examined using MuLV pseudotype HIV-1. As shown in Fig. 7(b), virus expression, suppressed by Z-100, was elevated by adding IFN-β.
**Table 1.** Inhibitory effect of MAPK or NF-κB inhibitors on HIV-suppressive activity of Z-100 in MDMs

Six hours after amphotropic MuLV pseudotype HIV-1 infection, MDMs were washed and maintained in the absence or presence of Z-100 at 100 μg ml⁻¹. Inhibitors were added to MDMs at the concentrations indicated 30 min before Z-100 treatment. Luciferase activity in MDMs was assayed with a luminometer at 4 days post-infection. Values represent means ± SEM of four replicates. Statistically significant differences are indicated by: ***, P<0.001 compared to medium control (t-test); ## and #, P<0.01 and P<0.05, respectively, compared to Z-100 without inhibitors (Dunnett’s test).

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Fig. 5. Z-100 inhibits transcription of HIV-1 in MDMs. MDMs were transfected with pNL43lucΔenv for 24 h using JetPEI, washed and incubated with fresh medium, without (open bar) or with (filled bar) Z-100 at 100 μg ml⁻¹. SB203580 (1 μM) was added to cultures 30 min before Z-100 treatment. The luciferase activity in MDMs was assayed with a luminometer at 4 days post-transfection. Values represent means ± SEM of four replicates. ***, P<0.01 compared to medium control (t-test); #, P<0.05 compared to Z-100 alone sample (t-test).

neutralizing antibodies (P<0.01, Fig. 7b). These results suggest that MDM-produced IFN-β was required for Z-100-mediated HIV suppression and induction of the inhibitory C/EBPβ isoform.

**Fig. 6.** Induction of inhibitory 16 kDa C/EBPβ isoform in Z-100-treated macrophages and its suppression by a p38 MAPK inhibitor. PMA-differentiated THP-1 macrophages (a) or MDMs (b) (4 × 10⁶) were incubated with Z-100 at 100 μg ml⁻¹ in the absence or presence of SB203580 (1 or 10 μM). After incubation for 4 days, nuclear proteins were extracted and subjected to Western blot analysis. Membrane-bound nuclear proteins were incubated with anti-C/EBPβ antibodies and visualized by ECL. Two isoforms of C/EBPβ, 37-kDa stimulatory C/EBPβ and 16-kDa inhibitory C/EBPβ, are indicated by arrows. The value below each band represents its density quantified using a Science Lab 98 Image Gauge.
Finally, we assessed whether IFN-β was produced by Z-100-treated MDMs. IFN-β levels were measured in culture supernatants from the pseudotype HIV-1-infected MDMs samples in Fig. 4(b). Z-100-treated MDMs produced about twice the amount of IFN-β compared to the untreated control (P<0-01; Fig. 7c). In addition, Z-100-induced IFN-β production was inhibited significantly by the addition of SB 203580 (P<0-01). These results indicate strongly that Z-100 induces MDMs to produce IFN-β via activation of p38 MAPK, leading to HIV-1 suppression by induction of the inhibitory C/EBPβ isoform.

**DISCUSSION**

In this study, we demonstrated that Z-100, an arabino-mannan extracted from *M. tuberculosis*, inhibits HIV-1 replication in macrophages. Z-100 significantly inhibited replication in MDMs of pseudotype HIV-1 that possess an amphotropic MuLV or a VSV-G envelope (Fig. 3a). These results indicate clearly that Z-100 suppressed post-entry stages of HIV-1 replication, because these pseudotype viruses do not require β-chemokine receptors for infection. Z-100 suppressed expression of pseudotype HIV-1 significantly, even when added at 24 h post-infection (Fig. 3b), suggesting that Z-100 inhibits virus replication at post-integration stages. Moreover, Z-100 substantially inhibited expression of the pNL43LucΔenv vector, transfected directly into MDMs (Fig. 5). These findings indicate strongly that Z-100 inhibits transcription of HIV-1.

Although Z-100 induced β-chemokines such as MIP-1α, MIP-1β and RANTES, the addition of their neutralizing antibodies had no effect on the HIV-suppressive activity of Z-100 (data not shown), corresponding with previous reports indicating that β-chemokines (Moriuchi et al., 1996) or pre-treatment of these (Kelly et al., 1998) cannot block the M-tropic HIV-1 infection of MDMs. However, Z-100 downregulated HIV-1 receptors (CD4 and CCR5) in MDMs (Fig. 2), suggesting that Z-100 might also inhibit M-tropic virus entry to a small degree. This result explains partially why expression of M-tropic-enveloped, but not VSV-enveloped, pseudotype HIV-1 was inhibited slightly more in Z-100-pretreated MDMs than in cells treated with Z-100 post-infection (Fig. 1b). The mechanism of CD4 and CCR5 receptor downregulation is unclear, but Z-100-induced β-chemokines might contribute to downregulation, at least for CCR5.
It has been reported that LPS inhibits HIV-1 replication in macrophages and that p38 MAPK is involved in this suppression, probably through a signalling pathway downstream of CD14 and TLR4 (Zybarth et al., 1999). As Z-100 HIV-suppressive activity was repressed significantly by SB 203580, a specific inhibitor of p38 MAPK (Fig. 4), Z-100 might suppress HIV-1 replication by a mechanism similar to that of LPS. In our preliminary studies, Z-100 induced MIP-1α production through activation of p38 MAPK (data not shown). Recent findings have demonstrated that LPS binds mainly TLR4 and activates MDMs through MyD88-dependent and -independent signalling pathways (Kaisho et al., 2001; Kawai et al., 1999). Although the major receptor for Z-100 is unclear, potentially its major component, arabinomannan, and the small amount of peptidoglycan found would bind the mannos receptor and TLR2/TLR6, respectively, and mediate various signalling pathways including the MAPK cascade. In this study, Z-100-mediated HIV-1 suppression was alleviated by a p38 MAPK inhibitor, but not by inhibitors of JNK or MKK1.

The p38 MAPK has been reported to consist of four known members of the p38 family, p38α, p38β, p38γ, and p38δ, all of which have different functions (Ono & Han, 2000). In macrophages, p38α and p38δ are abundant, but p38β is undetected (Hale et al., 1999). The pyridyl imidazole p38 inhibitors, SB 203580 and SB 202190, nearly are equipotent for p38α and p38β, but do not inhibit p38γ or p38δ (Kumar et al., 1997; Wang et al., 1997). In this study, Z-100-mediated, HIV suppression was alleviated by SB 203580 (Fig. 4 and Table 1), indicating that Z-100 might inhibit HIV-1 replication by activation of the p38α isoform, but not p38δ. Ro 318220, a potent inhibitor of MSK1/MAPKAP-K1 located downstream of MAPK, could not alleviate Z-100-mediated HIV suppression (Table 1). As p38α and p38β, but not p38γ or p38δ, are known to phosphorylate MAPKAP-K2 (Keesler et al., 1998; Kumar et al., 1997; Wang et al., 1997), HIV-suppressive activity by Z-100 might be mediated through the p38α MAPK–MAPKAP-K2 pathway.

In monocytes/macrophages, viral gene expression using the HIV-1 LTR is regulated by its interactions with C/EBPβ, which is also known to be an important regulator of inflammation among the C/EBP transcription factor family (Henderson et al., 1995; Natsuka et al., 1992). Three C/EBP-binding sites in the negative regulatory element (NRE) of the HIV-1 LTR (Tesmer et al., 1993) have been identified as DNA elements responsible for LPS-mediated HIV-1 suppression in macrophages (Bernstein et al., 1991). The stimulatory 37-kDa C/EBPβ isoform promotes transcription, whereas an inhibitory 16-kDa isoform acts in a dominant-negative manner, competing for C/EBP sites when expressed even at levels lower than that of the stimulatory isoform (Descomes & Schibler, 1991). In Z-100-treated macrophages, the inhibitory 16-kDa C/EBPβ isoform was induced more strongly than the stimulatory 37-kDa C/EBPβ isoform (Fig. 6), suggesting that Z-100 might repress HIV-1 LTR activity by altering the balance between the C/EBPβ isoforms towards suppression. In addition, SB 203580 inhibited Z-100-induction of the inhibitory isoform, indicating that p38 MAPK-dependent, Z-100-mediated HIV-1 suppression was through 16-kDa C/EBPβ induction by Z-100.

Interferons are known to inhibit HIV-1 replication in macrophages at various stages, including early stages (Meylan et al., 1993) and transcription (Tissot & Mecht, 1995). In the present study, we found that Z-100 induced IFN-β production from MDMs. Moreover, neutralizing antibodies for IFN-β inhibited Z-100-mediated, HIV suppression and induction of inhibitory C/EBPβ (Fig. 7a, b). These findings indicate that Z-100-mediated suppression of HIV-1 transcription is dependent partly on IFN-β induction. In addition, IFN-β production by Z-100 was inhibited by a p38 MAPK inhibitor, SB 203580 (Fig. 7c), indicating that Z-100 induces IFN-β through activation of the p38 MAPK pathway. These findings are consistent with reports demonstrating that M. tuberculosis infection, or LPS, induced the 16-kDa inhibitory C/EBPβ isoform through IFN-β induction and, coincidentally, repressed HIV-1 transcription in THP-1 macrophages (Honda et al., 1998). The precise mechanisms of how IFN-β induces 16-kDa C/EBPβ remain to be clarified.

In response to Z-100, TLR2 and the mannos receptor potentially mediate signals to induce IFN-β. The transcription enhancer of the IFN-β promoter contains four positive regulatory domains (PRD1 to PRDIV), which activate IFN-β expression cooperatively in response to virus infection (Maniatis et al., 1998). The transcription factors that bind to these elements include NF-κB, which binds to PRDII, IRF3, which binds to adjacent PRDIII and PRDI (PRDIV-I), and the heterodimeric transcription factor (ATF)-2-c-Jun, which binds to PRDIV. Although the mechanisms for IFN-β induction in Z-100-treated MDMs are not clear, p38 MAPK might contribute to activation of NF-κB and ATF-2 transcription factors, because these transcription factors are located downstream from p38 and bind to PRDII and PRDIV, respectively, in the IFN-β enhancer.

In conclusion, Z-100 suppressed the replication of HIV-1 in macrophages, mainly at virus transcription and partially at virus entry. This suggests that Z-100 might be a useful immunomodulator for control of HIV-1 infection.

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