VLPs at a high concentration (40 μg/ml) to obtain a strong signal, and fractionated. The level of HIV-1 Gag protein in the cytosolic and membrane/organelle fractions was analyzed by Western blot (Fig. 2B). As control, the calpain was detected mainly on the cytosolic fraction, whereas GRP78 was detected exclusively on the membrane/organelle fraction. There was no clear difference in the amount of Gag protein present in the cytosolic fractions in MDDCs pulsed with HmVLPs versus LmVLPs, as well as in the membrane/organelle fraction. We concluded that the mannosylation level of VLPs does not affect the internalization or the intracellular amount of Gag antigens in MDDCs.

3.3. LmVLPs are more efficiently cross-presented than HmVLPs for CD8$^+$ T cell (CTLs) activation by MDDCs

We next tested the efficiency of these VLPs on CTL activation by MDDC-mediated cross-presentation. We utilized HIV-1 Gag28 peptide-specific CTL lines established from HLA-A24$^+$ HIV-infected patients as an indicator cell for cross-presentation efficiency. Frozen HLA-A24$^+$ healthy donor-derived MDDCs were pulsed either with 10 μg/ml of HmVLPs or LmVLPs, cocultured with Gag-specific CTL lines for 40 h, and analyzed by IFN-γ ELISPOT.

For this experiment, three independent CTL lines (CTL #9, #21, and #31) were cocultured with VLP-pulsed MDDCs derived from two donors each (Fig. 3). Despite the MDDCs' donor variation, LmVLP-pulsed MDDCs were able to activate higher numbers of Gag28-specific CTL lines compared to HmVLP-pulsed MDDCs in four of six MDDCs. Thus, our results suggest that the level of mannose on antigens can modulate the cross-presenting activity of VLPs by MDDCs.

3.4. Cytokine production by MDDCs after uptake of VLPs is influenced by the level of VLP mannosylation

For effective induction of CTL activity, it is important to polarize DCs toward Th1-type cytokine secretion during the DC–T cell interaction. We previously showed that MDDCs stimulated with yeast VLPs produced a higher level of IL-12 than those stimulated with LPS, while IL-10 production is limited [14]. We therefore measured cytokine production by 10 μg/ml of HmVLP- or LmVLP-pulsed MDDCs from eight healthy donors. Two patterns of IL-12 production were identified: IL-12 responders ($n = 4$), and IL-12 non-responders ($n = 4$) (Fig. 4).

In the case of the IL-12 responders, IL-12 production induced by LmVLPs tended to be higher than that by HmVLP (Fig. 4A), though the difference was not statistically significant. In contrast, the level of IL-10 production was very low in these donors (data not shown).

In IL-12 non-responders, both IL-12 and IL-10 production by LmVLP- or HmVLP-pulsed MDDCs were consistently low (data not shown). When we stimulated the MDDCs from these donors with LPS, the production of IL-10, but not IL-12, was increased, and the LPS-induced IL-10 production

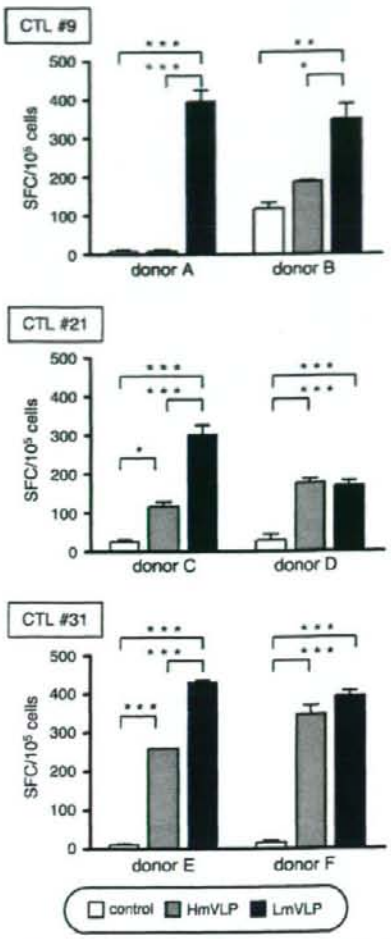

Fig. 3. The influence of yeast VLPs on HIV Gag-specific CD8$^+$ T cell activation. Immature MDDCs were pulsed overnight with 10 μg/ml HmVLPs (gray bar), LmVLPs (filled bar), or CS (open bar). Next day, MDDCs (1×10^4 cells per well) were mixed with MHC class I-matched allogenic CTL clones ($1–2 \times 10^4$ per well). Two days after cocultivation, the number of IFN-γ-producing cells was determined by ELISPOT analysis. The longitudinal axis shows the analysis spot forming cells (SFC) producing IFN-γ per 10^5 cells. Results are presented as the means + SEM. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, compared between two groups; one-way ANOVA followed by Bonferroni's *t*-test ($n = 3$).

was upregulated further in the presence of HmVLPs, while it was not true for LmVLPs (Fig. 4B). The level of IL-12 production remained undetectable or low even after stimulation with LPS plus HmVLPs or LPS plus LmVLPs (data not shown). The production of other cytokines (including IL-1, IL-6, IL-8, and TNF-alpha) was very low or showed little difference in response to HmVLPs and LmVLPs. These results suggest that a high level of mannosylation modulates the cytokine production by MDDCs toward a Th2-type response.

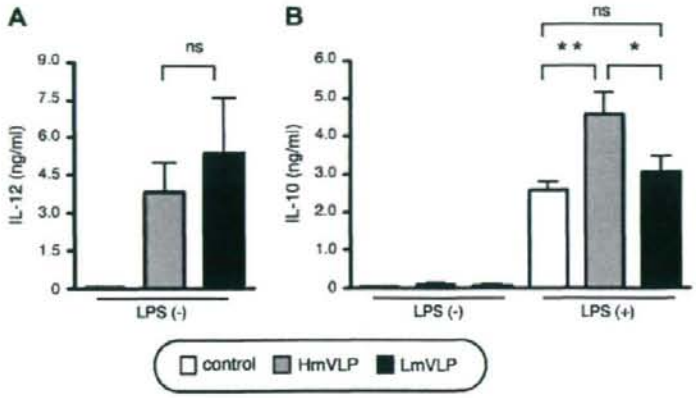

Fig. 4. The effect of yeast VLPs on cytokine production. (A) In the cases of IL-12 responders ($n = 4$), immature MDDCs (2×10^5 cells/200 μl) were incubated with 20 μg/ml HmVLPs (gray bar), LmVLPs (filled bar), or CS (open bar) for 24 h. (B) The MDDCs (2×10^5 cells/200 μl) of IL-12 non-responders ($n = 4$) were incubated with 20 μg/ml HmVLPs (gray bar), LmVLPs (filled bar), or CS (open bar) with or without 100 ng/ml LPS for 24 h. The cytokines produced by these cells were analyzed. Results are presented as the means + SEM. $^*P < 0.05$; $^{**}P < 0.01$; ns, not significant, compared between two groups; one-way ANOVA followed by Bonferroni's t-test ($n = 4$, respectively).

4. Discussion

Antigens expressed by yeast and insect cells contain abundant carbohydrate modifications and are perceived to be excellent vaccine antigens. Consistent with these observations, we previously demonstrated that MDDCs loaded with yeast-derived HIV-1 $p55^{gag}$ VLPs activated Gag-specific $CD8^+$ T cells in chronically-infected HIV patients [14]. Mannosylated antigens taken up by DCs are more efficiently presented to T cells than antigens internalized via fluid phase [15]. However, recent studies have indicated that excessive mannosylation leads to a Th2-dominant response or even suppresses the immune response via a signal from the lectin receptors [17–19]. Therefore, it is important to determine whether the level of mannosylation on VLPs positively or negatively affects the DC-based immune response.

The cell wall of *Saccharomyces cerevisae* is rich in mannoproteins. In this work, we used a yeast mnn9 mutant of the mannosyltransferase, which cannot synthesize completely glycosylated mannoproteins, and demonstrated that VLPs with much reduced mannose induced stronger CTL activation than more heavily mannosylated VLPs derived from wild-type yeast did. Because we used the same amount of Gag protein composed of these VLPs, the difference should be only at the level of glycosylation on VLPs. The efficiency of CTL activation by VLP-pulsed MDDCs was analyzed at 10–20 μg/ml concentration of VLPs. At this concentration, we detected no difference of uptake by FACS analysis. We speculate that the difference, if any, may be not at the level of uptake, but rather during antigen processing or loading to MHC class I. Although we failed to show the difference in the intracellular fate of these VLPs by the current technology, it is still possible that they are differently processed somewhere in the cytoplasm. In this context, it has been demonstrated previously that only a small amount (200 molecules) of peptide and MHC class I complex is enough to be recognized by CTLs [26]. Therefore, even a small difference in the antigen processing process may affect the efficiency of cross-presentation by DCs.

The balance of the Th1/Th2 immune response is influenced by elements such as host conditions and the antigen formulation [27]. LmVLPs induced IL-12 production slightly better than HmVLPs in some donors, whereas in those who do not produce a substantial level of IL-12, LPS-induced IL-10 production was increased by adding HmVLPs, but not by LmVLPs. A number of studies have demonstrated that cytokine production can be altered by the interaction of antigen with lectin receptors and/or toll-like receptors [17–19,28]. For example, Nigou et al. reported that the IL-12 production of DCs induced by LPS was negatively regulated by the engagement of an MR using mannose-capped lipoarabinomannans (the ManLAMs) of *Mycobacteria* [28]. Gringhuis et al. reported that stimulation of DC-SIGN by pathogens such as *Mycobacteria*, fungi, and viruses enhanced toll-like receptor signaling via Raf-1 kinase-dependent acetylation of the transcription factor NF-kB, resulting in strong augmentation of IL-10 mRNA expression by DCs [19]. Thus, stimulation of lectin receptors may preferentially induce IL-10 production in low IL-12 responders. We speculate that a mannosylation-dependent signal affects the Th1/Th2 balance by modulating the cytokine production of DCs, leading to the alternation of CTL activation.

In conclusion, a high level of mannosylation on an antigen may not necessarily be beneficial for CTL induction by DCs. The quantity of carbohydrate chain may alter the Th1/Th2 cytokine balance. DC-based immune therapy that aims to induce CTLs requires optimizing the antigen for the most effective clinical results. Further studies are required to develop the technology for modulating the antigenic property so that it can efficiently induce Th-1 type immune response.

One possible technology may be the modification of the outer structure, amount and variation of sugars moieties on vaccine antigens.

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References

[1] J. Banchereau, R.M. Steinman, Dendritic cells and the control of immunity, Nature 392 (1998) 245–252.
[2] J. Banchereau, A.K. Palucka, Dendritic cells as therapeutic vaccines against cancer, Nat. Rev. Immunol. 5 (2005) 296–306.
[3] C.G. Figdor, I.J. de Vries, W.J. Lesterhuis, C.J. Melief, Dendritic cell immunotherapy: mapping the way, Nat. Med. 10 (2004) 475–480.
[4] A.G. Thompson, R. Thomas, Induction of immune tolerance by dendritic cells: implications for preventative and therapeutic immunotherapy of autoimmune disease, Immunol. Cell Biol. 80 (2002) 509–519.
[5] T.E. Ichim, R. Zhong, W.P. Min, Prevention of allograft rejection by in vitro generated tolerogenic dendritic cells, Transpl. Immunol. 11 (2003) 295–306.
[6] H. Matsue, M. Kusuhara, K. Matsue, A. Takashima, Dendritic cell-based immunoregulatory strategies, Int. Arch. Allergy Immunol. 127 (2002) 251–258.
[7] J. Colino, C.M. Snapper, Dendritic cells, new tools for vaccination, Microbes Infect 5 (2003) 311–319.
[8] W. Lu, L.C. Arraes, W.T. Ferreira, J.M. Andrieu, Therapeutic dendritic-cell vaccine for chronic HIV-1 infection, Nat. Med. 10 (2004) 1359–1365.
[9] W. Lu, X. Wu, Y. Lu, W. Guo, J.M. Andrieu, Therapeutic dendritic-cell vaccine for simian AIDS, Nat. Med. 9 (2003) 27–32.
[10] R.J. Pomerantz, D.L. Horn, Twenty years of therapy for HIV-1 infection, Nat. Med. 9 (2003) 867–873.
[11] D. Finzi, J. Blankson, J.D. Siliciano, J.B. Margolick, K. Chadwick, T. Pierson, K. Smith, J. Lisziewicz, F. Lori, C. Flexner, T.C. Quinn, R.E. Chaisson, E. Rosenberg, B. Walker, S. Gange, J. Gallant, R.F. Siliciano, Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy, Nat. Med. 5 (1999) 512–517.
[12] B.D. Walker, B.T. Korber, Immune control of HIV: the obstacles of HLA and viral diversity, Nat. Immunol. 2 (2001) 473–475.
[13] A. Duerr, J.N. Wasserheit, L. Corey, HIV vaccines: new frontiers in vaccine development, Clin. Infect. Dis. 43 (2006) 500–511.
[14] Y. Tsunetsugu-Yokota, Y. Morikawa, M. Isogai, A. Kawana-Tachikawa, T. Odawara, T. Nakamura, F. Grassi, B. Autran, A. Iwamoto, Yeast-derived human immunodeficiency virus type 1 p55(gag) virus-like particles activate dendritic cells (DCs) and induce perforin expression in Gag-specific CD8(+) T cells by cross-presentation of DCs, J. Virol. 77 (2003) 10250–10259.
[15] A.J. Engering, M. Cella, D. Fluitsma, M. Brockhaus, E.C. Hoefsmit, A. Lanzavecchia, J. Pieters, The mannose receptor functions as a high capacity and broad specificity antigen receptor in human dendritic cells, Eur. J. Immunol. 27 (1997) 2417–2425.
[16] M.C. Tan, A.M. Mommaas, J.W. Drijfhout, R. Jordens, J.J. Onderwater, D. Verwoerd, A.A. Mulder, A.N. van der Heiden, D. Scheidegger, L.C. Oomen, T.H. Ottenhoff, A. Tulp, J.J. Neefjes, F. Koning, Mannose receptor-mediated uptake of antigens strongly enhances HLA class II-restricted antigen presentation by cultured dendritic cells, Eur. J. Immunol. 27 (1997) 2426–2435.
[17] E. Caparros, P. Munoz, E. Sierra-Filardi, D. Serrano-Gomez, A. Puig-Kroger, J.L. Rodriguez-Fernandez, M. Mellado, J. Sancho, M. Zubiaur, A.L. Corbi, DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production, Blood 107 (2006) 3950–3958.
[18] T.B. Geijtenbeek, S.J. Van Vliet, E.A. Koppel, M. Sanchez-Hernandez, C.M. Vandenbroucke-Grauls, B. Appelmelk, Y. Van Kooyk, *Mycobacteria* target DC-SIGN to suppress dendritic cell function, J. Exp. Med. 197 (2003) 7–17.
[19] S.I. Gringhuis, J. den Dunnen, M. Litjens, B. van Het Hof, Y. van Kooyk, T.B. Geijtenbeek, C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-kappaB, Immunity 26 (2007) 605–616.
[20] A. Kawana-Tachikawa, M. Tomizawa, J. Nunoya, T. Shioda, A. Kato, E.E. Nakayama, T. Nakamura, Y. Nagai, A. Iwamoto, An efficient and versatile mammalian viral vector system for major histocompatibility complex class I/peptide complexes, J. Virol. 76 (2002) 11982–11988.
[21] S. Sakuragi, T. Goto, K. Sano, Y. Morikawa, HIV type 1 Gag virus-like particle budding from spheroplasts of Saccharomyces cerevisiae, Proc. Natl. Acad. Sci. USA 99 (2002) 7956–7961.
[22] C.E. Ballou, Isolation, characterization, and properties of *Saccharomyces cerevisiae* mnn mutants with nonconditional protein glycosylation defects, Methods Enzymol 185 (1990) 440–470.
[23] Y. Morikawa, S. Hinata, H. Tomoda, T. Goto, M. Nakai, C. Aizawa, H. Tanaka, S. Omura, Complete inhibition of human immunodeficiency virus Gag myristoylation is necessary for inhibition of particle budding, J. Biol. Chem. 271 (1996) 2868–2873.
[24] Y. Tsunetsugu-Yokota, K. Akagawa, H. Kimoto, K. Suzuki, M. Iwasaki, S. Yasuda, G. Hausser, C. Hultgren, A. Meyerhans, T. Takemori, Monocyte-derived cultured dendritic cells are susceptible to human immunodeficiency virus infection and transmit virus to resting T cells in the process of nominal antigen presentation, J. Virol. 69 (1995) 4544–4547.
[25] C.E. Ballou, Yeast cell wall and cell surface, in: J.N. Stranthern, E.W. Jones, J.R. Broach (Eds.), The Molecular Biology of the Yeast Saccharomyces, Metabolism and Gene Expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1982, pp. 335–360.
[26] E.R. Christinck, M.A. Luscher, B.H. Barber, D.B. Williams, Peptide binding to class I MHC on living cells and quantitation of complexes required for CTL lysis, Nature 352 (1991) 67–70.
[27] P. Kidd, Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease, Altern. Med. Rev. 8 (2003) 223–246.
[28] J. Nigou, C. Zelle-Rieser, M. Gilleron, M. Thurnher, G. Puzo, Mannosylated lipoarabinomannans inhibit IL-12 production by human dendritic cells: evidence for a negative signal delivered through the mannose receptor, J. Immunol. 166 (2001) 7477–7485.

Erythromycin derivatives inhibit HIV-1 replication in macrophages through modulation of MAPK activity to induce small isoforms of C/EBPβ

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Macrophages (MΦs) are a major source of HIV-1 especially in patients with tuberculosis. There are MΦs that are permissive and those that restrict HIV-1. Regulation of hematopoietic cell kinase (Hck) activity and selective expression of CCAAT enhancer binding protein β (C/EBPβ) isoforms greatly contribute to determine distinct susceptibility of MΦs to HIV-1. Resistance is attributable to reduced expression of Hck and augmented expression of an inhibitory small isoform of C/EBPβ. Derivatives of erythromycin A (EMA) EM201 and EM703 inhibit the replication of HIV-1 in tissue MΦs, at posttranscriptional and translational levels. We demonstrate that EM201 and EM703 convert tissue MΦs from HIV-1 susceptible to HIV-1 resistant through down-regulation of Hck and induction of small isoforms of C/EBPβ. These drugs inhibit p38MAPK activation which is expressed only in susceptible tissue MΦs. Activated CD4+T cells stimulate the viral replication in HIV-1 resistant MΦs through down-regulation of small isoforms of C/EBPβ via activation of ERK1/2. EM201 and EM703 can inhibit the MAPK activation and inhibit the burst of viral replication produced when CD4+T cells and MΦs interact. These EM derivatives may be highly beneficial for repression of residual HIV-1 in the lymphoreticular system of HIV-1-infected patients and offer great promise for the creation of new anti-HIV drugs for the future treatment of AIDS patients.

AIDS | macrolides | Hck

At least 65 million people have been infected with HIV and AIDS has killed 25 million people since 1981. By 2007, worldwide, 39.5 million individuals were living with HIV, with 4.3 million new infections and 2.9 million deaths occurring in 2006 (http://data.unaids.org/pub/EpiReport/2006/02-Global_Summary_2006_EpiUpdate_eng.pdf). In developed countries, anti-HIV-1 therapy—highly active antiretroviral therapy (HAART)— potently inhibits HIV-1 replication, reduces viral antigenemia, and prolongs the survival of patients (1, 2). In contrast, patients in developing countries generally cannot use HAART therapy because of its high cost and the sheer number of patients. Furthermore, HAART therapy cannot remove HIV-1-infected latent memory T cells and monocytes (Mos)/macrophages (MΦs) in some lymphoreticular tissue, residual cells having the potential to become a viral resource capable of spreading new viral particles (3, 4). Therefore, the development of new drugs to improve and extend HAART therapy, particularly in countries in the developing world, is greatly and urgently needed.

Mos/MΦs are a major target of HIV-1 infection and serve as a reservoir for viral persistence and a chronic source of infectious virus *in vivo* (5). Most tissue MΦs are permissive to M-tropic virus entry and release a small number of virus particles in the asymptomatic carrier but they occasionally produce a large number of viral particles in the AIDS patients or HIV-1 patients with pulmonary tuberculosis (TB) or those whose conditions are complicated with opportunistic infection (3). TB markedly increases HIV-1 replication and mutation in the lung and is associated with an acceleration of AIDS (6, 7). The alveolar MΦ is the major cell type in which HIV-1 replication occurs during TB (8, 9). Thus MΦ is a key factor in the control of HIV-1 suffering.

We and others have previously demonstrated that expression of tyrosine kinase hematopoietic cell kinase (Hck) and relative amounts of a large isoform (37-kDa) to a small isoform (23-kDa) (L/S ratio) of transcription factor CCAAT enhancer binding protein β (C/EBPβ) play critical roles in M-tropic HIV-1 production in tissue MΦs (8, 10–13). We have also reported that modulation of the expression of Hck and the L/S ratio of C/EBPβ by treatment with antisense oligonucleotides can convert the phenotype of HIV-1 susceptibility in MΦs (10). These studies suggest that, not only anti-HIV-1 drugs that directly affect the virus (such as RT inhibitor or protease inhibitors), but also drugs that can convert the phenotype of tissue MΦs from "susceptible" to "resistant" by down-regulating the expression of Hck and enhancing the expression of small isoforms of C/EBPβ may be useful to help control HIV-1 replication in AIDS patients.

Macrolides with a 14-membered ring structure, such as erythromycin A (EMA), clarithromycin (CAM), or roxithromycin (RXM), are well known antibacterial drugs. Recently, these antibiotics have been shown to be efficacious against incurable chronic inflammatory airway disease, such as diffuse panbronchiolitis (DPB) (14, 15). This therapeutic efficacy is thought to be caused by either anti-inflammatory or immunomodulatory activity of the macrolide antibiotics, which can act on many cells, including epithelial cells, neutrophiles, monocytes/MΦs, and T cells (16–23). On the basis of this knowledge, we chemically modified EMA to obtain derivatives with both stronger capability for promoting monocyte-to-MΦ differentiation and no antibacterial activity. Among the derivatives, 8,9-anhydroerythromycin A 6,9-hemiketal (EM201), obtained by mild acid treatment of EMA, already known as an internal metabolite of EMA, showed a strong promotional effect on MΦ differentiation and possessed weak antimicrobial activity (24). Furthermore, the 12-membered pseudoerythromycin A (EM703) was both remarkably active and free of any antibacterial activity (25) and was known to exhibit a prophylactic effect on lung injury *in vivo* against a bleomycin-induced acute lung injury in the rat model, similar to EMA (26).

In this study, we show that both EM201 and EM703 are good lead candidates for drugs that can inhibit M-tropic HIV-1 replication in tissue MΦs by a new way of converting their phenotype from HIV-1-susceptible to HIV-1-resistant, through down-regulation of Hck and the induction of small isoforms of C/EBPβ via modulation of the activation of MAPKs.

Author contributions: T.S., K.S.A., and S.Ō. designed research; I.K., T.S., and K.S.A. performed research; T.S., Y.Y., and S.Ō. contributed new reagents/analytic tools; I.K., T.S., K.S.A., A.I., and S.Ō. analyzed data; and I.K., T.S., K.S.A., and S.Ō. wrote the paper.

The authors declare no conflict of interest.

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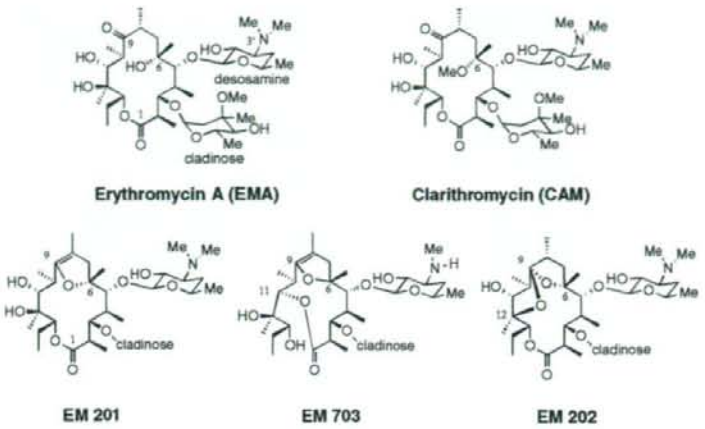

Fig. 1. Structure of EM derivatives.

Results

Effects of EM Derivatives on Viral Replication and Multinucleated Giant Cell Formation in M-Tropic HIV-1-Infected M-MΦs. We first examined whether EM derivatives (Fig. 1) have an ability to inhibit M-tropic HIV-1 replication in macrophage colony-stimulating factor (M-CSF)-induced monocyte-derived MΦs (M-MΦs), which express a high level of Hck and a large isoform of C/EBPβ, are susceptible to M-tropic HIV-1 replication, and whether they form multinucleated giant cells (MGC) by cell-to-cell fusion at 4–7 d after infection (10, 27). EM201 and EM703 (30 μM) completely inhibited viral replication and MGC formation at 7 d after infection, while EMA, CAM, and EM202 did not (Fig. 2*A*). PCR using a primer pair designed from the HIV-1 LTR region of HIV-1_{BaL} DNA at 2 d after infection showed that the DNA replication at first replicon was observed at similar levels in all of the M-MΦs. At 7 d after infection, however, the levels of viral DNA in M-MΦs treated with EMA, EM202, or DMSO (solvent) alone increased, whereas those in M-MΦs treated with EM201and EM703 remained low, at levels similar to those observed at 2 d after infection (Fig. 2*A*).

EM201 and EM703 (30 μM) persistently inhibited viral replication at 14 d (Fig. 2*B*), and inhibition was observed even at 21 d after infection (data not shown). EM201 and EM703 strongly inhibited HIV-1_{BaL} replication, even at 3 μM, and p24 levels were ~4% of those in cells treated with DMSO alone at 14 d after infection (Fig. 2*C*). EMA and EM202 induced inhibition of viral replication at higher concentration (>300 μM). However, the reduction curves in cells were similar to those in DMSO-treated cells (Fig. 2*C*), indicating the effects are mainly because of DMSO toxicity. In contrast, CAM partially but significantly inhibited HIV-1_{Bal} replication at 10–30 μM at 10 and 14 d after infection (Figs. 2 *B* and *C*), and it is impossible to deny that CAM itself can inhibit HIV-1 replication.

EM201 and EM703 Modulate the Expression of Hck and C/EBPβ Proteins in HIV-1_{BaL} Infected M-MΦs. To examine the possibility that EM201 and EM703 inhibit HIV-1 replication in M-MΦs via modulation of the expression of Hck and C/EBPβ, expression of these proteins in HIV-1 infected M-MΦs treated with 30 μM EM derivatives was examined by immunoblots at 2 d after infection. The levels of Hck protein in M-MΦs treated with EM201 and EM703 strongly decreased to one-seventh and one-ninth of that in M-MΦs treated with DMSO alone, respectively (Fig. 3*A*). Conversely, the small isoform of C/EBPβ protein was strongly induced in M-MΦs treated with EM201 and EM703, the levels increasing to 25- to 40-fold of that in M-MΦs treated with DMSO alone, with the L/S ratio of C/EBPβ markedly decreasing from 12.6 to 0.3 and 0.5, respectively (Fig. 3*A*).

EMA and EM202 did not affect the expression of Hck and C/EBPβ and consequently did not inhibit viral replication (Fig. 3*A*). Similarly CAM, which did not show inhibitory activity during the early phase of infection, did not significantly affect expression of either Hck or C/EBPβ at 2 d after infection.

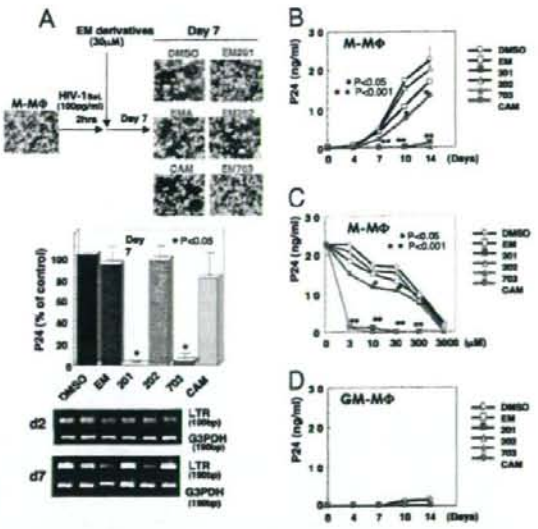

Fig. 2. Screening of EM derivatives that show an inhibitory effect on HIV-1_{BaL} replication in M-MΦs. (*A*) Effects of 30 μM of EMA (EM), CAM, EM201 (201), EM202 (202), and EM703 (703) on viral replication and MGC formation (Magnification, ×100) at 7 d after infection. The data of viral production were shown as the percentage of p24 antigen in control (DMSO alone) M-MΦs. The levels of viral DNA were assayed at 2 and 7 d after infection. (*B*) Kinetics of viral production in HIV-1 infected M-MΦs treated with 30 μM of EM derivatives. (*C*) Dose-response effects of EM derivatives on HIV-1 replication in M-MΦs at 14 d after infection. (*D*) EM derivatives (30 μM) do not change the resistant phenotype against HIV-1 infection in GM-MΦs. The data shown are representative one of five independent experiments.

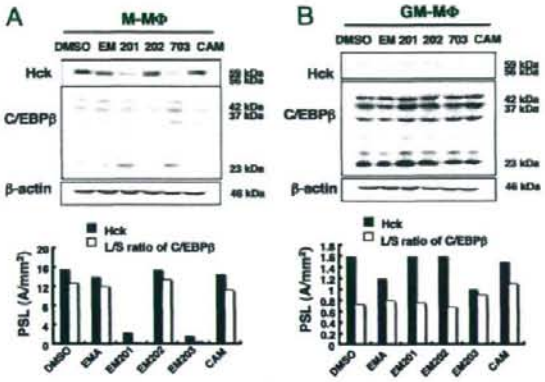

Fig. 3. Effects of EM derivatives on the expression of Hck and C/EBPβ in HIV-1_{BaL}-infected M-MΦs and GM-MΦs. Immunoblots of Hck and C/EBPβ in M-MΦs (*A*) and GM-MΦs (*B*) at 2 d after infection. EMA (EM), EM201 (201), EM202 (202), EM703 (703), and CAM were added at 30 μM. The relative amounts of Hck and C/EBPβ were measured using National Institutes of Health image software (PSL; photo stimulating luminescence, A/mm²). The relative amounts of the large band to the small band (L/S ratio) of C/EBPβ were calculated using PSL values of 37 kDa and 23 kDa of C/EBPβ isoforms and are shown at the bottom of each figure. The data shown are representative of one of three independent experiments.

EM201 and EM703 Change Neither the Expression of Hck and C/EBPβ Proteins Nor the Resistant Phenotype Against HIV-1 Infection in GM-MΦs. Granulocyte-macrophage CSF (GM-CSF)-induced monocyte-derived MΦ (GM-MΦ) is HIV-1 resistant and does not stimulate the replication of M-tropic HIV-1and MGC formation. This is because GM-MΦs express a high level of short isoforms of C/EBPβ and a low level of Hck, and HIV-1 infection drastically increases the expression of a short isoform of C/EBPβ but decreases that of Hck (10). We examined the effects of EM derivatives on viral replication and the expression of Hck and C/EBPβ in HIV-1_{BaL}-infected GM-MΦs. Even at 14 d after infection, we found that GM-MΦs treated with various kinds of EM derivatives (including EM201 and EM703) did not stimulate viral replication (Fig. 2*D*) or MGC formation (data not shown). Consistent with the lack of change in HIV-1 resistant phenotype, all of the EM derivatives did not affect the expression of Hck and C/EBPβ protein in GM-MΦs (Fig. 3*B*).

p38MAPK Inhibitor, but Not ERK1/2 Inhibitor, Inhibits Viral Replication in M-Tropic HIV-1 Infected M-MΦs via Reduced Expression of Hck and Increased Expression of a Small Isoform of C/EBPβ. Previous reports have shown that the replication of M-tropic HIV-1 in tissue MΦs requires the activation of p38MAPK (28) and that ERK1/2 mediates the activation of C/EBPβ (29, 30). We consequently examined the activation of MAPKs in HIV-1 susceptible M-MΦs and HIV-1 resistant GM-MΦs. Expressions of total and phosphorylated forms of p38MAPK in M-MΦs were higher than those in GM-MΦs before HIV-1_{BaL} infection (Fig. 4*A*). After infection, the phosphorylated form was augmented in M-MΦs but not in GM-MΦs (Fig. 4*A*). In contrast to p38MAPK, the expressions of total and phosphorylated forms of ERK1/2 in M-MΦs were lower than those in GM-MΦs before infection, but the expression was unchanged in both MΦs after infection (Fig. 4*A*). Consistent with the augumented activation of p38MAPK in M-MΦs, addition of p38MAPK inhibitor SB203580 (at 10 μM) completely suppressed viral replication and MGC formation in HIV-1_{BaL}-infected M-MΦs (Fig. 4*B*).

We subsequently investigated whether the inhibitory activity of SB203580 on viral replication in HIV-1_{BaL}-infected M-MΦs is mediated through modulation of the expression of Hck and C/EBPβ protein. SB203580 not only inhibited the phosphorylation of p38MAPK but also reduced the expression of Hck and increased the expression of the small isoform of C/EBPβ to mimic the inhibitory effect on viral replication (Fig. 4 *C–E*). Conversely, the ERK1/2 inhibitor PD98059 affected neither viral replication nor the expression of Hck and C/EBPβ protein (Fig. 4 *B–E*).

EM201 and EM703 Inhibit Viral Replication in M-Tropic HIV-1 Infected M-MΦs via Inhibition of p38MAPK Activation. The above results suggest that EM201 and EM703 inhibit M-tropic HIV-1 replication

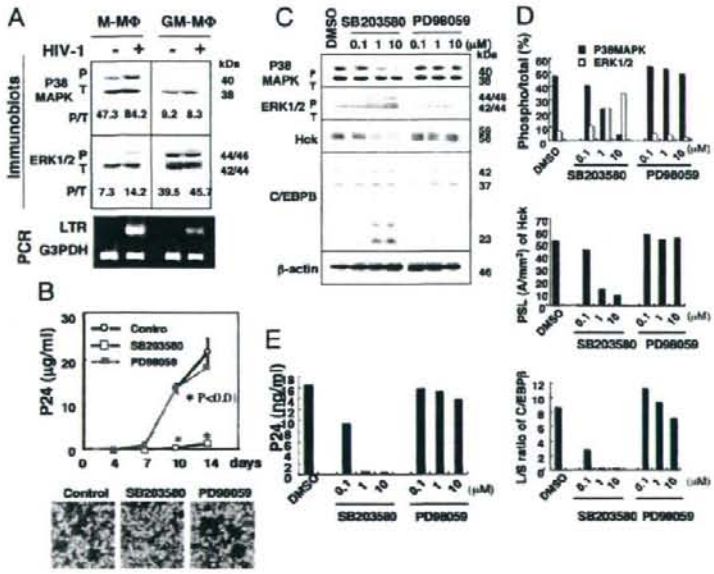

Fig. 4. Effects of p38 MAPK inhibitor and ERK1/2 inhibitor on viral replication and expression of Hck and C/EBPβ in HIV-1_{BaL}infected M-MΦs. (*A*) Immunoblot analysis of total and phosphorylated forms of p38MAPK and ERK1/2 in M-MΦs and GM-MΦs before and 2 d after infection. (*B*) Kinetic analysis of viral replication and morphology in HIV-1_{BaL}-infected M-MΦs treated with 10 μM of SB203580 or PD98059. (*C*) Immunoblot analysis of Hck and C/EBPβ in HIV-1_{BaL}-infected M-MΦs treated with various concentrations of SB203580 or PD98059 at 2 d after infection. (*D*) The relative amounts of Hck and L/S ratio of C/EBPβ in the cells or the phosphorylated protein *P* to the total protein *T* (P/T ratio) of p38 MAPK and ERK1/2 in immunoblot analysis shown in *c* calculated as described in Fig. 3. (*E*) Viral production in HIV-1_{BaL}-infected M-MΦs treated with various concentrations of SB203580 or PD98059. The data shown are representative of one of three independent experiments.

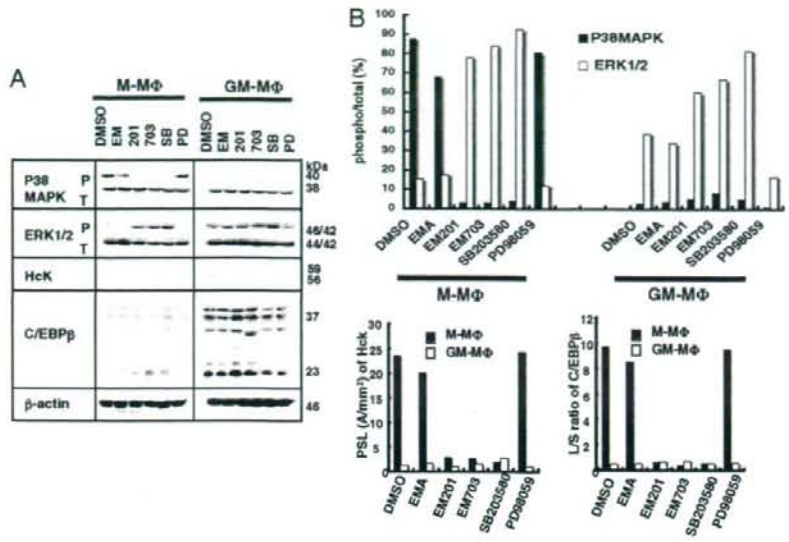

Fig. 5. Effects of EM derivatives on the phosphorylation of p38 MAPK and ERK1/2 in M-MΦs and GM-MΦs. (*A*) Immunoblot analysis of Hck and C/EBPβ, and the phosphorylation of p38 MAPK and ERK1/2 in M-MΦs and GM-MΦs treated with 30 μM of EMA (EM), EM201 (201), EM703 (703), 10 μM of SB203580 (SB), or PD98059 (PD), and DMSO alone at 2 d after HIV-1 infection. (*B*) The relative amounts of Hck and L/S ratio of C/EBPβ or P/T ratio of p38 MAPK and ERK1/2 in immunoblot analysis shown in *a* are calculated as described in Fig. 3. The data shown are representative of one of three independent experiments.

via inhibition of p38MAPK activation. To help confirm this hypothesis, we examined the effect of EM201 or EM703 (30 μM) on the phosphorylation of p38MAPK and ERK1/2 in M-MΦs by immunoblot. EM201 and EM703, but not EMA, reduced phosphorylation of p38MAPK but enhanced the phosphorylation of ERK1/2 in HIV-1-infected M-MΦs at 2 d after treatment (Fig. 5).

As described above, EM201 and EM703 did not affect the HIV-1-resistant phenotype of GM-MΦs (Figs. 2*D* and 3*B*). Consistent with the results, none of the EM derivatives significantly affected the phosphorylation pattern of p38MAPK and ERK1/2 in HIV-1-infected GM-MΦs (Fig. 5).

Activated CD4⁺T Cells Stimulate Viral Replication in M-Tropic HIV-1 Infected GM-MΦs via Down-Regulation of a Small Isoform of C/EBPβ. Recently, Hosino *et al.* (9) reported a phenotypical change of human alveolar MΦs (A-MΦs) from resistant to susceptible for HIV-1 replication caused by the addition of activated lymphocytes. The change was brought about by decreased expression of a small isoform of C/EBPβ (9). In line with this report, the addition of activated CD4⁺T cells to HIV-1_{BaL}-infected GM-MΦs stimulated marked viral replication (Fig. 6*A*), with MGC formation and clusters of GM-MΦs with CD4⁺ T cells (data not shown) at 10–14 d after infection. The amounts of viral DNA in the GM-MΦs increased at 2–7 d after infection (Fig. 6*B*). In GM-MΦs stimulated with activated CD4⁺ T cells, expression of the small isoform of C/EBPβ protein significantly decreased whereas the L/S ratio of C/EBPβ increased (from 0.57 to 3.6) at 2 d after infection (Fig. 6 *C* and *D*). Expression of Hck in the GM-MΦs, however, did not change significantly, even after stimulation with activated T cells and was very low compared with that in M-MΦs (Fig. 6 *C* and *D*).

Activated CD4⁺T Cells Down-Regulate the Small Isoform of C/EBPβ in M-Tropic HIV-1-Infected GM-MΦs via Augmentation of ERK1/2 Phosphorylation. As described above, activation of p38MAPK but not ERK1/2 is critical for HIV-1 replication in M-MΦs. However, the p38MAPK inhibitor, SB203580, did not inhibit viral replication in GM-MΦs stimulated with activated CD4⁺T cells (Fig. 6*A*). Instead, the ERK1/2 inhibitor PD98059 completely inhibited viral replication (Fig. 6*A*) and suppressed the level of viral DNA to that observed in the culture of GM-MΦs alone in which viral replication was absent (Fig. 6*B*). Upon examination of the phosphorylation of p38MAPK and ERK1/2 in HIV-1_{BaL}-infected GM-MΦs stimulated with activated CD4⁺ T cells, the phosphorylation ratio of ERK1/2 but not of p38MAPK significantly increased in GM-MΦs stimulated with activated CD4⁺ T cells, compared with that in GM-MΦs alone. Addition of PD98059 not only inhibited the phosphorylation of ERK1/2 but also increased expression of the small isoform of C/EBPβ, while markedly decreasing the L/S ratio of C/EBPβ from 3.6 to 0.82 (Fig. 6 *C* and *D*). The addition of SB203580 did not affect the expression of C/EBPβ. The expression of Hck was unaffected by treatment with either of the two inhibitors (Fig. 6 *C* and *D*).

EM201 and EM703 Inhibit M-Tropic HIV-1 Replication in GM-MΦs Stimulated with Activated CD4⁺ T Cells via Inhibition of the Activation of ERK1/2 and Augmentation of the Expression of the Small Isoform of C/EBPβ. In examining whether EM201 and EM703 can inhibit viral replication in M-tropic HIV-1-infected GM-MΦs stimulated with activated CD4⁺T cells, addition of EM201 and EM703 (30 μM) completely inhibited viral replication (Fig. 6*A*) and MGC formation (data not shown). The levels of HIV-1 DNA observed were very low, the same as those seen in the culture of GM-MΦs alone (Fig. 6*B*).

We subsequently examined the effects of EM201 and EM703 on the expression of Hck and C/EBPβ and on the phosphorylation of p38MAPK and ERK1/2 in HIV-1_{BaL}-infected GM-MΦs stimulated with activated CD4⁺ T cells at 2 d after infection by immunoblot. Treatment with EM201 and EM703 did not change the levels of Hck protein, but increased levels of the small isoform of C/EBPβ protein and the L/S ratio of C/EBPβ decreased from 3.6 to 0.45 (EM201) and 0.44 (EM703) (Fig. 6 *C* and *D*). The phosphorylation level of ERK1/2 decreased following treatment with EM201 and EM703, but that of p38MAPK remained unchanged (Fig. 6 *C* and *E*).

Discussion

In this study, we demonstrated that two EMA derivatives, EM201and EM703, can inhibit the replication of M-tropic HIV-1 in tissue MΦs at the posttranscriptional and translational levels, but do not affect viral entry and first DNA replicon. The inhibition is

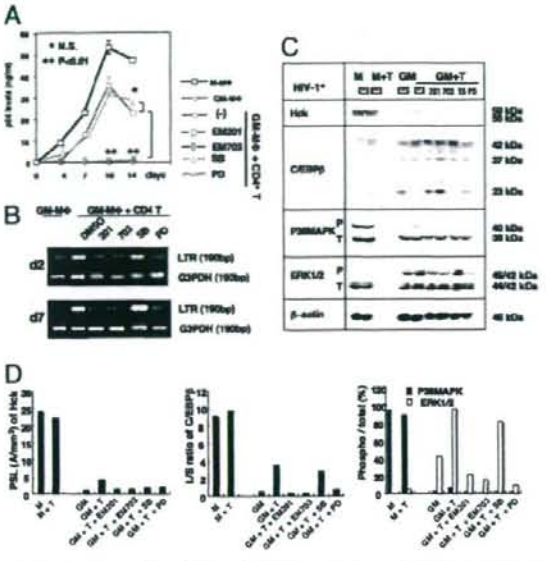

Fig. 6. Augmentation of M-tropic HIV-1 production in GM-MΦs stimulated with CD4+ T cells, and the suppressive effects of EM201 and EM703 on viral replication through the induction of small isoforms of C/EBPβ via inhibition of phosphorylation of ERK1/2. M-MΦs and GM-MΦs were infected with HIV-1_{BaL}. Part of HIV-1-infected GM-MΦs were stimulated with activated CD4+ T cells and incubated with or without 30 μM of EMA (EM), EM201 (201), or EM703 (703), 10 μM of SB203580 (SB), or PD98059 (PD), and DMSO alone. (*A*) Kinetic analysis of HIV-1 replication. (*B*) Levels of viral DNA at 2 and 7 d after infection. (*C*) Immunoblot analysis of Hck, C/EBPβ, and phosphorylation of p38 MAPK and ERK1/2 at 2 d after infection. (*D*) The relative amounts of Hck and L/S ratio of C/EBPβ or P/T ratio of p38 MAPK and ERK1/2 in immunoblot analysis shown in *c* calculated as described in Fig. 3. (*M*) M-MΦ; GM, GM-MΦ; T, T cells; 201, EM201; 703, EM703; SB, SB203580; and PD, PD98059. The data shown are representative of one of three independent experiments.

caused by a new means of converting the phenotype of tissue MΦs, from HIV-1 susceptible to HIV-1 resistant, via down-regulation of Hck and induction of the small isoform of C/EBPβ through modulation of the activation of MAPKs. Consistent with the previous report (9), we showed that HIV-1-resistant GM-MΦs, require stimulation with activated CD4+T cells to produce vigorous virus production. This is mediated by down-regulation of the expression of the small isoform of C/EBPβ. Both EM201 and EM703 potently inhibit viral replication, not only in M-MΦs but also in GM-MΦs stimulated with activated CD4+T cells via inhibiting the down-regulation of the expression of the small isoform of C/EBPβ.

Both EM201 and EM703 change the phenotype only of HIV-1-susceptible MΦs. They do not affect the phenotype of HIV-1-resistant GM-MΦs. HIV-1 susceptibility and the expression of Hck and C/EBPβ proteins in A-MΦs from normal healthy volunteers are the same as those in GM-MΦs (8, 10). Therefore EM201 and EM703 do not change the resistant phenotype of A-MΦs. This would be beneficial for healthy A-MΦs in the HIV-1 carrier, by maintaining resistance against HIV-1 replication.

In the present study, we demonstrated that activation of p38MAPK and ERK1/2 play a critical role in HIV-1 production via down-regulation of the small isoform of C/EBPβ in HIV-1-infected-M-MΦs and -GM-MΦs stimulated with activated CD4+T cells, respectively. This study shows that different MAPKs play crucial roles in HIV-1 production in different types of tissue MΦs. P38MAPK activation in HIV-1-infected M-MΦs link to the augmented expression of Hck and the maintenance of the low level of the small isoform of C/EBPβ. We previously reported that reduced expression of Hck in M-MΦs with antisense oligonucleotide for Hck stimulates the induction of the short isoform of C/EBPβ and inhibits the viral replication (10). Our present results, taken together with the previous study, show the unique evidence that the p38MAPK signal cascade is upstream of Hck expression and is linked to down-regulation of the small isoform of C/EBPβ in HIV-1-susceptible M-MΦs. However, ERK1/2-mediated down-regulation of the small isoform of C/EBPβ in HIV-1-infected GM-MΦs stimulated with activated CD4+T cells does not link to Hck expression.

Interestingly, EM201 and EM703, in contrast to existing MAPK inhibitors, can inhibit viral replication via prevention of the activation of respective MAPKs in both HIV-1-infected-M-MΦs and -GM-MΦs stimulated with activated T cells, where different MAPKs play a critical role for viral replication. Such a novel and unique suppressive mechanism of EM201 and EM703 on HIV-1 replication in tissue MΦs may be useful for the future treatment of AIDS patient.

The anti-HIV-1 activity of EM201 and EM703 does not relate to their antibiotic activity, because they have only weak (EM201) or completely lack (EM703) such antibiotic activity (24, 25). At present, we do not know what kind of structure activity relationships exist in EM201 and EM703. Recently, calmodulin- and calmodulin-dependent protein kinase-II (CaMK-II)-dependent activation of p38MAPK has been reported in HIV accessory protein, Tat-induced IL-10 expression in normal human monocytes (31). EM201 and EM703 are known to act as inhibitors of intracellular Ca^{2+} level and Ca^{2+} oscillation (21, 32, 33). These characteristics may contribute to the novel anti-HIV-1 mechanism of EM201 and EM703.

Macrolides, such as EMA and CAM, are known to be specifically accumulated into tissue MΦs and stay stable at high levels for long periods because of a low rate of breakdown and excretion (34). EM201 and EM703 potently inhibit HIV-1 replication in MΦs at low levels, such as 30 μM, that correspond to the concentration of EMA or CAM in MΦs after oral intake of EMA, 400 mg or CAM 200 mg/day (usual doses are 1600 mg and 400 mg/day, respectively). Furthermore, inhibition of viral replication can be observed at lower concentrations, such as 3 μM, which is sustained for 2–14 days after infection. These findings offer advantages with respect to drug specificity and reduction of drug toxicity. In addition, these new macrolides are derived from EMA (24, 25) and would be very inexpensive. Thus, these substances offer great potential for the creation of new anti-HIV-1 drugs for the future treatment of AIDS patients.

Materials and Methods

Erythromycin Derivatives. EMA was purchased from Sigma-Aldrich. CAM was supplied by Taisho Pharmaceutical. EM201, EM202, and EM703 were prepared as described previously (24, 25).

Preparation and Culture of MΦs. Monocytes (Mos) and Mo-derived Mp were prepared as described previously (10, 35). M-CSF-induced monocyte-derived MΦs and GM-CSF-induced monocyte-derived MΦs were called M-MΦs and GM-MΦs, respectively. [see supporting information (SI) *Materials and Methods* for a detailed description].

HIV-1 Strain and Infection. M-tropic HIV-1 strain, HIV-1_{BaL}, was collected from culture supernatant of the HIV-1 strain-infected M-MΦs as a viral resource. Mo-derived MΦs were incubated for 2 h at 37°C with 100 pg/ml p24 antigen of DNase-treated viral supernatant (p24, the 50% tissue culture infective dose ($TCID_{50}$) and multiplicity of infection (MOI) are 50 ng/ml, ~3,000 and 0.05, respectively) and then cultured in RPMI MEDIUM 1640 containing 10% FCS and CSF. If necessary, the viral inoculum was pretreated with 100 μM AZT for 2 h at 4°C (5). Fresh culture medium containing CSF was added every 3–4 d (20% of the volume). Heat-inactivated virus (1 h, 56°C) was used as negative control. Viral production was assayed by sequential measurement of p24 antigen in superna-

tants by an ELISA using a combination of two antibodies; anti-gag-p24 monoclonal antibody (Nu24) and peroxidase-labeled 1085 (36), or the RETRO-TEK HIV-1 p24 antigen ELISA kit for high-affinity detection of low levels of p24 antigen (ZeptoMetrix, Buffalo, New York).

Coculture of HIV-1 Infected GM-MΦs with the Activated CD4⁺T Cells. $CD4^+$ T cells were positively isolated from $CD14^-$ PBMCs using a MACS with anti-CD4 mAb coated microbeads. The selected population was >93% positive for CD3 and CD4. Activated $CD4^+$ T cells were prepared by stimulation with PHA and cultured with IL-2 (30 unit/ml) (Genzyme). GM-MΦs were incubated for 2 h at 37°C with 100 pg/ml p24 antigen of DNase-treated viral supernatant, washed twice, and then cocultured with the activated $CD4^+$ T cells in the presence of IL-2.

Detection of HIV-1 DNA by Nested PCR. Detection of HIV-1 DNA by nested PCR was performed as described previously (10). HIV LTR and *gag* primers were JAM 62 and JAM 65. For the nested PCR, JAM 63 and JAM 64 were used as internal primers (36). (see *SI Materials and Methods* for a detailed description).

Immunblot Analysis. Immunoblot analysis was performed as described in ref. 10. Antibodies against the following proteins were used: rabbit polyclonal antibody against Hck (N-30), C/EBPβ (C-19) (Santa Cruz Biotechnology), phospho-specific (Tyr 182) p38 mitogen-activated protein kinase (P38MAPK) (no. 9211), p38 MAPK (no. 9212), phospho-specific (Tyr 204) phosphorylated extracellular signal-regulated kinases (ERK)1/2 (no. 9101), ERK1/2 antibody (no. 9102) (New England BioLabs), or normal rabbit IgG. Horseradish peroxidase-conjugated goat anti-rabbit IfG (sx-2030)(Santa Cruz Biotechnology) was used as secondary Ab. The blots were visualized with Amersham ECL Reagent on Hyper ECL-film (Amersham). (see *SI Materials and Methods* for a detailed description).

Statistical Analysis. Statistical analysis of the data were performed using Student's *t test*. *P*-values <0.01 were considered significant. The experiments shown are representatives of three to seven independent experiments.

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1. Cavert W, *et al.* (1997) Kinetics of response in lymphoid tissues to antiretroviral therapy of HIV-1 infection. *Science* 276:960–964.
2. Perelson AS, *et al.* (1997) Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature* 387:188–191.
3. Orenstein JM, Fox C, Wahl SM (1997) Macrophages as a source of HIV during opportunistic infections. *Science* 276:1857–1861.
4. Crowe SM, Sonza S (2000) HIV-1 can be recovered from a variety of cells including peripheral blood monocytes of patients receiving highly active antiretroviral therapy: A further obstacle to eradication. *J Leukoc Biol* 68:345–350.
5. Orenstein JM (2001) The macrophage in HIV infection. *Immunobiol* 204:598–602.
6. Nakata K, *et al.* (1997) Mycobacterium tuberculosis enhances human immunodeficiency virus-1 replication in the lung. *Am J Resp Crit Care Med* 155:996–1003.
7. Whalen C, *et al.* (1995) Accelerated course of human immunodeficiency virus infection after tuberculosis. *Am J Respir Crit Care Med* 151:129–135.
8. Honda Y, *et al.* (1998) Type I interferon induces inhibitory 16-kD CCAAT/enhancer binding protein (C/EBP)beta, repressing the HIV-1 long terminal repeat in macrophages: Pulmonary tuberculosis alters C/EBP expression, enhancing HIV-1 replication. *J Exp Med* 188:1255–1265.
9. Hoshino Y, *et al.* (2002) Maximal HIV-1 replication in alveolar macrophages during tuberculosis requires both lymphocyte contact and cytokines. *J Exp Med* 195:495–505.
10. Komuro I, Yokota Y, Yasuda S, Iwamoto A, Akagawa KS (2003) CSF-induced and HIV-1-mediated distinct regulation of Hck and C/EBPbeta represent a heterogeneous susceptibility of monocyte-derived macrophages to M-tropic HIV-1 infection. *J Exp Med* 198:443–453.
11. Henderson AJ, Calame KL (1997) CCAAT/enhancer binding protein (C/EBP) sites are required for HIV-1 replication in primary macrophages but not CD4(+) T cells. *Proc Natl Acad Sci USA* 94:8714–8719.
12. Welden M, *et al.* (2000) Differentiation of monocytes to macrophages switches the mycobacterium tuberculosis effect on HIV-1 replication from stimulation to inhibition: Modulation of interferon response and CCAAT/enhancer binding protein beta expression Type I interferon induces inhibitory 16-kD CCAAT/enhancer binding protein (C/EBP)beta, repressing the HIV-1 long terminal repeat in macrophages: Pulmonary tuberculosis alters C/EBP expression, enhancing HIV-1 replication. *J Immunol* 165:2028–2039.
13. Hogan TH, Krebs FC, Wigdahl B (2002) Regulation of human immunodeficiency virus type 1 gene expression and pathogenesis by CCAAT/enhancer binding proteins in cells of the monocyte/macrophage lineage. *J Neurovirol* 8 Suppl 2:21–26.
14. Kudoh S, Uetake K, Hagiwara K (1987) Clinical effect of low dose long term erythromycin chemotherapy on diffuse panbronchiolitis. *Jap J Thorac Dis* 25:632–642.
15. Kadota J, *et al.* (1993) A mechanism of erythromycin treatment in patients with diffuse panbronchiolitis. *Am Rev Respir Dis* 147:153–159.
16. Sugawara E (1997) Effect of macrolide antibiotics on neutrophil function in human peripheral blood. *Kansenshogaku Zasshi* 71:329–336.
17. Sunazuka T, *et al.* (1999) Effects of erythromycin and its derivatives on interleukin-8 release by human bronchial epithelial cell line BEAS-28 cells. *J Antibiot* 52:71–74.
18. Oohori M, *et al.* (2000) Effect of 14-membered ring macrolide compounds on rat leucocytes chemotaxis and the structure-activity relationships. *J Antibiot* 53:1219–1222.
19. Keicho N, Kudoh S, Yotsumoto H, Akagawa KS (1993) Erythromycin promotes monocytes to macrophage differentiation. *J Antibiot* 47:80–89.
20. Keicho N, Kudoh S, Yotsumoto H, Akagawa KS (1993) Antilymphocytic activity of erythromycin distinct from that of FK506 or cyclosporin A. *J Antibiot* 46:1406–1413.
21. Kudoh S, *et al.* (2002) Novel activity of erythromycin and its derivatives. *Macrolide Antibiotics, Chemistry, Biology, and Practice*, eds Omura S (Academic), 2nd Ed, pp 533–569.
22. Culic O, Erakovic V, Parnham MJ (2001) Anti-inflammatory effects of macrolide antibiotics. *Eur J Pharmacol* 429:209–229.
23. Labro MT (2001) Anti-inflammatory activity of macrolides: A new therapeutic potential? *J Antimicrob Chemother* 41(Suppl B):37–46.
24. Sunazuka T, *et al.* (2003) Effect of 14-membered macrolide compounds on monocyte to macrophage differentiation. *J Antibiot* 5:721–724.
25. Yoshida K, *et al.* (2005) Macrolides with promotive activity of monocyte to macrophage differentiation. *J Antibiot* 58:79–81.
26. Ying JL, *et al.* (2006) EM703 improves bleomycin-induced pulmonary fibrosis in mice by the inhibition of TGF-beta signaling in lung fibroblasts. *Respir Res* 7:16:1–13.
27. Matsuda S, *et al.* (1995) Suppression of HIV replication in human monocyte-derived macrophages induced by granulocyte/macrophage colony-stimulating factor. *AIDS Res Hum Retroviruses* 11:1031–1038.
28. Muthumani K, *et al.* (2004) Suppression of HIV-1 viral replication and cellular pathogenesis by a novel p38/JNK kinase inhibitor. *Aids* 18:739–748.
29. Nakajima T, *et al.* (1993) Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc Natl Acad Sci USA* 90:2207–2211.
30. Giltiay NV, Karakashian AA, Alimov AP, Ligthie S, Nikolova-Karakashian MN (2005) Ceramide- and ERK-dependent pathway for the activation of CCAAT/enhancer binding protein by interleukin-1beta in hepatocytes. *J Lipid Res* 46:2497–2505.
31. Gee K, Angel JB, Mishra S, Blahoianu MA, Kumar A (2007) IL-10 regulation by HIV-Tat in primary human monocytic cells: Involvement of calmodulin/calmodulin-dependent protein kinase-activated p38 MAPK and Sp-1 and CREB-1 transcription factors. *J Immunol* 178:798–807.
32. Sunazuka T, Omura S (2004) Creation of a new macrolide derivative, EM703. *Jpn J Antibiot* 57(Suppl A):114–116.
33. Hattori R, Shimizu T, Shimizu S, Majima Y (2004) Effect of EM703, a new macrolide derivative, on mucus secretion from the airway epithelial cells. *Jpn J Antibiot* 57(Suppl A):123–125.
34. Fietta A, Merlini C, Gialdroni GG (1997) Requirements for intracellular accumulation and release of clarithromycin and azithromycin by human phagocytes. *J chemotherapy* 9:23–31.
35. Akagawa KS (2002) Functional heterogeneity of colony-stimulating factor induced human monocyte-derived macrophages. *Int J Hematol* 76:27–34.
36. Tsunetsugu-Yokota Y, *et al.* (1995) Monocyte-derived cultured dendritic cells are susceptible to human immunodeficiency virus infection and transmit virus to resting T cells in the process of nominal antigen presentation. *J Virol* 69:4544–4547.

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Oral Attenuated *Salmonella enterica* Serovar *typhimurium* Vaccine Expressing Codon-Optimized HIV Type 1 *Gag* Enhanced Intestinal Immunity in Mice

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ABSTRACT

Oral immunization is a safe and easily applicable route to induce mucosal immunity to HIV infection. We examined the ability of oral attenuated *Salmonella typhimurium* (ST) vaccine expressing Gag for the efficiency of generating Gag-specific mucosal IgA and CD8$^+$ T cell responses in intestinal lymphoid tissues. By optimizing the codon of HIV-1 *gag* to the preferred codon bias of *Salmonella*, the expression of Gag in *Salmonella* was dramatically improved. The oral ST-Gag vaccine by itself was not so powerful and induces little Gag-specific CD8$^+$ T cell responses in the intestine. Nevertheless, we found that it potentiates otherwise weak intestinal CD8$^+$ T cell responses in nasally primed mice with Gag p24 and cholera toxin adjuvant. Thus, the oral delivery of *Salmonella* expressing Gag would be utilized in combination with other parenteral vaccine to direct and strengthen intestinal HIV-specific CTL responses.

INTRODUCTION

TRANSMISSION OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) occurs mainly through mucosal surfaces during sexual contact. Recent studies revealed that the gastrointestinal and vaginal mucosa serve as sites for virus entry and are the initial and predominant sites where the virus replicates and amplifies itself; they are also the initial sites of CD4$^+$ T cell depletion.[1] Also in the macaque AIDS model, the quite recent reports that the massive destruction of resting memory CD4$^+$ T cells occurs early during simian immunodeficiency virus (SIV) infection further supports the importance of mucosal immunity.[2,3] Thus, it is currently believed that the new generation of candidate anti-HIV vaccines should elicit mucosal immune responses, including mucosal secretory IgA and, especially, CD8$^+$ cytotoxic T lymphocyte (CTL) responses, especially in intestinal and vaginal tissues. Furthermore, because of the economic concerns in many resource-poor countries, an ideal vaccine should be safe, have needle-free delivery, be cheap and easy to handle, and not need cold chain maintenance.

Mucosal immunization, especially by the oral route, has in recent years attracted great interest as a means of eliciting protective immunity against a variety of infectious diseases. The oral polio vaccine, which induces both systemic and mucosal humoral responses, is the most successful mucosal vaccine known to date.[4] Oral, live, attenuated *Salmonella* vaccine, Ty21a, is also known to induce strong and sustained humoral as well as cellular immune responses both in the mucosal and systemic compartments and now is licensed in 56 countries in Asia, Africa, Europe, and the Americas,[5] though a further improved vaccine is desired.[6] *Salmonella typhimurium* (ST) causes a self-limiting gastroenteritis in a wide range of mammals, including humans, and a systemic typhoid-like disease in mice. ST preferentially binds to the M cells in the follicular-associated epithelium (FAE) that is located above the Peyer's patch, and enters the submucosa either via the transcytosis of M cells or by disturbing the seal of the epithelial cell layer (which of these options mainly occurs is unclear at present).[7] ST is then phagocytosed by macrophages and dendritic cells (DCs) that are local to the area or recruited there by the infection. The bacteria taken up by these cells are known to persist in an intracellular membrane compartment and initiate T cell responses.[8,9] Thus, live *Salmonella* serves as an ideal carrier for the delivery of antigen to mucosa-associated lymphoid tissues in the intestine.

In animal models, attenuated ST has been used as a carrier to orally deliver the truncated HIV-1 env in mice[10,11] or SIV Gag in macaques.[12] However, these studies revealed that the

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expression level of foreign proteins in ST was low and, therefore, oral ST induced only weak immune responses. We have previously studied the effect of recombinant p24 Gag protein with cholera toxin (CT) adjuvant via the nasal and rectal routes on mucosal immune responses against HIV-1 Gag, with a particular focus on CTL responses.[13] We showed that although nasal immunization potently elicits both systemic and mucosal immunity in nasal-associated lymphoid tissue (NALT), its effect on intestinal/rectal mucosal immune responses was relatively weak and it was enhanced by rectal immunization of the same regimen, which was consistent with the report by Belyakov *et al.*[14] These results indicate that the intestinal tissue is a primary target site for mucosal vaccine to elicit immune responses against HIV. In this study, we developed ST expressing the Gag-EGFP fusion protein at a high level and tested the immunogenicity of orally administered live attenuated ST as an alternative and safe intestinal mucosal vaccine.

MATERIALS AND METHODS

Mice and immunization protocols

Female BALB/c mice at 7–8 weeks of age were purchased from Japan SLC Inc. (Shizuoka, Japan). All experimental procedures were approved and carried out in accordance with the guidelines of the Laboratory Animal Care Unit at the National Institute of Infectious Diseases. Mice were anesthetized by an intraperitoneal injection of sodium isomytal (Nihon Shinyaku Co. Ltd, Tokyo, Japan) before antigen was administered nasally. For nasal immunizations, recombinant Gag p24 antigen (5 μg) mixed with 2 μg CT (a whole toxin, Sigma Chemical Co., St. Louis, MO) in 10 μl of phosphate-buffered saline (PBS) was inoculated into the nasal cavity of each mouse as described previously.[13] Each nasal immunization was carried out at 3 week interval.

Attenuated ST (*aroA* mutant strain SL7207) was kindly provided by Dr. Bruce Stocker (Department of Microbiology and Immunology, Stanford University), which was successfully utilized as a delivery system for oral DNA immunization.[15] The EGFP-Gag expression or empty vector DNA was electroporated into ST at 1750 V, 25 μF by using a Gene Pulser (Bio-Rad, Hercules, CA). The bacteria were grown in LB media containing ampicillin overnight, collected, and then resuspended in PBS at $1\sim2 \times 10^{10}$ CFU/ml. For oral administration, the mice were left without food early in the morning and given 200 μl of the ST suspension through a gastric tube directly into the lower stomach in the evening. We avoided to use the sodium bicarbonate solution, because it reduced the viability of bacteria too severely. We were able to detect live bacteria in fecus with this method. Four oral immunizations were carried out twice a week over 2 weeks.

Reagents

Recombinant Gag p24 was prepared in house from p24-expressing bacteria as described previously.[16] The H-2^d-restricted Gag197–205 (Gag197)[17] and control peptides (Gag77 or Gag239, which were derived from HIV-1 Gag, but not a CTL epitope of BALB/c mice) were produced by Sawaday Technology Co. Ltd. (Tokyo, Japan). The FITC-labeled-anti-CD3, APC-labeled-anti-CD8, PE-labeled CD11c, and PE-labeled-anti-interferon (IFN)-γ antibodies were purchased from e-Bioscience Inc. (San Diego, CA). Collagenase A (Sigma) and Hanks' balanced salt solution (HBSS: Invitrogen Corp., Carlsbad, CA) were also purchased.

Construction of a Gag-expression plasmid for Salmonella vaccine

A truncated gene encoding Gag p24 [nucleotide numbers ranging from 1213 to 1707 of pNL432 (GenBank: M19921), which encodes a 166 amino acid stretch that is designated as N1C2] was amplified by PCR and subcloned into a *Hin*dIII–*Sal*I site of pEGFP-C2 (BD Bioscience, San Jose, CA) under the CMV promoter. The whole *EGFP–N1C2* fragment was then inserted into the *EGFP* gene region of pEGFP (BD Bioscience) via its *Nco*I and *Eco*RI sites. This resulting pEGFP–N1C2 plasmid expresses the *EGFP–Gag* fusion gene under the lac promoter (*lacP*).

To maximize the production of Gag p24 in *Salmonella*, we changed the codons of the viral *gag* gene to reflect the codon usage of *Salmonella* and it was synthesized with the *Hin*dIII and *Bam*HI site at both ends (GenScript Corp., Scotch Plains, NJ). This gene, which encodes 157 amino acids, was supplied as a clone in a pUC57 plasmid (GenBank No. Y14837) and was further subcloned into a multicloning site (*Hin*dIII and *Bam*HI) of pEGFP; this construct was designated as $pN1C2_{ST}$–EGFP.

Western blot analysis

Escherichia coli and *Salmonella typhimurium* transformed with pEGFP–N1C2 or $pN1C2_{ST}$–EGFP were lysed in B-PER protein extraction reagent (Pierce Biotechnology Inc., Rockford, IL). The proteins were separated by 10% SDS–PAGE and transferred to a PVDF membrane (Immunobilon-P, Millipore Ltd., Bedford, MA). The membrane was blocked with PBS containing 5% nonfat dried milk, washed with PBS-Tween (0.05%), and then reacted with anti-EGFP mAb (BD Bioscience) or anti-Gag p24 mAb [Nu24:IgG_1, a kind gift from Dr. T. Sata, Department of Pathology, National Institute of Infectious Diseases (NIID), Tokyo] for 1 h at room temperature (RT), followed by incubation with HRP-labeled antimouse IgG antibody. The signal was visualized by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech Inc., Uppsala, Sweden) using an LAS 3000 analyzer (Fuji film, Co. Ltd., Tokyo, Japan).

Cell preparation

Cells were prepared from pooled spleens, NALT, posterior cervical lymph nodes (CLN), mesenteric lymph nodes (MLN), and a lymphoid follicle in the appendix (App) of immunized BALB/c mice as described previously.[13] Splenocytes were enriched for T cells by lysing the erythrocytes and then passing the suspension through a nylon wool column. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, a mixture of penicillin and streptomycin (Invitrogen Corp., Carlsbad, CA), and 50 μM 2-mercaptoethanol (Sigma). A20.2J murine B cell line cells were served as antigen-presenting cells (APCs).

To enrich $CD11c^+$ DCs, the MLNs and Peyer's patch (PP) of two to three immunized mice were pooled and treated with

collagenase A (50 U/ml) for 30 min at 37°C. After washing with HBSS supplemented with 5% FBS and 5 mM EDTA, the cells were labeled with anti-CD11c-conjugated magnetic beads (Miltenyi) in MACS buffer (0.5% BSA/5 mM EDTA) for 15 min at 10°C, and then run through the column under a magnetic field followed by five washes. The trapped cells were finally flushed out and subjected to a second round of column purification. The yield of DCs from MLNs and PPs were from $1\sim5 \times 10^5$ cells, depending on the experiments.

ELISA

Anti-p24 antibodies in mouse sera were measured by ELISA as described previously.[13] Briefly, serially diluted mouse sera were added to the wells of an ELISA plate (MaxiSorb F96; Nunc Inc., Roskilde, Denmark) that had been coated with 1 μg/ml recombinant Gag p24. After incubation for 2 h at RT, the plate was washed and incubated with biotinylated rat anti-mouse IgG1 mAb (Southern Biotechnology Associates Inc., Birmingham, AL), followed by reaction with HRP-streptavidin (Behringer-Roche, Basel, Switzerland). After washing, *o*-phenylenediamine (Sigma) was added to the wells as a peroxidase substrate and the optical density (OD) at 490 nm in each well was determined. The level of p24-specific IgG1 antibodies in each biological sample was determined by using the purified mouse anti-Gag p24 mAb (Nu24) standard.

To measure the intestinal anti-p24 IgA antibody levels, mice were sacrificed, canulated at a duodenal bulb, and intestinal fecal remnants were collected by flushing out the whole intestine down to the upper colon with cold 5 ml HBSS. Fecal clumps were mashed and the intestinal fluid containing the feces was transferred into plastic tubes and centrifuged at 2000 rpm for 10 min. The anti-Gag IgA antibodies were detected as described above but by using an HRP-antimouse IgA antibody (Cosmo-Bio Co., Ltd., Tokyo, Japan) instead of the rat antimouse IgG1 mAb and the TMB(+) reagent (Dako Corp., Carpinteria, CA) as the substrate. Optical density at OD_{450} was then determined.

Analysis of DC antigen presentation

The antigen-presenting activity of DCs was analyzed by using a Gag-specific $CD8^+$ CTL cell line[13] as the indicator cells. Briefly, CTL line cells (2×10^5 cells) and $CD11c^+$ DCs were plated together in the presence of Gag197 peptide (1 μg/ml) into 96-well round bottom plates at a 10:1 ratio and cultured overnight in a CO_2 incubator at 37°C. Monensin (2 μM) was then added and the cells were incubated further for 6 h. They were stained first with ethidium monoazide bromide (EMA: 5

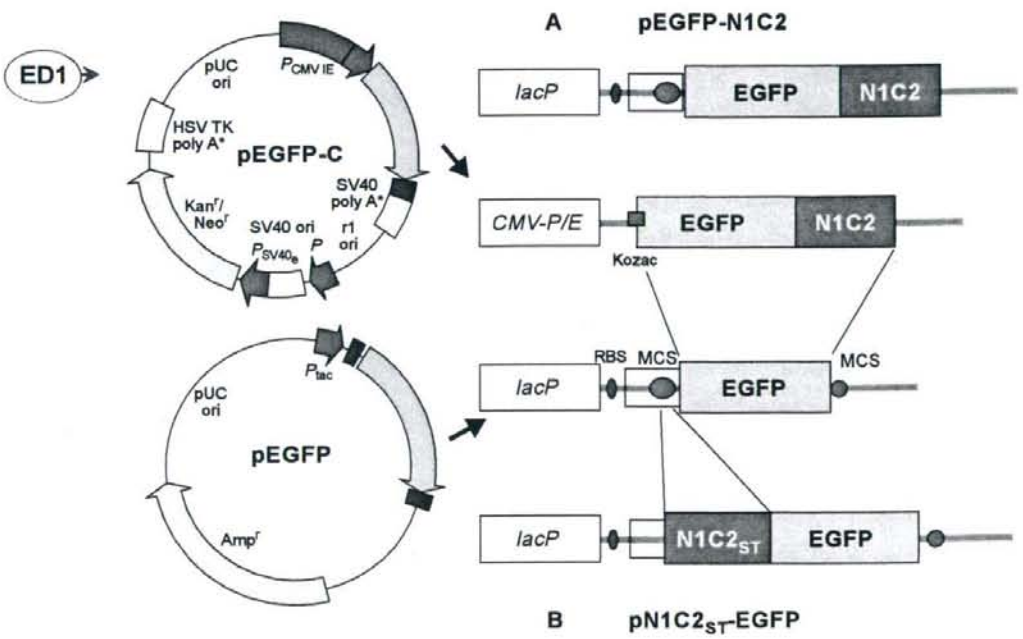

FIG. 1. Structure of plasmids expressing Gag–EGFP fusion proteins. (A) A truncated *gag* gene encoding a Gag p24 protein denoted as N1C2 was PCR amplified and inserted into a multiple cloning site of the eukaryote expression plasmid pEGFP-C2 to produce the *egfp–N1C2* fragment. The whole *egfp* gene of pEGFP was then replaced with *egfp–N1C2*, resulting in the plasmid designated as pEGFP–N1C2. *lacP*, lac promoter; RBS, ribosomal binding site; MCS, multiple cloning sites. (B) The synthetic *N1C2* gene that employed the codon usage of *Salmonella* was cloned into the pEGFP vector at a multiple cloning site, resulting in a plasmid designated as $pN1C2_{ST}$–EGFP.

μg/ml) for 20 min on ice and then incubated with FITC-antimouse CD3 and APC-antimouse CD8 on ice for 20 min. After washing, fixing, and permeabilizing, intracellular IFN-γ staining was carried out as described previously.[13] The stained cells were analyzed by FACScalibur (BD Bioscience) using the Cell Quest program. The data were reanalyzed and depicted by gating on FSC/SSC low, $CD3^+$ T cells using Flowjo software (Tree Star Inc., San Carlos, CA).

ELISPOT assay

HA-Multiscreen plates (Millipore, Burlington, MA) were precoated with 100 μl of PBS containing 2 μg/ml rat antimouse IFN-γ mAb (clone R4-6A2; Endogen, Woburn, MA), incubated overnight at 4°C, washed, and blocked with RPMI 1640/10% FBS for 2–3 h at 37°C. Lymphocytes from various tissues were cocultured in triplicate with irradiated A20.2J in the presence of Gag197 or control peptide at 1 μg/ml. After an overnight incubation, the cells were washed with tap water and the wells were washed three times with PBS-Tween 20 (0.05%) and incubated with biotin-conjugated antimouse IFN-γ mAb (clone XMG1.2, Endogen) in PBS-Tween (0.5%) containing 0.5% BSA for 2 h at 37°C. After extensive washes, HRP-conjugated streptavidin (Boehringer-Roche) was added for 1 h at RT, and then the spots were stained with AEC (Sigma-Aldrich). The colored spots representing IFN-γ-producing cells were counted by using the KS ELISPOT compact system (Carl Zeiss Inc., Jena, Germany).

Statistical analyses

Intergroup comparisons were performed by using the one-way ANOVA test followed by the posttest of Tukey. p values less than 0.05 were considered to be significant.

RESULTS

Expression of a Gag-EGFP fusion protein by Escherichia coli and attenuated Salmonella typhimurium

To monitor the tissue distribution of antigen after inoculation of attenuated *Salmonella*, we constructed a Gag–EGFP gene fusion. First, we amplified a part of the gag gene, N1C2, by PCR and cloned into the CMV-driven expression plasmid, pEGFP-C (Fig. 1). The *N1C2* gene encodes a truncated HIV-1 Gag p24 protein that contains the H-$2K^d$-restricted immunodominant epitope Gag-197 (AMQMLKETI).[17] When we transfected COS cells with this plasmid, the fluorescence of the fusion protein could be readily detected by fluorescent microscopy and by anti-Gag and anti-EGFP mAb on Western blots of the cell extracts, suggesting that the fusion protein is correctly folded and that the fusion did not disturb the fluorescence of EGFP (data not shown). Next, we constructed the pEGFP–N1C2 plasmid by replacing the EGFP gene in pEGFP with the EGFP–N1C2 fusion gene (Fig. 1A) After transformation into *E. coli* and ST, the expression of the Gag–EGFP fusion protein in *E. coli* (Fig. 2, lane 1) and ST (Fig. 2, lane 4) was assessed by Western blot analysis using anti-EGFP (Fig. 2, left panel) and anti-p24 mAbs (Fig. 2, right panel). The expression level of the truncated Gag–EGFP protein was poor in ST compared to that in *E. coli*.

F1

F2

Although we attempted to utilize ST transformed with pEGFP–N1C2 as an oral vaccine in mice, a significant immune response could not be achieved. Therefore, we constructed an additional *N1C2*-bearing plasmid after altering the codons of the HIV1 *gag* gene to reflect the codon usage of *Salmonella*. To do this, synthetic *N1C2* DNA was cloned into the 5′ multiple cloning site (MCS) of pEGFP to avoid further modifica-

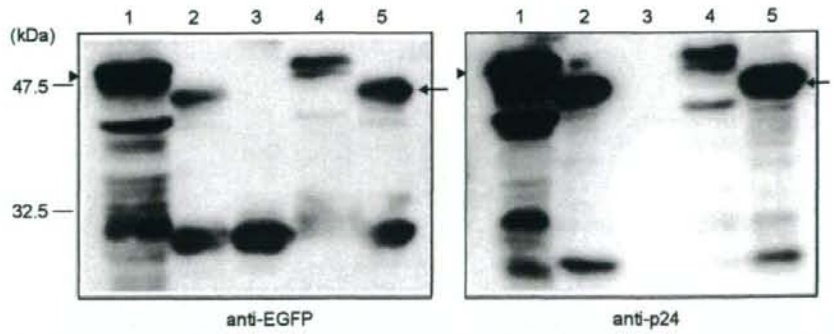

FIG. 2. Expression of the Gag–EGFP fusion proteins in *E. coli* and ST. Cell lysates prepared from *E. coli* (lanes 1 and 2) or ST (lanes 3–5) transformed with pEGFP–N1C2 (lanes 1 and 4), $pN1C2_{ST}$–EGFP (lanes 2 and 5), or pEGFP (lane 3) were separated by 10% SDS–PAGE in duplicate, and Western blot analysis was carried out. One membrane was reacted with anti-EGFP mAb (1:10,000 dilution) (left) and the other with anti-Gag p24 mAb (1:500 dilution) (right) for 1 h at RT, followed by incubation with biotinylated antimouse IgG polyclonal antibody (1:1000 dilution). The membranes were then washed and further incubated with HRP-conjugated streptavidin (1:20,000 dilution) for 30 min at RT. The signal was visualized by an enhanced chemiluminescence detection system (Amersham Pharmacia) using LAS 3000 analyzer (Fuji film). The Gag–EGFP fusion protein encoded by pEGFP–N1C2 (50.4 kDa) is shown by an arrowhead while that encoded by $pN1C2_{ST}$–EGFP (47 kDa) is shown by an arrow.

tion of the pEGFP vector itself. Furthermore, we reasoned that it would be beneficial to translate Gag first to increase the reliability of Gag protein production in the bacteria, because many extra bands were detected in *E. coli* transformed with the original pEGFP–N1C2 (Fig. 2, lane 1). These extra bands are presumably truncated fusion proteins caused by aberrant initiation of translation within the fusion protein gene. The resulting plasmid was designated as pN1C2$_{ST}$–EGFP (Fig. 1B). This plasmid encodes a Gag protein that is nine amino acids shorter than the original N1C2 protein.

The pN1C2$_{ST}$–EGFP plasmid expressed considerably more Gag–EGFP fusion protein upon transfection into ST than the original plasmid, as shown in Fig. 2 (compare lanes 4 and 5). Note that both pEGFP–N1C2 and pN1C2ST–EGFP express Gag–EGFP bearing several additional amino acids encoded by the *β-galactosidase* gene located in the 5′ region of the expression vector. This results in proteins with predicted sizes of 50.4 and 47 kDa, respectively. As a control, *E. coli* and ST were transfected with the original pEGFP plasmid; this plasmid expressed the 27-kDa EGFP protein at similar levels in both bacteria without codon optimization (Fig. 2, lane 3 and data not shown), suggesting that sequences that inhibit transcription/translation in *Salmonella* are present in the original HIV-1 *gag* gene. Therefore, by altering the codons of HIV-1 *gag* to reflect codon usage in *Salmonella*, recombinant Gag expression in ST was enhanced by approximately 20-fold, though direct comparisons of the levels of EGFP–N1C2 and N1C2ST–EGFP fusion proteins were difficult. Of note, we observed that ST transformed with pN1C2ST–EGFP was more visibly green in color than ST transformed with pEGFP–N1C2, suggesting that pN1C2$_{ST}$–EGFP was more stable in ST. The ST strains transfected with pN1C2$_{ST}$–EGFP and pEGFP are denoted henceforth as ST-coGag, and ST-cont, respectively.

Gag-presenting DCs are present in the MLNs after oral administration of Salmonella expressing Gag–EGFP

We designed the pN1C2–EGFP and pN1C2$_{ST}$–EGFP plasmids because the EGFP signal of the resulting Gag–EGFP fusion proteins could be used to identify Gag-bearing APCs by flow cytometry. In a preliminary experiment, the green fluorescence of ST-coGag taken up by macrophage cell line cells (J774.1) was detectable by a fluorescent microscope. Therefore, to evaluate the function of DCs localized in intestinal lymphoid tissues, three mice per group were given ST-cont or ST-coGag orally for 2 consecutive days and sacrificed the next day. The MLNs and PP of these mice were pooled, treated with collagenase A, and the CD11c$^+$ cell fraction was obtained by using anti-CD11c-conjugated magnetic beads. Although the experiment was repeated several times, we failed to detect the EGFP signal in this fraction of cells (data not shown).

We then examined whether the orally immunized mice with ST-coGag nevertheless had a small, undetectable fraction of CD11c$^+$ DCs that can present Gag antigen to T cells by utilizing a Gag-specific CD8$^+$ CTL cell line that produces IFN-γ upon stimulation with the Gag197 peptide.[13] This Gag-specific CD8$^+$ T cell line was cocultured with CD11c$^+$ cells derived from the MLNs or PP of ST-coGag- or ST-cont-immunized mice. Note that when these T cells were cocultured with Gag197 peptide-pulsed CD11c$^+$ cells of normal mouse, approximately 14~18% of them produced IFN-γ, while IFN-γ$^+$ cells were <0.01% without peptide (data not shown). The representative results of two experiments are shown in Fig. 3. By coculturing with CD11c$^+$ cells derived from MLNs of ST-coGag-immunized mice, a low but substantial number of T cells responded to produce IFN-γ (0.13%) (Fig. 3B), while only a very few T cells produced IFN-γ by coculturing with CD11c$^+$ cells derived from MLNs of ST-cont-immunized mice (0.03%) (Fig. 3C), and no T cells were activated when cultured without APCs (Fig. 3A). CD11c$^+$ cells similarly enriched from the PP of ST-coGag- or ST-cont-immunized mice failed to activate the T cell line (<0.01%) (data not shown). Thus, it is possible that the MLNs, but not PP, contain a very small number of CD11c$^+$ DCs that present Gag to T cells shortly after oral immunization with ST-Gag.

F3

Salmonella expressing coGag induce Gag-specific antibodies in the intestinal mucosa

It is believed that *Salmonella* efficiently induces specific IgA responses in the intestinal mucosa by selectively binding to M cells in the mucosal epithelium.[7] We showed previously that nasal immunization twice with recombinant Gag p24 plus CT efficiently induced a high level of IgA secretion in the nasal cavity as well as serum IgG; however, it was relatively inefficient to elicit IgA in the rectal mucosa.[13] Therefore, we first examined the systemic and mucosal antibody responses by oral immunization of mice with ST-coGag. As a positive control of the mucosal anti-Gag response, a group of mice was nasally immunized twice with Gag p24 plus CT. In preliminary experiments, we were unable to detect an anti-Gag antibody response by two or three oral ST-coGag immunizations (data not shown). However, when mice were orally immunized with ST-coGag four times, twice a week, two of four mice immunized with ST-coGag, but not with ST-cont, developed Gag-specific IgG in the serum (Fig. 4A). As expected, when mice were immunized twice, at 3 week interval, with Gag p24 plus CT a high level of Gag-specific IgG was elicited in all the mice. In contrast, while orally immunized mice with ST-coGag secreted the Gag-specific IgA in the intestine (intestinal wash), it was not detected in orally immunized mice with ST-cont and even in nasally immunized mice (Fig 4B). Therefore, the anti-Gag IgA antibody response was preferentially induced in intestinal mucosa by oral ST-coGag immunization. Our results suggest that oral ST-coGag administration is useful to direct Gag-specific immunity to the intestinal mucosa.

F4

Oral Salmonella vaccine enhances Gag-specific CD8$^+$ T cell responses both in the intestinal and nasal tissues

It was shown that long-lasting protection against mucosal HIV transmission could be achieved by CD8 CTLs induced in the mucosal site of exposure.[18] Our previous study showed that while nasal immunizations with Gag p24 plus CT potently elicit specific CTL responses in NALT, they induce only a weak CTL response in intestinal lymphoid tissues.[13] We then examined the ability of the oral ST vaccine to elicit Gag-specific T cell responses in intestinal tissues by IFN-γ ELISPOT analysis using the Gag197 epitope peptide. Mice received four consecu-

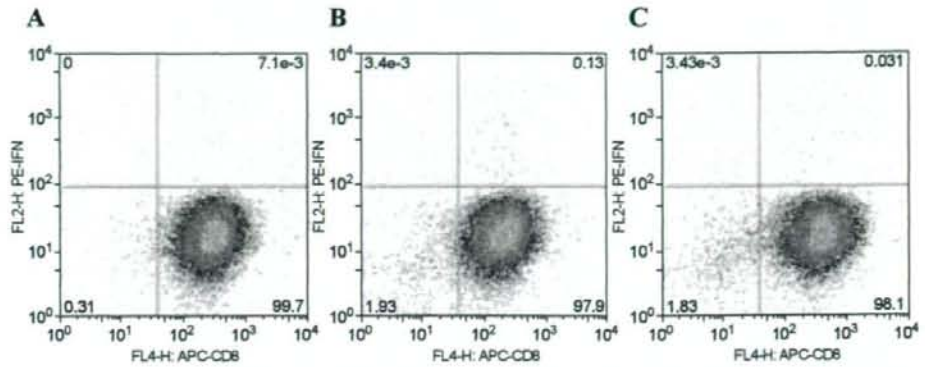

FIG. 3. Gag-presenting DCs are present in MLNs after oral administration of *Salmonella* expressing Gag. Four mice per group were orally immunized four times with ST-coGag (**B**) or ST-cont (**C**) twice a week over 2 weeks and sacrificed 24 h later. The pooled MLNs of these mice were treated with collagenase and the cell suspension was enriched for $CD11c^+$ DCs. These DCs were cocultured with a Gag-197-specific NALT-derived $CD8^+$ CTL cell line overnight. The next day, monensin was added to a final concentration of 2 μM. After 6 h incubation, the cells were stained for CD8 expression, fixed, subjected to intracellular IFN-γ staining, and analyzed by FACScalibur using the Cell Quest program. The data were then reanalyzed and depicted by gating on FSC/SSC low, $CD3^+$ T cells by using Flowjo software. (**A**) The NALT-derived $CD8^+$ CTL cell line was cultured in the absence of APC. The percentage of T cells expressing IFN-γ is shown in the top righthand quadrant. No IFN-γ production was detected in (**A**) and (**C**). In contrast, the DCs from ST-Gag-immunized animals induced weak but substantial IFN-γ production in Gag-specific $CD8^+$ T cells (**B**).

tive oral ST-coGag immunizations; however, this did not elicit $CD8^+$ T cell responses in MLN, suggesting that oral ST immunization is not strong enough with respect to the inducibility of cellular immunity. Evans *et al.*[19] utilized oral ST vaccine expressing SIV Gag in their macaque model and showed its priming effect followed by a peripheral boost with modified vaccine virus Ankara expressing SIV Gag.

Therefore, to examine whether the oral ST vaccine is able to direct cellular immunity to intestinal mucosa as in the case of the antibody response described above, we primed mice nasally with Gag p24 plus CT, only once, followed by four oral administrations of ST-coGag or ST-cont immunization. As shown in Fig. 5A, a single injection of Gag p24 plus CT elicited only weak $CD8^+$ T cell responses in NALT but not in MLN. F5

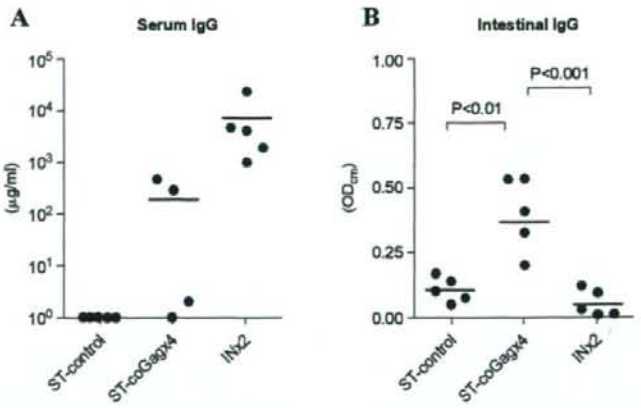

FIG. 4. *Salmonella* expressing coGag elicits both systemic and mucosal Gag-specific antibody responses. Mice (five or four mice) received either two nasal immunizations of Gag p24 plus CT (INx2) at 3 week interval, or four oral immunizations with ST-coGag or ST-control (ST-cont) twice a week over 2 weeks. Seven days after the last immunization, ELISA was used to measure the serum Gag-specific IgG (**A**) or intestinal Gag-specific IgA (**B**) levels in each mouse. The individual results are represented by closed circles. The bars indicate the mean antibody levels in the mouse groups. *Significant difference between the two groups indicated ($p < 0.05$).

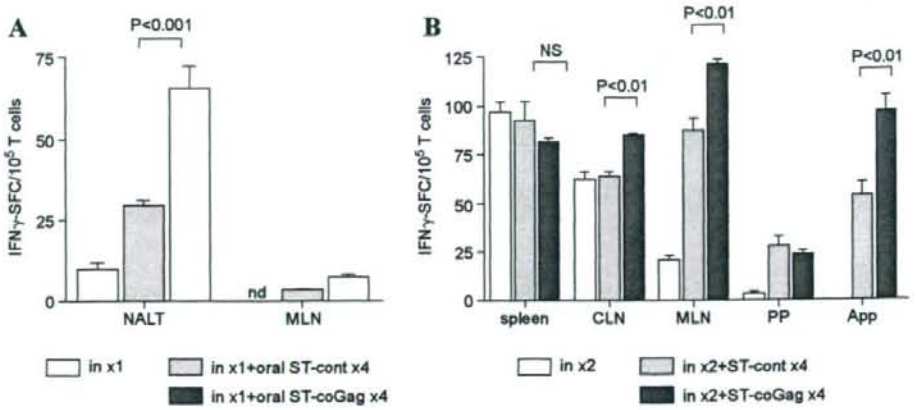

FIG. 5. Generation of Gag-specific $CD8^+$ T cells in MLNs by nasal priming and oral boosting. (**A**) One group of five mice was immunized with a single nasal application of Gag p24 plus CT alone (white columns). Two groups of mice were immunized nasally once followed by four oral immunizations with ST-cont (gray columns) or ST-coGag (black columns) 1 month later. The frequency of Gag197 peptide-specific $CD8^+$ T cells in the NALT and MLNs of the mice was determined by ELISPOT. Standard deviations (SD) of triplicate wells are shown, as are the p values of the indicated comparisons. nd, not detected. SFC, spot-forming cells. (**B**) Three groups of mice were immunized nasally two times at 3 week interval with Gag p24 plus CT alone (white column). Two groups were boosted 1 month later by four oral immunizations with ST-cont (gray columns) or ST-coGag (black columns), twice a week over 2 weeks. Seven days after the last immunization, the mice were sacrificed and T cells were enriched from the pooled spleens, CLN, MLN, PP, and App. The frequency of Gag197 peptide-specific $CD8^+$ T cells in each tissue was determined by ELISPOT. SDs of triplicate wells are shown, as are the p values of the indicated comparisons. NS, not significant. SFC, spot-forming cells.

However, when four consecutive oral ST-coGag immunizations were added 1 month later, an enhanced $CD8^+$ T cell response in NALT was observed, and a $CD8^+$ T cell response became detectable in MLN. Note that oral immunization with ST-cont also enhanced the $CD8^+$ T cell response in nasally primed mice in NALT, although the level is significantly lower than that of the booster effect by ST-coGag ($p < 0.001$). This prime-booster protocol also induced MLN $CD8^+$ T cell responses, though its level was still very low. To study further the booster effect by oral ST vaccine in different tissues, we primed mice with two nasal administrations of Gag p24 plus CT at 3 week interval and boosted this with four oral administrations of ST-coGag or ST-cont 1 month later (Fig. 5B). The $CD8^+$ T cell responses in the spleen, CLN, and intestinal tissues (MLN, PP, and a single lymphoid follicle in the App)[20] were analyzed by ELISPOT 1 week later. The two consecutive intranasal immunizations on their own induced strong Gag-specific $CD8^+$ T cell responses in the spleen and CLN, but not in the intestinal tissues. This verifies our earlier results.[13] When these nasally primed mice were then immunized four times with oral ST-coGag, strong Gag-specific $CD8^+$ T cell responses in the MLN and App were detected. Again, in this immunization schedule, oral administration with ST-cont also increased the Gag-specific $CD8^+$ T cell response in the MLN and App, although not as profoundly as ST-coGag ($p < 0.01$). This non-Gag197 peptide-specific effect of oral ST may be related to the selective uptake of ST by DCs. Thus, it is possible that ST infection of DCs triggers an innate immune response or inflammation that increases the basal level of immune responses[21,22] in nasally primed mice. Of note, the nasally primed $CD8^+$ T cell response in PP was only slightly increased by both the ST-coGag and ST-cont booster immunizations (both to a similar extent). The disparity between PP and App may reflect the fact that the bacteria stay longer in the mouse appendix than in the small intestine. We also observed that the App was enlarged in mice that received oral ST immunizations.

These results indicate first, that nasal priming is not sufficient to elicit a $CD8^+$ T cell response in intestinal tissues. Second, oral ST-coGag immunization on its own generates only weak cellular immune responses in intestinal tissues. Third, following nasal immunizations with oral ST-Gag, oral immunization enhances the Gag-specific $CD8^+$ T cell response not only in intestinal tissues but also in remote mucosal sites, namely, NALT and CLN. Thus, oral vaccination using ST as a delivery system has the advantage of enhancing the T cell responses, especially in the intestine.

DISCUSSION

To develop an effective, safe, and inexpensive vaccine that can protect people from HIV infection/transmission through the lower intestinal/rectal and genital mucosa would be a goal for mucosal HIV vaccine. One of the candidate vaccines that can target antigen directly to the lower intestine is the oral live attenuated ST vaccine. The oral vaccine has an advantage in its easy and safe applicability. In this study we showed that by altering the codons of the HIV-1 *gag* gene to reflect the codon

usage of *Salmonella*, the expression of Gag in *Salmonella* was markedly improved. This optimized ST vaccine induced both systemic and intestinal Gag-specific immune responses, albeit at a low level. Although this vaccine did not by itself induce Gag-specific $CD8^+$ T cell responses in the intestine, it markedly boosted weak intestinal $CD8^+$ T cell responses in mice that had been primed by nasal immunization with p24 and CT adjuvant. In an earlier report, Valentine *et al.*[23] used a recombinant ST, in which the SIV-*gag* gene driven by the *E. coli* groEL promoter was integrated into the *aroA* locus. They showed that a single dose of this vaccine elicited a local mucosal IgA response and a CTL response 4 weeks later. Unfortunately, we have found that at least four consecutive oral administrations of ST-coGag were necessary to induce detectable mucosal immune responses. Note that the stability of the expression plasmid may not be a problem, because both ST-coGag and ST-control were visibly colored green before oral inoculation. The disparity between our study and theirs is that the ST used by the latter is relatively weakly attenuated, thus making more antigen available and thereby more strongly provoking the intestinal immune system. In fact, while they could detect the bacteria in the spleens and liver of the recipient mice more than a month later, we were unable to detect EGFP expressed by ST in intestinal tissues even 2 days after oral immunization, even though live bacteria was recovered from the intestinal wash (data not shown). Therefore, it is an important issue to consider the balance between the safety and immunogenicity of oral *Salmonella* vaccine.

Regarding the prime-boost regimens, Evans *et al.*[19] showed in their macaque model that an attenuated *Salmonella* vaccine expressing a large polypeptide antigen of SIV Gag induced transient and only low-level systemic Gag-specific $CD8^+$ T cell responses, which was enhanced by boosting with vaccina virus expressing Gag. Thus, this study, like ours, demonstrated the potential benefit of weak oral *Salmonella* vaccines with respect to directing specific cellular immune responses to the intestinal mucosa. From the data available at present, it seems that the limitation of oral attenuated *Salmonella* vaccine in inducing potent immune responses may be best overcome by a combined prime-boost immunization regimen.[24]

Marriott *et al.*[25] reported that *Salmonella* efficiently enters and survives within $CD11c^+$ DCs and stimulates DCs to produce cytokines. Yrlid *et al.*[26] also showed that intravenous or intraperitoneal administration of GFP-expressing *Salmonella* generated GFP-positive splenic DCs that could mature and activate T cells. Despite a visible green fluorescence of ST-coGag, we could not detect $EGFP^+$ DCs in the MLN and PP after oral administration, probably because the viability of attenuated ST is severely reduced by the oral route of administration. Alternatively, the fluorescent bacteria may be quickly degraded in DCs. However, we did notice an enlarged lymphoid follicle in the appendix of mice that had been orally immunized with ST; this is the site where high levels of Gag-specific T cells were detected. This observation suggests that most EGFP-expressing *Salmonella* may primarily enter the intestinal mucosa through the M cells above this follicle in the appendix, after which DCs harboring the bacteria migrate to the regional MLNs. Although we did not analyze DC in this follicle, the appendix, not PP, would be the site where the immune response to *Salmonella* infection is initiated. Intestinal DCs were recently shown to induce T cells to express a gut-homing receptor after the T cells become activated in the intestinal lymphoid tissues.[27,28] Moreover, Lundin *et al.*[29] showed that the oral immunization of humans with *Salmonella* induced antigen-specific $CD4^+$ and $CD8^+$ T cells that express a gut-homing receptor. Thus, ST-bearing DCs are likely to play a crucial role in intestinal immunity.

We showed that oral vaccination with *Salmonella* expressing HIV-1 Gag was able to generate antigen-presenting DCs in MLNs. These DCs then activate $TcR\alpha\beta^+$ $CD8\alpha\beta$ T cells, which subsequently enter the circulation and then home back to the intestinal tissues (lamina propria or intestinal epithelium). Once back in the intestine, these T cells execute their effector functions to eliminate HIV-infected cells. In this context, Belyakov *et al.*[30] showed that mice immunized intrarectally with an HIV peptide vaccine that had measurable CTL activity in their LPLs were protected from intrarectal challenge with a recombinant HIV gp160-expressing vaccinia virus. Although we did not analyze LPLs, we expect that the increased number of Gag-specific $CD8^+$ T cells in the MLN and App by the booster effect of the oral ST-coGag vaccine may help to enhance protective immunity in the intestinal mucosa by combining with other vaccine regimens.

In summary, we found that optimizing the codon usage of the target gene in the ST vaccine elevated target protein expression by individual bacteria and elicited an intestinal IgA response. Futhermore, the orally administered ST vaccine expressing HIV-Gag was able to enhance intestinal cellular immunity in nasally primed mice. Thus the oral attenuated *Salmonella* vaccine is attractive as an economical and safe antigen delivery system. However, it definitely needs to be improved, for example, by selecting an ST mutant strain (different from the *aroA* mutant we used) that can replicate at least a few cycles in DCs. Alternatively, this vaccine may be improved by genetically engineering ST to secrete antigenic epitopes into the cytosol of DCs as described by others,[31] or by utilizing a vector that expresses chemokines that recruit more DCs to the intestinal submucosa upon ST entry. We believe that such oral ST vaccines will, using either a priming or booster regimen, be effective in preventing HIV infection, especially by enhancing intestinal cellular immunity.

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REFERENCES

1. Belyakov IM and Berzofsky JA: Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. Immunity 2004;20:247–253.
2. Li Q, Duan L, Estes JD, *et al.*: Peak SIV replication in resting memory $CD4^+$ T cells depletes gut lamina propria $CD4^+$ T cells. Nature 2005;434:1148–1152.

3. Mattapallil JJ, Douek DC, Hill B, *et al.*: Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature 2005;434:1093–1097.
4. Onorato IM, Modlin JF, McBean AM, *et al.*: Mucosal immunity induced by enhance-potency inactivated and oral polio vaccines. J Infect Dis 1991;163:1–6.
5. Girard MP, Steele D, Chaignat CL, and Kieny MP: A review of vaccine research and development: Human enteric infections. Vaccine 2006;24:2732–2750.
6. von Seidlein L: The need for another typhoid fever vaccine. J Infect Dis 2005;192:357–359.
7. Sirard J-C, Niedergang F, and Kraehenbuhl J-P: Live attenuated *Salmonella*: A paradigm of mucosal vaccines. Imm Rev 1999; 171:5–26.
8. Finlay BB: Molecular and cellular mechanisms of *Salmonella* pathogenesis. Curr Top Microbiol Immunol 1994;192:163–185.
9. Wick MJ: The role of dendritic cells during *Salmonella* infection. Curr Opin Immunol 2002;14:437–443.
10. Hone DM, Wu S, Powell RJ, *et al.*: Optimization of live oral *Salmonella*-HIV-1 vaccine vectors for the induction of HIV-specific mucosal and systemic immune responses. J Biotechnol 1996;44: 203–207.
11. Wu S, Pascual DW, Lewis GK, and Hone DM: Induction of mucosal and systemic responses against human immunodeficiency virus type 1 glycoprotein 120 in mice after oral immunization with a single dose of a *Salmonella*-HIV vector. AIDS Res Hum Retroviruses 1997;13:1187–1194.
12. Steger KK, Valentine PJ, Heffron F, So M, and Pauza CD: Recombinant, attenuated *Salmonella* typhimurium stimulate lymphoproliferative responses to SIV capsid antigen in rhesus macaques. Vaccine 1999;17:923–932.
13. Yoshizawa I, Mizuochi T, Ogata A, *et al.*: Studies on the generation and maintenance of mucosal cytotoxic T lymphocytes against human immunodeficiency virus type 1 Gag in mice. AIDS Res Hum Retroviruses 2003;19:469–479.
14. Belyakov IM, Wyatt LS, Ahlers JD, *et al.*: Induction of a mucosal cytotoxic T-lymphocyte response by intrarectal immunization with a replication-deficient recombinant vaccinia virus expressing human immunodeficiency virus 89.6 envelope protein. J Virol 1998;72:8264–8272.
15. Darji A, Guzman CA, Gerstel B, *et al.*: Oral somatic transgene vaccination using attenuated S. typhimurium. Cell 1997;91:765–775.
16. Yasuda S, Iwasaki M, Oka S-I, *et al.*: Detection of HIV-gag p24-speicific antibodies in sera and saliva of HIV-infected adults and in sera of infants born to HIV-1-infected mothers. Microbiol Immunol 1998;42:305–311.
17. Mata M, Travers PJ, Liu Q, Frankel FR, and Paterson Y: The MHC class I-restricted immune response to HIV-gag in BALB/c mice selects a single epitope that does not have a predictable MHC-binding motif and binds to Kd through interactions between a glutamine at P3 and pocket D. J Immunol 1998;161:2985–2993.
18. Belyakov IM, Ahlers JD, Brandwein BY, *et al.*: The importance of local mucosal HIV-specific CD8(+) cytotoxic T lymphocytes for resistance to mucosal viral transmission in mice and enhancement of resistance by local administration of IL-12. J Clin Invest 1998;102:2072–2081.
19. Evans DT, Chen LM, Gillis J, *et al.*: Mucosal priming of simian immunodeficiency virus-specific cytotoxic T-lymphocyte responses in rhesus macaques by the *Salmonella* type III secretion antigen delivery system. J Virol 2003;77:2400–2409.
20. Mizuochi A, Mizuochi E, Chiba C, and Bhan AK: Role of appendix in the development of inflammatory bowel disease in TCR-γ mutant mice. J Exp Med 1996;184:707–715.
21. Strindelius L, Filler M, Sjoholm I, *et al.*: Mucosal immunization with purified flagellin from *Salmonella* induces systemic and mucosal immune responses in C3H/HeJ mice. Vaccine 2004;22:3797–3808.
22. Weiss DS, Raupach B, Takeda K, Akira S, and Zychlinsky A: Toll-like receptors are temporally involved in host defense. J Immunol 2004;172:4463–4469.
23. Valentine PJ, Meyer K, Rivera MM, *et al.*: Induction of SIV capsid-specific CTL and mucosal sIgA in mice immunized with a recombinant S. typhimurium aroA mutant. Vaccine 1996;14:138–146.
24. Lasaro MO, Luiz WB, Sbrogio-Almeida ME, *et al.*: Combined vaccine regimen based on parenteral priming with a DNA vaccine and administration of an oral booster consisting of a recombinant *Salmonella* enterica serovar Typhimurium vaccine strain for immunization against infection with human-derived enterotoxigenic Escherichia coli strains. Infect Immun 2004;72:6480–6491.
25. Marriott I, Hammond TG, Thomas EK, and Bost KL: *Salmonella* efficiently enter and survive within cultured CD11c+ dendritic cells initiating cytokine expression. Eur J Immunol 1999;29:1107–1115.
26. Yrlid U, Sevensson M, Hakansson A, *et al.*: In vivo activation of dendritic cells and T cells during *Salmonella* enterica serovar Typhimurium infection. Infect Immun 2001;69:5726–5735.
27. Stagg AJ, Kamm MA, and Knight SC: Intestinal dendritic cells increase T cell expression of alpha4beta7 integrin. Eur J Immunol 2002;32:1445–1454.
28. Mora JR, Bono MR, Manjunath N, *et al.*: Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. Nature 2003;424:88–93.
29. Lundin BS, Johansson C, and Svennerrholm A-M: Oral immunization with a *Salmonella enterica* serovar Typhi vaccine induces specific circulating mucosa-homing CD4+ and CD8+ T cells in humans. Infect Immun 2002;70:5622–5627.
30. Belyakov IM, Derby MA, Ahlers JD, *et al.*: Mucosal immunization with HIV-1 peptide vaccine induces mucosal and systemic cytotoxic T lymphocytes and protective immunity in mice against intrarectal recombinant HIV-vaccinia challenge. Proc Natl Acad Sci USA 1998;95:1709–1714.
31. Russmann H, Shams H, Poblite F, *et al.*: Delivery of epitopes by the *Salmonella* type III secretion system for vaccine development. Science 1998;281:565–568.

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Lentivirus vectors expressing short hairpin RNAs against the *U3*-overlapping region of HIV *nef* inhibit HIV replication and infectivity in primary macrophages

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Although successful attempts to inhibit HIV-1 replication in T cells using RNAi have been reported, the effect of HIV-specific RNAi on macrophages is not well known. Macrophages are key targets for anti–HIV-1 therapy because they are able to survive long after the initial infection with HIV and can spread the virus to T cells. In this study, we identified a putative RNAi target of HIV, consisting of the portion of the *nef* gene overlapping the U3 region (Nef366), and generated a lentivirus-based short hairpin RNA (shRNA) expression vector (Lenti shNef366). We show that Lenti shNef366 inhibits (1) HIV-1 replication in a monocytic cell line and in primary monocyte-derived macrophages (MDMs), (2) reactivation of latent HIV-1 infection, and (3) the production of secondary HIV-1 from MDMs harboring a genomic copy of Nef366. Moreover, we found that the up-regulated production of macrophage inflammatory protein 1β (MIP-1β), but not MIP-1α, in MDMs by Nef expression was considerably suppressed by Lenti shNef366, which suggests that HIV-1 dissemination to T cells through its interaction with HIV-1–infected MDMs can also be controlled by Lenti shNef366. Thus, lentivirus-mediated shRNA expression targeting the U3-overlapping region of HIV *nef* represents a feasible approach to genetic vaccine therapy for HIV-1. (Blood. 2006;108:3305-3312)

Introduction

HIV Nef, which is uniquely conserved among HIV-1, HIV-2, and SIV, is essential for viral replication in vivo.[1] Nef is located at the 3′ end of the viral genome, partially overlapping the 3′ long terminal repeat (LTR). The *nef* gene is one of the earliest expressed genes during HIV-1 replication and is transcribed at particularly high levels, often accounting for up to 80% of HIV-1–specific RNA in the early stages of viral replication. The Nef protein is multifunctional, having been shown to be involved in the down-regulation of CD4 receptor molecules, cell apoptosis, and signal transduction.[2-6] From studies of HIV-infected individuals, accumulating evidence indicates that Nef plays an important, albeit currently not clearly understood, role in the pathogenesis of AIDS.[1,2,6,7]

Recent investigations have shown that Nef has evolved macrophage-specific functions, such as the recruitment of T cells to sites of infection.[8] Macrophages expressing Nef secrete a high level of macrophage inflammatory protein 1α (MIP-1α) and MIP-1β, thus recruiting peripheral T cells to lymph nodes. More recently it was shown that Nef regulates the release of paracrine factors from macrophages[9]; at least 2 proteins have been identified, which enhance lymphocyte susceptibly to HIV-1 infection in the absence of cell-cycle progression. These results provide ample evidence that Nef functions as a virulence factor that contributes to the manifestation of the clinical symptoms of immunodeficiency. Thus, any therapeutic intervention aimed at either completely blocking or at least partially reducing the expression of *nef* during HIV infection would likely enhance the ability of the immune system to fight HIV infection.

Sequence-specific degradation of viral mRNA by the process of RNAi is a mechanism for selectively inhibiting the synthesis of viral proteins that are critical for HIV-1 replication. RNAi therapy is based on an existing mechanism of gene regulation that is ubiquitous in plants and animals, in which targeted mRNAs are degraded in a sequence-specific manner.[10] Quite recently, several groups reported the use of RNAi to successfully inhibit HIV-1 replication.[11-15]

To study the effect of stable expression of short hairpin RNA (shRNA) against the U3-overlapping region of HIV-1 *nef* on virus replication and Nef-mediated cytokine regulation in primary macrophages, we established a lentivirus vector system expressing HIV-specific shRNAs. We show that HIV replication in primary macrophages was considerably suppressed following transfection of shRNAs targeting the U3-overlapping region of genomic HIV *nef*. Moreover, RNAi was able to control CC-chemokine

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T.Y. and Y.T.-Y. performed laboratory experiments, data management, and the biostatistical analysis; T.Y., Naoki Y., J.-i.I., and Y.T.-Y. were responsible for the general design of the study; H.M. and Norio Y. were responsible for the design of the specific parts on lentivirus vectors and quantitative PCR analysis, respectively; T.Y., Y.T.-Y., and J.-i.I. were involved in the interpretation of the results and general outline of the paper; and T.Y. and Y.T.-Y. wrote the article.

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production associated with Nef expression in HIV-1–infected macrophages. Thus, lentivirus-vector–based RNAi of the *U3*-overlapping region of HIV-1 *nef* might have potential usefulness as a genetic vaccine against HIV-1 infection.

Materials and methods

Construction of plasmids

To express gene-specific shRNAs under the human U6-RNA promoter, sense and antisense oligonucleotides 47 bp in length were ligated into pENTR/U6 (Invitrogen, Carlsbad, CA). The sequences of the oligonucleotides were as follows: *lacz*, sense oligonucleotide, 5′-caccgctacacaaatcagcgatttcgaaaaatcgctgatttgtgtag-3′, and antisense oligonucleotide, 5′-aaaactacacaaatcagcgatttttcgaaatcgctgatttgtgtagc-3′; *Nef366* (nucleotides 366-385 of the HIV-1_{NL432} *nef* ORF overlapping the 3′ LTR), sense oligonucleotide, 5′-caccgattggcagaactacacaccaagagagtgtgtagtttctgccaatc-3′, and antisense oligonucleotide, 5′-aaaagattggcagaactacacactctct-tggtgtgtagtttctgccaatc-3′. The resulting entry vectors were termed pENTR/shLacZ and pENTR/shNef366, respectively.

A Gateway-compatible (Invitrogen) HIV-1–based vector, pCS-RfA, containing elongation factor 1α promoter (EF-1α)–driven green fluorescent protein (EGFP) (pCS-RfA-EG),[16] was used to construct the lentivirus vectors, pCS-EG/shLacZ and pCS-EG/shNef366, according to the manufacturer's instructions (Invitrogen).

Cell culture and transfection

The human cell line 293T and human monocytic cell lines U937 and U1[17] were maintained in Dulbecco modified Eagle medium (DMEM) and RPMI 1640 medium (Gibco, Grand Island, NY), respectively, supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 μg/mL), and streptomycin (100 μg/mL). To establish CCR5$^+$ CEMx174 cells expressing EGFP driven by HIV-LTR, CEMx174 cells were transfected with pEF-BOSbst-HuCCR5 and pHIV-1 LTR-EGFPpuro (kind gifts from M. Tatsumi, National Institute of Infectious Diseases, Tokyo, Japan) and CEMx174 CCR5/LTR-EGFP cells were established.

HeLa-CD4 cells (obtained from the National Institutes of Health AIDS Reagent Program) were transfected with pEF-Nef bst, and Nef-expressing HeLa-CD4 cells were established (HeLa-CD4-Nef).

RNAi target site selection

A Web-based program for designing siRNA targets (Promega, Madison, WI), BLOCK-iT RNAi Target Designer (Invitrogen), and the National Center for Biotechnology Information Web site were used for the selection of siRNA and shRNA sequences, and for BLAST searches. Stealth siRNAs were synthesized (Figure 1) and HeLa-CD4-Nef cells were transfected with 2.5 μL stealth siRNA complexed to 2.5 μL Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Total RNA was extracted and analyzed by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) using specific LUX primers (Invitrogen) and the SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen). The sequences of the qRT-PCR primers were as follows: *nef* forward, labeled at its 3′ terminus with a reporter fluorophore 6-carboxyfluorescein (FAM), 5′-cagcagagtgtgattggatggcctgcFAMg-3′; *nef* reverse, 5′-tggctcagctcgtctcattctt-3′; *ef-1α* forward labeled at its 3′ terminus with a reporter fluorophore 6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxyfluorescein (JOE), 5′-gaaccacaagtgctaacatgccttggJOEtc-3′; *ef-1α* reverse, 5′-agcgtggttccactggcatt-3′. The reactions were performed using an Mx3000P (Stratagene, La Jolla, CA).

For Western blot analysis, cell lysates were prepared, subjected to 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with anti-Nef monoclonal antibody (mAb; F3, a kind gift from Dr Y. Fuji, Graduate School of Pharmaceutical Science, Nagoya City University, Nagoya, Japan). The blot was reacted with biotinylated goat anti–mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA), then with streptavidin-POD (Roche, Indianapolis, IN).

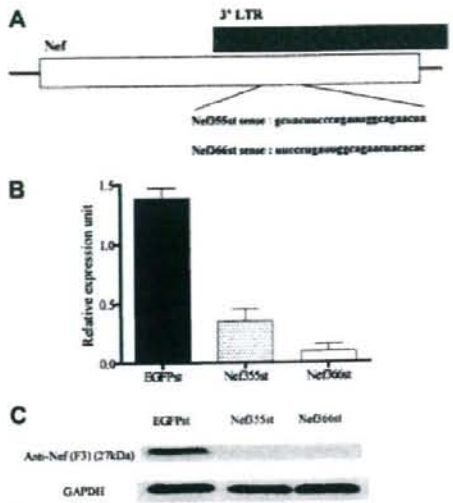

Figure 1. siRNA target sequences in *nef*. (A) Targets of siRNAs against the U3-overlapping region of HIV-1_{NL432} Nef and their sequences. Nef-expressing HeLa CD4 cells were transfected either with 2.5 μM *egfp* siRNAs (control: EGFPst) or *nef* siRNAs (Nef355st or Nef366st). At 48 hours after transfection, these cells were lysed to obtain total RNA and protein. (B) Total RNA was extracted and analyzed by qRT-PCR. The level of *nef* mRNA expression was normalized with that of elongation factor 1α (EF-1α) mRNA expression (*nef*/EF-1α). The data represent the expression level of *nef* mRNA relative to that of the control as 100%. The data represent the average ± SD of 3 independent experiments. (C) The cell lysates were subjected to 12.5% SDS-PAGE and immunoblotted with anti-Nef mAb.

Proteins were visualized by the SuperSignal Western Dura Extended Duration Substrate (Pierce, Rockford, IL) using an LAS3000 analyzer (Fuji Film, Tokyo, Japan).

Preparation of lentivirus vector

The lentivirus shRNA expression vectors were produced by transient transfection of 293T cells with a self-inactivating (SIN) vector construct, VSV-G– and Rev-expressing plasmid pCMV-VSV-G-RSV-Rev, and the packaging construct pCAG-HIVgp using the calcium phosphate precipitation method.[16] The lentiviral vector was concentrated by ultracentrifugation and the final solution was assayed for p24 antigen by an in-house enzyme-linked immunosorbent assay (ELISA).[18] The infectivity was determined by using 293T cells based on the EGFP expression.

Preparation of HIV-1 virus stocks

To prepare HIV-1, COS-7 cells were transfected with either pNL432, pNF462 (a kind gift from A. Adachi, Tokushima University, Tokushima, Japan), or pNF462dNef, in which the *nef* gene was deleted by digestion with *Xho*I and *Kpn*I, as described previously.[18]

Primary MDM culture

From peripheral blood mononuclear cells (PBMCs) of healthy, HIV-1$^-$ donors, CD14$^+$ monocytes were enriched using a magnetic-activated cell sorter (MACS; Miltenyi Biotec, Cologne, Germany) as described.[18] Monocytes were cultivated in RPMI 1640 medium supplemented with 10% FCS, 5% human AB plasma, and 10 ng/mL macrophage colony-stimulating factor (M-CSF) for 1 week to allow differentiation into monocyte-derived macrophages (MDMs).

Kinetics of virus production in stable shRNA-expressing U937 cells

Stable shRNA-expressing cells were infected with HIV-1_{NL432} for 2 hours, then cells were washed 5 times. Culture supernatants were harvested at 3- or