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

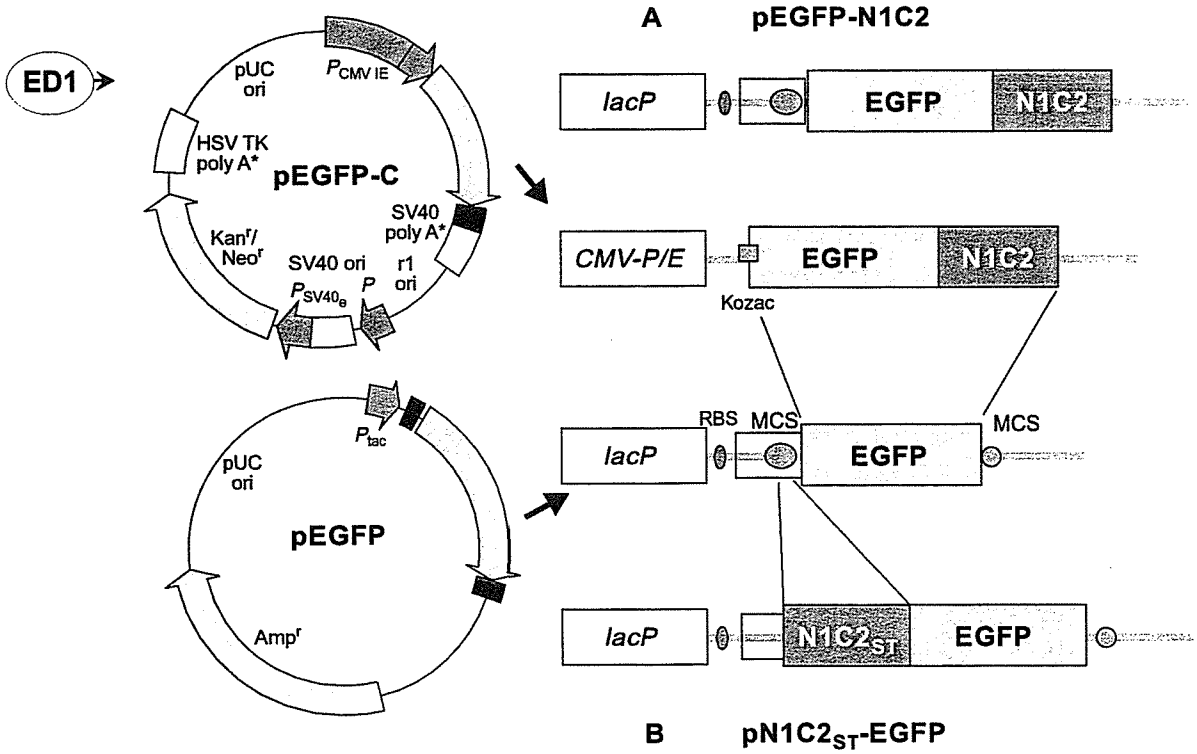

FIG. 1. Structure of plasmids expressing Gag–EGFP fusion proteins. (A) A truncated *gag* gene encoding a Gag p24 protein denoted as N1C2 was PCR amplified and inserted into a multiple cloning site of the eukaryote expression plasmid pEGFP-C2 to produce the *egfp–N1C2* fragment. The whole *egfp* gene 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-

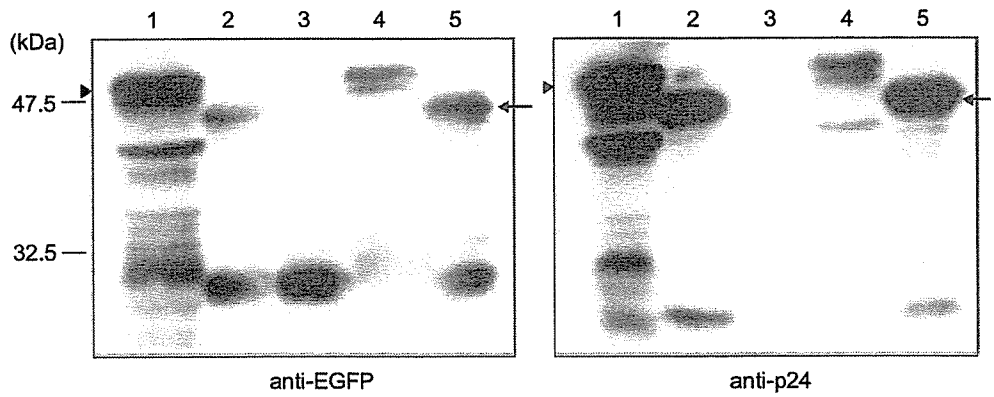

FIG. 2. Expression of the Gag–EGFP fusion proteins in *E. coli* and ST. Cell lysates prepared from *E. coli* (lanes 1 and 2) or ST (lanes 3–5) transformed with pEGFP–N1C2 (lanes 1 and 4), $pN1C2_{ST}$–EGFP (lanes 2 and 5), or pEGFP (lane 3) were separated by 10% SDS–PAGE in duplicate, and Western blot 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-

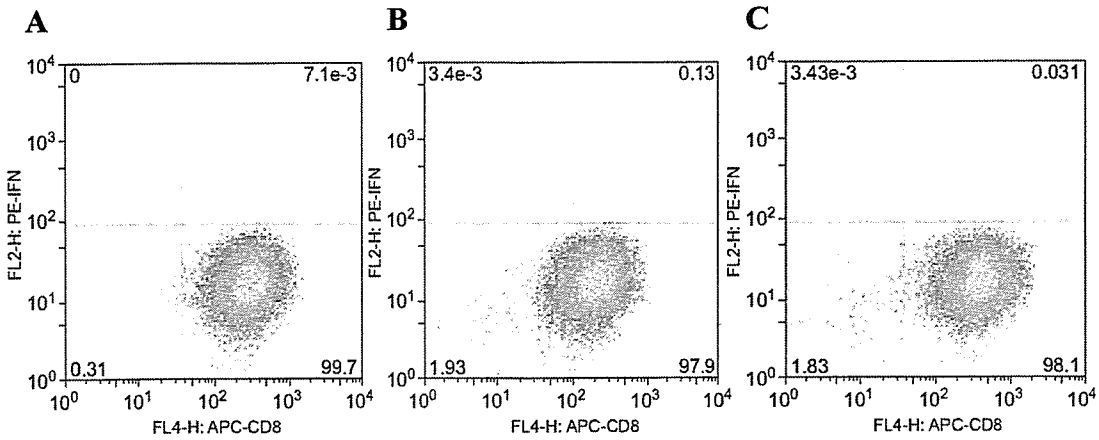

FIG. 3. Gag-presenting DCs are present in MLNs after oral administration of *Salmonella* expressing Gag. Four mice per group were orally immunized four times with ST-coGag (**B**) or ST-cont (**C**) twice a week over 2 weeks and sacrificed 24 h later. The pooled MLNs of these mice were treated with collagenase 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

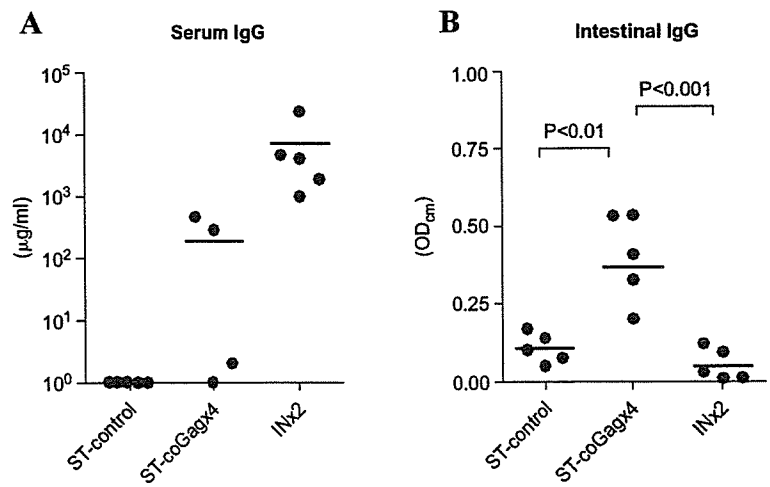

FIG. 4. *Salmonella* expressing coGag elicits both systemic and mucosal Gag-specific antibody responses. Mice (five or four mice) received either two nasal immunizations of Gag p24 plus CT (INx2) at 3 week interval, or four oral immunizations with ST-coGag or ST-control (ST-cont) twice a week over 2 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 ($p < 0.05$).

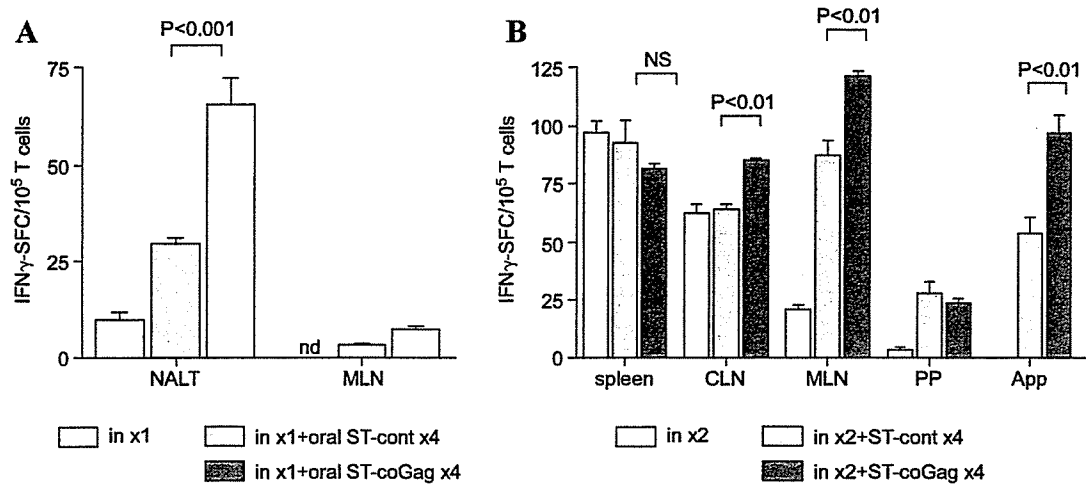

FIG. 5. Generation of Gag-specific CD8$^+$ T cells in MLNs by nasal priming and oral boosting. (A) One group of five mice was immunized with a single nasal application of Gag p24 plus CT alone (white columns). Two groups of mice were immunized nasally once followed by four oral immunizations with ST-cont (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 Svennerholm 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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Original article

High and inducible expression of human immunodeficiency virus type 1 (HIV-1) Nef by adenovirus vector does not disturb potent antigen presentation by monocyte-derived dendritic cells

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Abstract

Numerous studies indicated that Nef is a pleiotropic factor. Although it has been shown that Nef impairs the antigen-presenting activity of dendritic cells, more recent studies have shown no such impairment. This issue is critical for designing a vaccine expressing Nef. To refine our knowledge regarding the effect of Nef on dendritic cells, we developed constitutive and inducible adenovirus vector systems that express high levels of Nef in monocyte-derived dendritic cells (MDDCs). We showed here that Nef expression clearly downregulated CD4 expression of MDDCs but had little or no effect on other surface molecules, including MHC class I. Nef also did not affect the functional maturation of MDDCs. Use of the inducible Nef-expression system clearly revealed that adenovirus infection per se modulates cytokine secretion and the expression of apoptosis-related molecules in MDDCs, whereas Nef had no effect on these functions. Moreover, the antigen-presenting activity of MDDCs was not disturbed by the presence of Nef. On the contrary, we found that Nef-expressing MDDCs generated from HIV-1-infected individuals efficiently activated Nef-reactive T cells. Therefore, although adenovirus vector may modulate some aspects of MDDC function, Nef-expressing adenovirus would be served as one of HIV vaccine candidates.

Keywords: HIV-1; Nef; Dendritic cells; Adenovirus vector

1. Introduction

The accessory protein Nef is encoded by a gene located at the 3′ end of the genomes of all primate lentiviruses, namely, human immunodeficiency virus type 1 (HIV-1), HIV-2, and simian immunodeficiency virus (SIV). A study with the macaque AIDS model has suggested that Nef is important for viral pathogenesis in vivo (reviewed in [1]), and it has been shown that Nef enhances the infectivity of HIV-1 [2] by interacting with cellular signal transduction molecules, which modulates the immune response to the virus in various ways [3]. One repeatedly observed effect of Nef is the down-regulation of CD4 and MHC class I molecules on the surface of $CD4^+$ T cells [1, 3]. However, many other proposed consequences of Nef, such as T-cell activation and apoptosis, remain controversial. Thus, the role Nef plays in the immuno-pathogenesis of HIV-1 infection is still not clear.

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Monocyte-derived macrophages and monocyte-derived dendritic cells (MDDCs) both express CD4 and chemokine receptors (CCR5 and CXCR4) and are susceptible to HIV-1 infection. Significantly, when HIV-1-infected MDDCs interact with resting antigen-specific $CD4^+$ T cells in their role as antigen-presenting cells, they efficiently infect the T cells with HIV-1 by inducing T-cell activation [4]. The transmission of the virus from the DC to the T cell requires the production of a so-called infectious synapse. Recently, it was reported that the DC-specific type 1 lectin, DC-SIGN, may play an important role in this infectious synapse because HIV preferentially binds to DC-SIGN, but that the virus transmission from DC to T cell occurs more efficiently in a DC-SIGN-independent manner [5]. On the other hand, DCs play an important role in opposing HIV-1 infection as they are potent initiators of anti-HIV immune responses in HIV infection; indeed, their antigen-presenting activity was found to be essential for the generation of effective immunity by vaccination [6]. Although it has been suggested that the antigen-presenting activity of MDDCs in HIV-1-infected individuals are unaltered by the infection [7], these observations are based on in vitro studies. Thus, it remains possible that HIV-1 infection may induce both qualitative and quantitative alterations of DCs in vivo that may contribute to the pathogenesis of HIV infection [6,8].

The *nef* gene is the most abundantly expressed molecule during HIV-1 infection. By decreasing the surface expression of MHC class I on HIV-1-infected T cells, Nef effectively interferes with the ability of CTLs to kill the infected T cells [9], which is a classic immune evasion mechanism. It has also been postulated that Nef may impair the function of DCs [10,11]. For example, *Vaccinia*-mediated Nef expression in MDDCs downregulated their expression of MHC class I, which made the Nef-expressing DCs resistant to CTL killing and probably would also limit the ability of these DCs to activate CTLs [10]. However, other studies have suggested Nef has minimal or no effect on DC function [12–14]. One study found that when $CD34^+$ progenitor cell-derived DCs were induced to express Nef by infection with a retrovirus vector, their ability to induce an alloresponse by PBMC was normal [14]. Moreover, it has been reported that adenovirus-mediated expression of Nef in DCs has no effect on their cell surface phenotypes [12,15], although it did induce their production of inflammatory cytokines [15]. Maccormac et al. also showed that adenovirus-mediated expression of HIV Nef did not impair the function of DCs [13]. However, it should be noted that the adenovirus-mediated Nef expression in their study was rather low and Nef was only expressed in part of the DC population.

To avoid the confusion regarding Nef effect on DC function, we developed two types of Nef-expressing adenovirus vectors. One induces the CAG promoter-driven constitutive expression of Nef while the other expresses Nef in a tetracycline-regulatable manner by using the T2 promoter. Both viruses induced high levels of Nef expression in most MDDCs. We show here that, despite high levels of Nef expression in MDDCs, their functions as antigen-presenting cells (APCs) are well preserved.

2. Materials and methods

2.1. Reagents

RPMI1640 supplemented with 10% FBS, 2 mM glutamine, and antibiotics (Invitrogen Corp., Carlsbad, CA) was used in all culture. *Escherichia coli* LPS was obtained from Sigma–Aldrich (St. Louis, MO). Labeled monoclonal antibodies (mAbs) against CD1a, CD40, CD83, CD86, HLA-ABC and HLA-DR were purchased from BD Bioscience (San Jose, CA). The anti-Nef mAb (clone F3, a kind gift from Dr. Y. Fuji, Graduate School of Pharmaceutical Science, Nagoya City University) was conjugated with Alexa Fluor 647 (Invitrogen).

2.2. Construction of adenovirus vectors and infection of DCs

The recombinant adenovirus vectors were generated by using adenovirus expression vector kit (Takara Co. Ltd., Kyoto, Japan). In this kit, the E1- and E3-deficient adenovirus type 5 sequence was incorporated into cosmids, which allowed a foreign gene under the CAG promoter [16] (pAxCAwt) or any promoter (pAxcw), along with the rabbit β-globin poly A sequence. The Tet-off system is composed of T2 promoter and tetracycline-controlled transactivator (tTA). The tTA fragment of pTA-Hyg (a kind gift from Dr. J. Yokota, Department of Biology, National Cancer Center, Tokyo, Japan [17]) was ligated with EF-1α promoter [18] to produce pAxEF-TA. The CMV promoter was replaced by T2-promoter (0.45 kbp) of pT2Xp53wt/neo [17] to produce pT2X-IRES1-EGFP. HIV-1 *nef* wt was PCR-amplified from pNL432 proviral DNA using specific primers containing *Eco*RI sites at both ends, subcloned and inserted into pT2X-IRES1-EGFP. The resulting DNA fragments with or without *nef* were inserted into pAxcw (designated as pAxT2 vectors). When both AdEF-TA and AdT2-Nef are infected to MDDCs, the transcription of Nef is activated by tTA bound to T2 promoter and it is shut-off in the presence of tetracycline.

Similar fragments without the T2 promoter were also inserted into pAxCAwt to generate the *nef* and control vectors driven by the CAG promoter. Adenoviruses produced from 293 cells using these cosmids were purified by two-step discontinuous CsCl gradients, followed by dialysis against PBS containing 10% glycerol as described by Kanegae et al. [19].

2.3. Preparation of MDDCs and T cells

MDDCs and T cells were prepared as described previously [20]. Briefly, the $CD14^+$ cells were enriched from PBMCs and cultured with 10 ng/ml interleukin (IL)-4 and 10 ng/ml GM-CSF (both from PeproTech Inc., London, UK) to generate the MDDCs. The $CD4^+$ T cells were negatively selected by depletion of CD8, CD14, CD11b, CD16, and CD20 positive cells by using magnetic beads and a magnetic cell sorter (MACS, Miltenyi Biotec, Cologne, Germany). The purity of

the resulting $CD4^+$ T-cell preparations was >96% as assessed by FACScalibur (BD Bioscience).

2.4. Surface and intracellular staining

Flow cytometric detection of surface and intracellular antigens were carried out as described [21]. To detect apoptosis, cells were suspended in an annexin-binding buffer (10 mM HEPES pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) and stained with Alexa 647-conjugated annexin (Invitrogen) for 5 min at room temperature, washed, and resuspended in the same buffer containing propidium iodide (PI). The stained cells were analyzed by FACScalibur (BD Bioscience) using the Cell Quest program. The FACS data were re-analyzed using Flowjo software (Tree Star Inc., San Carlos, CA).

2.5. Mixed allo-lymphocyte reaction (alloMLR) and T-cell activation

Adenovirus-uninfected or -infected MDDCs (1×10^4) were cocultured with autologous or allogeneic $CD4^+$ T cells (1×10^5) for 5 days in 96-well flat-bottom culture plates. [^{3}H]Thymidine (0.5 μCi/well) was added to the culture for the final 18 h. The cells were harvested onto UltraFilter®-96 GF/C® filter membrane plates (Perkin–Elmer Japan, Yokohama, Japan), which were then soaked with 30 μl microscintillator per well and measured by using a Top-counter (Perkin–Elmer Japan). To measure T-cell activation, adenovirus-uninfected or -infected MDDCs (1×10^5) were placed into 24-well plates and purified $CD4^+$ T cells (2×10^6) were added to the culture in the presence or absence of 25 μg/ml PPD (purified protein derivative of *Mycobacterium tuberculosis*). The next day, cells were harvested and surface CD69 and intracellular interferon (IFN)-γ were stained.

2.6. Detection of HIV-specific T cells in HIV-infected individuals by ELISPOT

Blood samples from HIV-infected individuals were collected with informed consent (kindly provided by Dr. A. Iwamoto, Department of Infectious Diseases, Institute of Medical Science, University of Tokyo). MDDCs of these donors were infected with AdCAG-cont, AdCAG-Nef, or AdCAG-Gag/Pol (a kind gift from Dr. A. Iwamoto), all at MOI 500. After 24 h infection, these MDDCs were washed, cocultured (1×10^4 cells) in triplicate with 1×10^5 autologous PBMCs (CD14 negative fraction) and IFN-γ ELISPOT analysis was carried out [20]. This study was approved by ethical committees both in our institute and in the Institute of Medical Science.

2.7. Western blot analysis

After 24 or 48 h of adenovirus infection, MDDCs were harvested and cell lysates were prepared. These lysates were subjected to 10% or 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a PVDF membrane (Immunobilon-P, Millipore Ltd., Bedford, MA). The membrane was reacted with antibodies against activated caspase 3, Fas Ligand (FasL), Bcl-2, or GAPDH (BD Bioscience) followed by incubation with HRP-conjugated $F(ab')_2$ fragment anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA). The signal was visualized by a Super Signal Western Dura Extended Duration Substrate (Pierce, Rockford, IL) using an LAS3000 analyzer (Fuji film Co. Ltd., Tokyo, Japan).

2.8. Cytokine/chemokine detection

The culture supernatant of adenovirus-infected MDDCs was collected 12 or 24 h after cultivation and kept frozen. The human cytomeric bead array (CBA) flex system (BD Bioscience) was utilized to simultaneously measure the levels of the cytokines/chemokines, FasL, Eotaxin, IFN-γ, IL-1β, IL-8, IL-10, IL-12p70, IP-10, MIG, MIP-1α, MIP-1β, and tumor necrosis factor (TNF).

2.9. Statistics

To determine how viral load correlated with the ELISPOT data, a Pearson correlation test was performed by using the GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Recombinant adenovirus vector-infected MDDCs show high levels of Nef expression

We constructed Nef-expressing adenovirus vectors in which *nef* expression is driven by the constitutive CAG promoter or the tetracycline-controlled T2 promoter and tTA expression is driven by EF-1α promoter. The resulting adenovirus vectors were designated as AdCAG-Nef, AdT2-Nef, and AdEF-TA, respectively. As a control, AdCAG-cont and AdT2-cont, which do not contain the *nef* gene, were generated. EGFP was not consistently expressed, presumably due to the low efficiency of the original *IRES1* gene (data not shown). However, this problem was not important since we were able to directly detect intracellular Nef expression by using the Alexa-647-labeled anti-Nef mAb. Fig. 1a shows FACS profiles of Nef expression in the MDDCs 48 h after infection. When MDDCs were infected with AdT2-Nef at MOI 500 in combination with AdEF-TA at MOI 250 (Fig. 1a, left), or with AdCAG-Nef at MOI 500 (Fig. 1a, right), high levels of Nef expression in the MDDCs were consistently achieved. The frequency of Nef^+ MDDCs varied considerably among donors but was above 80% in all the experiments below (82–97%). The addition of 1.0–0.01 μg/ml doxycycline completely shut off the Nef expression by AdT2-Nef/AdEF-TA (see Fig. 2b).

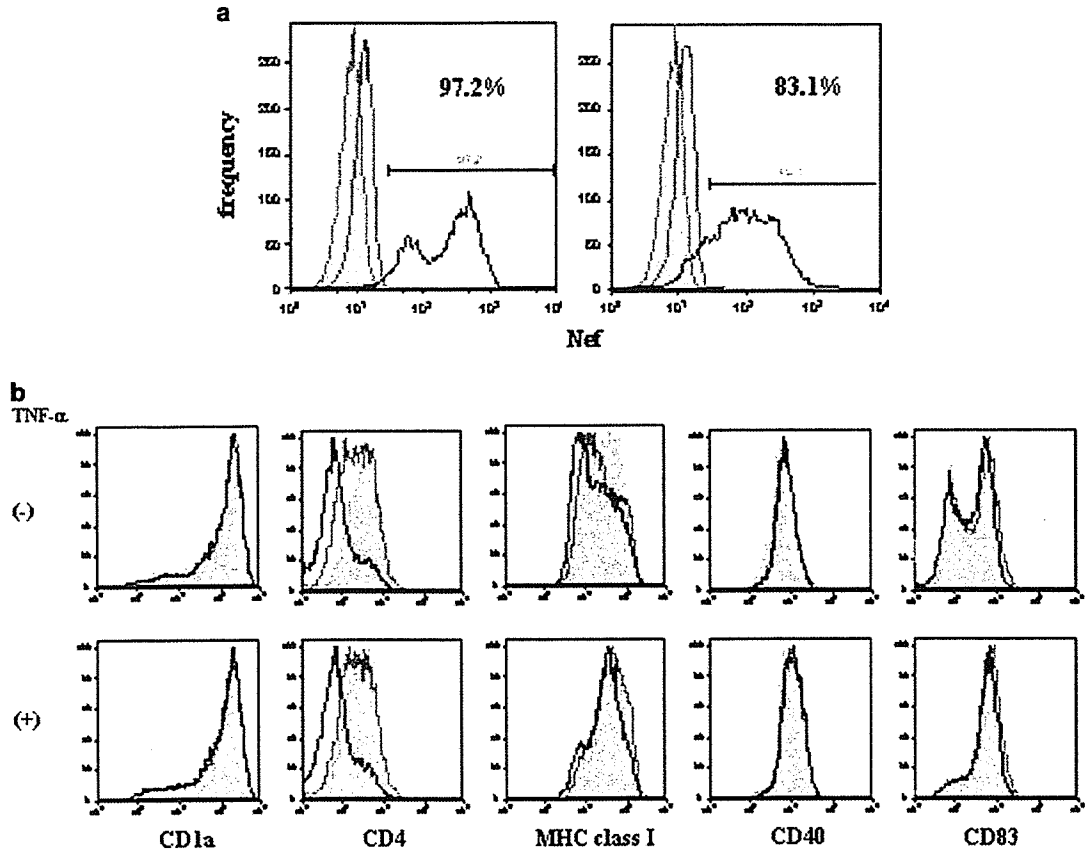

Fig. 1. MDDCs infected with adenovirus vectors show high levels of Nef expression but few changes in cell surface phenotype. (a) MDDCs were left uninfected (shaded peak) or were infected with AdT2-Nef/EF-TA (black line) or AdT2-cont/EF-TA (grey line) (left panel), or AdCAG-Nef (black line) or AdCAG-cont (grey line) (right panel), and cultured for 48 h. The cells were then fixed with 4% formaldehyde in PBS for 20 min at room temperature, stained with Alexa 647-labeled anti-Nef mAb for 30 min, and analyzed by FACScalibur. The FSC^{high}/SSC^{high} cells were gated and are depicted. (b) TNFα-untreated (−) immature and TNFα-treated (+) mature MDDCs were left uninfected (shaded peak) or were infected with AdCAG-cont (grey line) or AdCAG-Nef (black line) and cultured for 48 h. The cells were then examined for CD1a, CD4, MHC class I, CD40 and CD83 expression as described above.

3.2. Effect of Nef on the surface expression profiles of immature and mature MDDCs

We examined the effect of Nef on the surface expression profiles of immature and mature MDDCs. MDDCs were left untreated, or were treated with 10 ng/ml TNF-α for 24 h, after which they were infected with AdCAG-Nef or control adenovirus (AdCAG-cont). The CD4, MHC class I, CD1a, CD83 and CD40 surface antigens were analyzed 48 h later by FACS. Representative results of the MDDCs

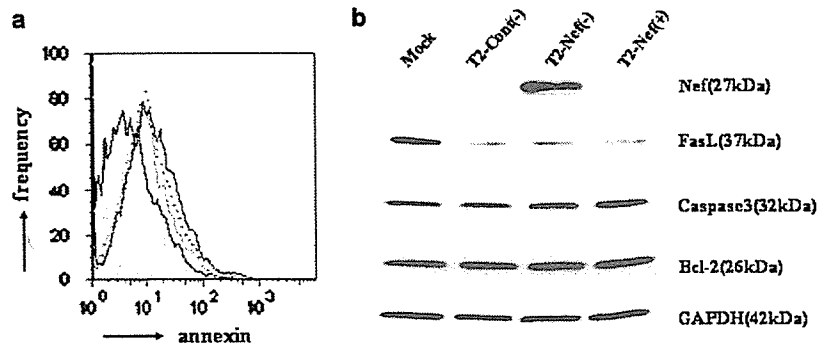

Fig. 2. Nef is neither an anti-apoptotic nor a pro-apoptotic factor. MDDCs were infected with T2-Cont or T2-Nef, cultured in the absence (−) or presence (+) of 1 μg/ml doxycycline and subjected to FACS (a) at 24 h or Western blotting (b) at 48 h. In (a), the cells were examined for the surface expression of annexin. The shaded peak indicates the staining pattern of the uninfected control MDDCs, while the grey line indicates the T2-Cont-infected cells, the solid line indicates the T2-Nef (−) cells, and the dotted grey line indicates the T2-Nef (+) cells. In (b), MDDC lysates were subjected to Western blot with anti-caspase3, anti-FasL, anti-Bcl-2 or anti Nef mAbs. GAPDH was served as a loading control. The molecular size of each protein is shown in parentheses.

from several donors are shown in Fig. 1b. The expression of CD4 by the MDDCs was clearly downregulated by Nef expression in both the immature [TNFα(−)] and mature [TNFα(+)] MDDCs. However, MHC class I expression was marginally downregulated in immature MDDCs only. It has been shown that the downregulation of MHC class I on $CD4^+$ T cells requires higher levels of Nef than the downregulation of CD4 [22]. Therefore, the levels of Nef generated by the AdCAG-Nef adenovirus may still not be sufficient to reduce MHC class I, which is abundantly expressed in MDDC (which is further upregulated upon maturation). Nef did not have a significant effect on the expression of important costimulatory molecules involved in DC function such as CD83, CD40 and CD1a. Thus, while a high level of Nef expression in MDDCs downregulates the CD4 expression, it has only marginal effects on the MHC class I expression and no effect on the expression of other molecules that are important for antigen-presenting function.

3.3. Nef is not involved in MDDC apoptosis

In $CD4^+$ T cells, Nef interacts with a variety of signaling molecules, including those involved in apoptosis (see for review [1]). Depending on the cells and the culture conditions used, Nef can be both an anti-apoptotic and a pro-apoptotic factor. We found that when MDDCs were infected with adenovirus, their numbers became reduced during cultivation (data not shown). To test whether Nef enhances the apoptosis of MDDCs, we infected MDDCs with the inducible Nef expression system, cultivated them for 24 h in the absence (−) or presence (+) of doxycycline, and then stained them with annexin. As shown in the representative result, Nef only slightly, if any, enhanced the annexin expression during cultivation of the MDDCs at 24 h after adenovirus infection (Fig. 2a).

We then assessed whether Nef alters the expression of various anti-apoptotic and pro-apoptotic proteins in MDDCs by Western blot. GAPDH served as an internal control of protein input. As shown in Fig. 2b, the active form of caspase 3 and Bcl-2 levels in all MDDCs were similar, regardless of the Nef expression. It was suggested that the upregulated FasL expression in HIV-1-infected monocyte-derived macrophages mediates the apoptosis of uninfected T cells [23]. However, we observed that FasL expression was somewhat suppressed by adenovirus infection but not by Nef expression. Therefore, we found no evidence to connect Nef expression and apoptosis in MDDCs.

3.4. Nef expression does not alter the cytokine/chemokine production profile of MDDCs

Because monocyte/macrophages expressing adenovirus-transduced Nef are shown to produce unknown chemokines that attract naïve $CD4^+$ T cells [24], we tested whether or not Nef expression influences the cytokine/chemokine production in MDDCs. The MDDCs of three donors were infected with AdT2-cont/AdEF-TA (T2-Cont) or AdT2-Nef/AdEF-TA (T2-Nef), washed, and cultivated in the absence or presence of doxycycline. Culture supernatants were collected 12 and 24 h later and the levels of 12 cytokines/chemokines (MIP-1α, MIP-1β, FasL, Eotaxin, IFN-γ, IL-1β, IL-8, IL-10, IL-12p70, IP-10, MIG, and TNF-α) were analyzed simultaneously. All these cytokines are more or less upregulated upon adenovirus infection. Only the MIP-1α and MIP-1β results of three donors are shown in Fig. 3,

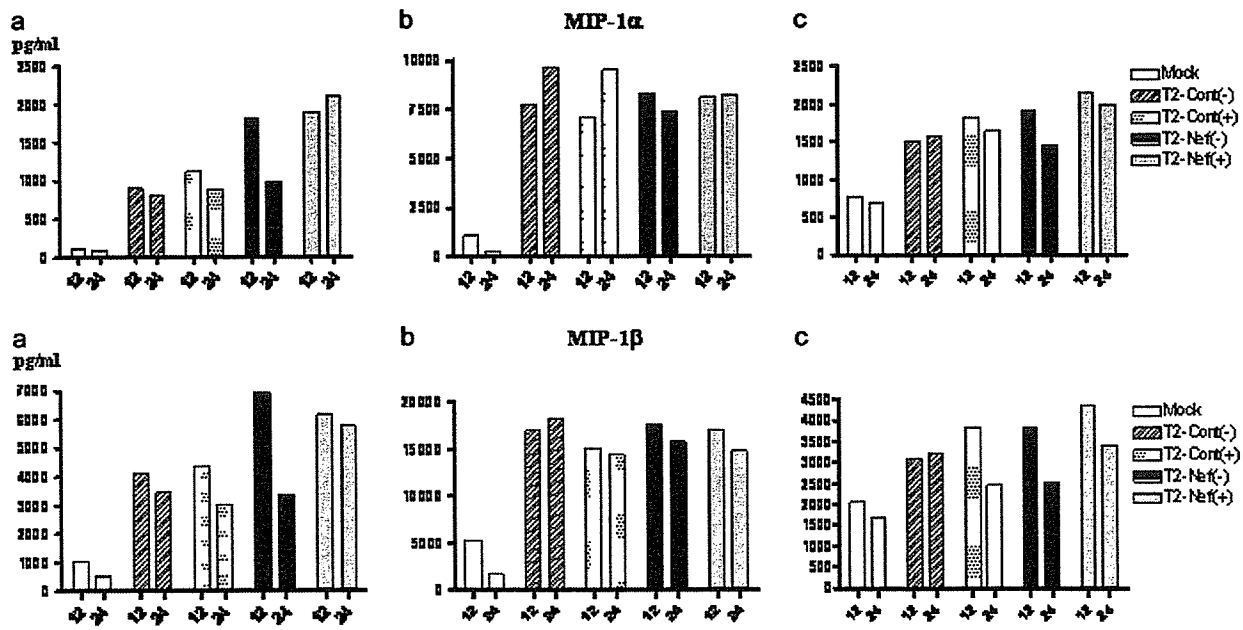

Fig. 3. Nef does not modulate MDDC cytokine/chemokine production. MDDCs were infected with T2-Cont or T2-Nef and cultured in the absence or presence of 1 μg/ml doxycycline for 12 or 24 h. The cytokine/chemokine levels in the culture supernatants were measured by using a FACS-based CBA Flex kit. The MIP-1α (a) and MIP-1β (b) results of three donors are shown.

since these are known to be important for HIV infection/replication and have been reported to be upregulated by Nef [15,24]. Although the donors showed marked variation, Nef expression did not further modulate the increased production of these cytokines/chemokine by adenovirus-infected MDDCs.

3.5. Nef expression has no effect on the ability of MDDCs to stimulate CD4$^+$ T-cell responses

We then analyzed the antigen-presenting activity of Nef-expressing MDDCs by testing their capacity to induce the allogeneic mixed lymphocyte reaction (alloMLR). T-cell

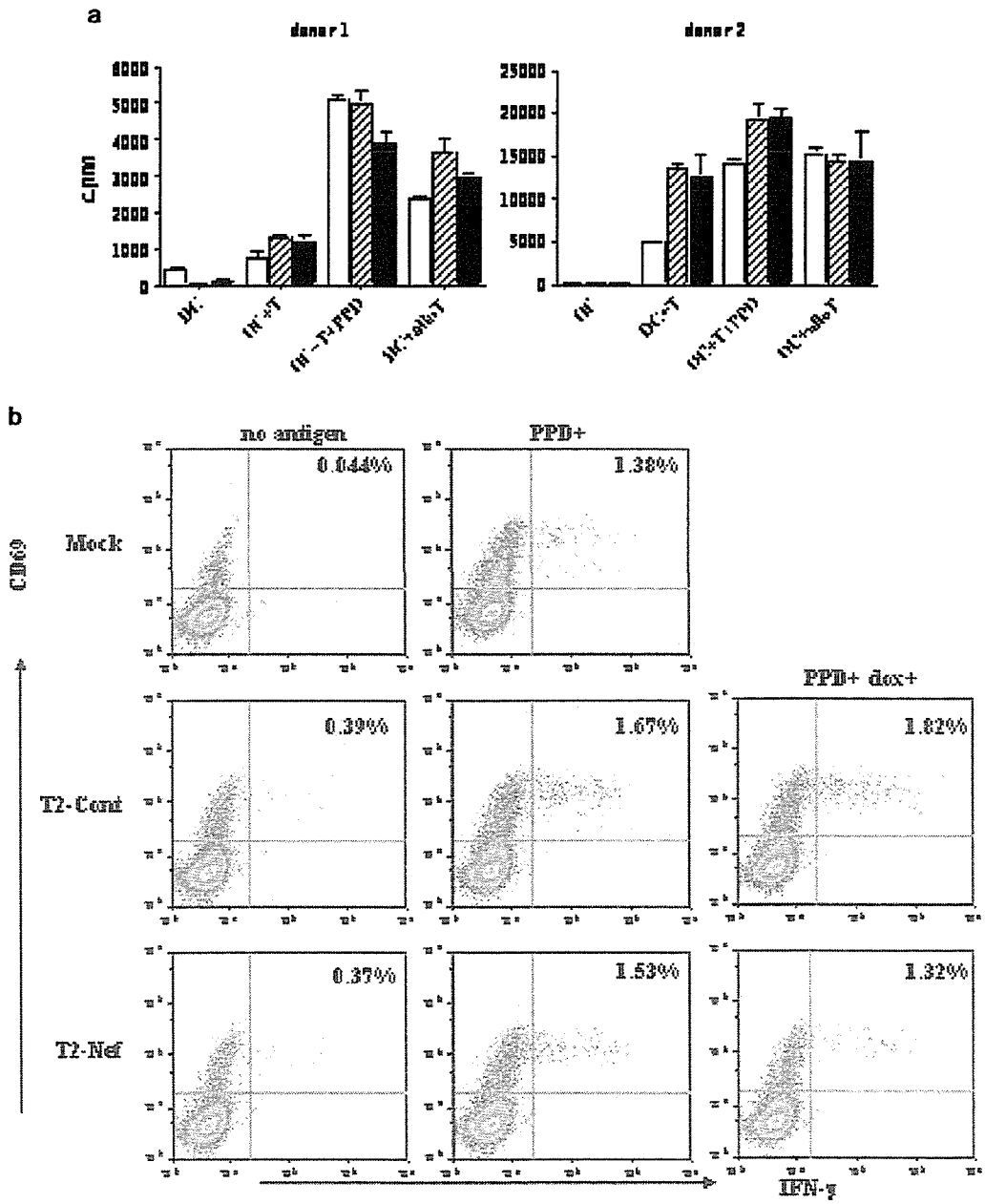

Fig. 4. The ability of MDDCs to activate T cells is not impaired by Nef. (a) MDDCs were left uninfected (white bar) or were infected with AdCAG-cont (grey bar) or AdCAG-Nef (black bar), cultured for 24 h, washed and cultured alone (DC) or cocultured with autologous CD4$^+$ T cells with (DC + T + PPD) or without (DC + T) PPD, or with allogeneic CD4$^+$ T cells (DC + alloT). T-cell proliferation was analyzed by [^{3}H]thymidine uptake after 5 days of cultivation. The results of two donors are shown. (b) MDDCs were left uninfected (Mock) or were infected with T2-Cont/EF-TA (T2-Cont) or T2-Nef/EF-TA (T2-Nef) and cultured for 24 h in the absence (left and middle panels) or presence (right panels) of doxycycline. The cells were washed and cocultured with autologous CD4$^+$ T cells in the absence (no antigen) or presence (PPD+) of PPD. On the next day, cell surfactant (CD69 and CD4) and intracellular IFN-γ staining was carried out. The frequencies of activated (CD69$^+$IFN-γ$^+$) CD4$^+$ T cells are shown in each panel.

proliferation of two donors is shown in Fig. 4a. The alloMLR was not affected by the presence of Nef, nor did the adenovirus infection significantly induce the proliferation of MDDCs. Notably, when autologous T cells were used instead of allogeneic T cells, they showed slight activation with uninfected MDDCs and quite a strong reaction with adenovirus-infected MDDCs regardless of Nef expression, especially in donor 2. The latter reaction may reflect previous infections by the T-cell donors with environmental adenoviruses. The autologous T cells of both donors proliferated well in the presence of PPD and MDDCs, which further suggests that the antigen-specific T-cell response of MDDCs is not impaired by Nef expression.

The inducible Nef-expression system was utilized to ensure that adenovirus infection, but not Nef expression has some effects on T-cell activation. The T2-Nef-infected MDDCs were cultured with T cells in the presence or absence of PPD or doxycycline, and then T cells were stained for CD4, CD69 and IFN-γ. Representative results are shown in Fig. 4b. PPD presented by uninfected MDDCs increased the frequency of activated $CD4^+$ T cells ($CD69^+IFN\text{-}\gamma^+$) from 0.044% to 1.38%. Adenovirus infection by itself induced a low level of T-cell activation that elevated the activated T-cell frequency to 0.39%. However, Nef expression in the MDDCs had no effect on either adenovirus- or PPD-induced T-cell activation. Thus, Nef has no apparent effect on the antigen-presenting function of MDDCs.

3.6. Adenovirus-infected MDDCs from HIV-1-infected individuals activate autologous Nef-specific T cells

Since we found adenovirus-mediated Nef expression has little effect on recall T-cell responses, we speculated that adenovirus vectors could be useful in an AIDS vaccine designed to stimulate Nef-specific T cells. To test this, we harvested the MDDCs from HIV-infected individuals, infected them with the AdCAG-Nef at MOI 500, and examined their ability to present Nef to their autologous T cells by IFN-γ ELISPOT analysis. The results of 15 patients on highly active antiretroviral therapy (HAART) and three patients without any therapy are shown in Table 1. Although the background level (i.e. reactivity to the adenovirus vector) differed significantly among patients, 16 of the 18 patients reacted to the Nef presented by their MDDCs. As a control, the MDDCs were infected with an adenovirus expressing Gag/Pol. All patients reacted to Gag/Pol. The frequency of Nef-reactive IFN-γ-expressing T cells did not correlate with viral load ($r^2 = 0.253$). Both $CD4^+$ and $CD8^+$ T cells are detectable by this ELISPOT analysis and indeed, FACS analysis revealed that both types of T cells (but predominantly $CD8^+$ T cells) were activated by the Nef-expressing MDDCs (data not shown). This supports the notion that a Nef-expressing adenovirus vaccine is able to activate Nef-reactive T cells.

Table 1
ELISPOT analysis of the IFN-γ response of PBMCs from HIV-infected individuals to autologous MDDCs that express Nef or Gag/Pol via adenovirus vectors

Patient no.	CD4 (/cm^3)	VL	Therapy	IFN-γ SFC/10^6 PBMC[a]		
				AdCAG-cont	AdCAG-Nef	AdCAG-Gag/Pol
1	819	<50	AZT/ddC	17	84	700
2	853	<50	HAART	7	27	1100
3	592	<50	HAART	237	220[b]	343
4	557	<50	HAART	3	33	83
5	489	<50	HAART	30	200	393
6	374	84	HAART	173	760	707
7	480	110	HAART	130	127[b]	630
8	816	140	HAART	113	217	737
9	397	220	HAART	120	233	158
10	457	290	HAART	20	53	333
11	491	480	HAART	550	717	990
12	418	2200	HAART	50	1505	4905
13	670	5000	HAART	0	17	117
14	451	8100	HAART	50	62	1193
15	486	150000	HAART	415	713	690
16	285	3300	No	37	63	287
17	547	5400	No	90	224	2300
18	339	86000	No	7	2667	2333

VL, viral load; HAART, highly active antiretroviral therapy; AZT, azathioprine; ddC, dideoxycytidine.
[a] The number of IFN-γ spot-forming cells (SFC) per 10^6 PBMCs generated upon coculture with MDDCs. Autologous MDDCs were infected with AdCAG-cont, AdCAG-Nef, or AdCAG-Gag/Pol at MOI 500 1 day prior to coculture. The average counts of three wells are shown. The standard errors were within 5%.
[b] Below background.

4. Discussion

The recent development of reverse genetics has allowed us to use various DNA and RNA virus vectors, such as *Vaccinia*, adenovirus, and retrovirus, to efficiently transfer genes of choice into various cell types, including MDDCs. Of these vectors, the adenovirus is an effective tool for transferring genes, particularly into monocyte-derived macrophages and MDDCs, since it lacks the ability to infect T cells. However, because the infection of the virus vector itself may influence the intrinsic host-cell biology, the interpretation of the gene function obtained by using virus vectors needs to be carefully assessed. For instance, it was known that recombinant adenovirus infection enhances immunostimulatory capacity of DCs, although CD40 triggering is required for their full maturation [25]. To evaluate the effect of Nef on MDDC function, while excluding the effect of adenovirus infection itself, we developed adenovirus vectors that express Nef inducibly in MDDCs. Our results suggest that Nef expression in MDDCs has little, if any, effect on the antigen presenting activity, but that adenovirus infection per se could be responsible to the altered function of MDDCs.

Collins et al. have suggested that Nef expressed during HIV-1 infection may downregulate MHC class I expression by infected $CD4^+$ T cells, therefore protecting them from being killed by CTLs and giving the virus enough time to replicate [9]. However, since these HIV-infected T cells eventually die due to virus-mediated cytopathy, even in the absence of CTL, the importance of such a Nef-mediated CTL escape

mechanism in vivo remains unclear. An alternative and much more effective immune escape mechanism that has been proposed frequently in the literature [10,11,24,26] is Nef-mediated dysfunction of APCs, especially DCs. Supporting this hypothesis, Andrieu et al. demonstrated that Nef downregulates MHC class I expression by DCs and impairs their APC function [10]. However, we and others have failed to show that Nef significantly downregulates DC expression of MHC class I [12–14]. This discrepancy may related to the *Vaccinia*-mediated Nef expression system used by Andrieu et al. as the *Vaccinia* vector itself is known to downregulate DC maturation markers [27].

Messmer et al. have reported that adenovirus transduction of *nef* into human or macaque DCs induces them to express several cytokines/chemokines, including TNF-α, IL-12, IL-6, MIP-1α, MIP-1β, and RANTES [15]. In contrast, Maccromac et al. have shown that while the low levels of MIP-1β secretion by uninfected MDDCs are upregulated upon adenovirus infection, this occurs regardless of whether Nef is present or absent [13]. Our data support the latter study, as we found that while adenovirus infection upregulates MIP-1α and MIP-1β expression, this occurred irrespective of whether Nef was expressed. However, it remains possible that Nef alters the production of any other unidentified factors by MDDCs.

Most importantly, we, like others [13,14], found that Nef expression by MDDCs had no apparent effect on their T-cell stimulatory functions in vitro. We showed here that our adenovirus system could be used to generate effective recall responses, and that the adenovirus-mediated expression of Nef (or Gag/Pol) by MDDCs efficiently stimulated IFNγ-producing T cells from HIV-infected individuals. Thus, the tetracycline-controlled Nef expression system with high transduction efficiency allowed us clearly discriminate the effect of adenovirus infection and Nef expression, which encourage us to use the Nef-expressing adenovirus vector as a potent vaccine to activate HIV-specific T cells.

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References

[1] O.T. Fackler, A.S. Baur, Live and let die: Nef functions beyond HIV replication, Immunity 16 (2002) 493–497.
[2] F. Peter, HIV nef: the mother of all evil? Immunity 9 (1998) 433–437.
[3] J.W. Marsh, The numerous effector functions of, Nef. Arch. Biochem. Biophys. 365 (1999) 192–198.
[4] 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.
[5] J.F. Arrighi, M. Pion, E. Garcia, J.M. Escola, Y. van Kooyk, T.B. Geijtenbeek, V. Piguet, DC-SIGN-mediated infectious synapse formation enhances X4 HIV-1 transmission from dendritic cells to T cells, J. Exp. Med. 200 (2004) 1279–1288.
[6] C.R. Rinaldo Jr., P. Piazza, Virus infection of dendritic cells: portal for host invasion and host defense, Trends Microbiol. 12 (2004) 337–345.
[7] Z. Fan, X.L. Huang, L. Zheng, C. Wilson, L. Borowski, J. Liebmann, P. Gupta, J. Margolick, C. Rinaldo, Cultured blood dendritic cells retain HIV-1 antigen-presenting capacity for memory CTL during progressive HIV-1 infection, J. Immunol. 159 (1997) 4973–4982.
[8] M.A. Barron, N. Blyveis, B.E. Palmer, S. MaWhinney, C.C. Wilson, Influence of plasma viremia on defects in number and immunophenotype of blood dendritic cell subsets in human immunodeficiency virus 1-infected individuals, J. Infect. Dis. 187 (2003) 26–37.
[9] K.L. Collins, B.K. Chen, S.A. Kalams, B.D. Walker, D. Baltimore, HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes, Nature 391 (1998) 397–401.
[10] M. Andrieu, D. Chassin, J.F. Desoutter, I. Bouchaert, M. Baillet, D. Hanau, J.G. Guillet, A. Hosmalin, Downregulation of major histocompatibility class I on human dendritic cells by HIV Nef impairs antigen presentation to HIV-specific CD8+ T lymphocytes, AIDS Res. Hum. Retrovir. 17 (2001) 1365–1370.
[11] E. Shinya, A. Owaki, M. Shimizu, J. Takeuchi, T. Kawashima, C. Hidaka, M. Satomi, E. Watari, M. Sugita, H. Takahashi, Endogenously expressed HIV-1 nef down-regulates antigen-presenting molecules, not only class I MHC but also CD1a, in immature dendritic cells, Virology 326 (2004) 79–89.
[12] A.L. Cramer, J.A. Frelinger, Dendritic cells transduced with HIV Nef express normal levels of HLA-A and HLA-B class I molecules, J. Acquir. Immun. Defic. Syndr. 27 (2001) 417–425.
[13] L.P. Maccormac, J.M. Jacque, B. Chain, The functional consequences of delivery of HIV-1 Nef to dendritic cells using an adenoviral vector, Vaccine 22 (2004) 528–535.
[14] B. Verhasselt, E. Naessens, C. Verhofstede, M. De Smedt, S. Schollen, T. Kerre, D. Vanhecke, J. Plum, Human immunodeficiency virus nef gene expression affects generation and function of human T cells, but not dendritic cells, Blood 94 (1999) 2809–2818.
[15] D. Messmer, J.M. Jacque, C. Santisteban, C. Bristow, S.Y. Han, L. Villamide-Herrera, E. Mehlhop, P.A. Marx, R.M. Steinman, A. Gettie, M. Pope, Endogenously expressed nef uncouples cytokine and chemokine production from membrane phenotypic maturation in dendritic cells, J. Immunol. 169 (2002) 4172–4182.
[16] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.
[17] J. Adachi, K. Ookawa, M. Shiseki, T. Okazaki, S. Tsuchida, K. Morishita, J. Yokota, Induction of apoptosis but not G1 arrest by expression of the wild-type p53 gene in small cell lung carcinoma, Cell Growth Differ 7 (1996) 879–886.
[18] I. Yoshizawa, Y. Soda, T. Mizuochi, S. Yasuda, T.A. Rizvi, T. Takemori, Y. Tsunetsugu-Yokota, Enhancement of mucosal immune response against HIV-1 Gag by DNA immunization, Vaccine 19 (2001) 2995–3003.
[19] Y. Kanegae, M. Makimura, I. Saito, A simple and efficient method for purification of infectious recombinant adenovirus, Jpn. J. Med. Sci. Biol. 47 (1994) 157–166.
[20] 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.
[21] Y. Tsunetsugu-Yokota, H. Tamura, M. Tachibana, K. Ogata, M. Honda, T. Takemori, Selective expansion of perforin-positive CD8+ T cells by immature dendritic cells infected with live Bacillus Calmette-Guerin mycobacteria, J Leukoc Biol. 72 (2002) 115–124.

[22] X. Liu, J.A. Schrager, G.D. Lange, J.W. Marsh, HIV Nef-mediated cellular phenotypes are differentially expressed as a function of intracellular Nef concentrations, J. Biol. Chem. 276 (2001) 32763–32770.

[23] A.D. Badley, J.A. McElhinny, P.J. Leibson, D.H. Lynch, M.R. Alderson, C.V. Paya, Upregulation of Fas ligand expression by human immunodeficiency virus in human macrophages mediates apoptosis of uninfected T lymphocytes, J. Virol. 70 (1996) 199–206.

[24] S. Swingler, A. Mann, J. Jacque, B. Brichacek, V.G. Sasseville, K. Williams, A.A. Lackner, E.N. Janoff, R. Wang, D. Fisher, M. Stevenson, HIV-1 Nef mediates lymphocyte chemotaxis and activation by infected macrophages, Nat. Med. 5 (1999) 997–1003.

[25] D. Rea, F.H. Schagen, R.C. Hoeben, M. Mehtali, M.J. Havenga, R.E. Toes, C.J. Melief, R. Offringa, Adenoviruses activate human dendritic cells without polarization toward a T-helper type 1-inducing subset, J. Virol. 73 (1999) 10245–10253.

[26] S. Swingler, B. Brichacek, J.M. Jacque, C. Ulich, J. Zhou, M. Stevenson, HIV-1 Nef intersects the macrophage CD40L signalling pathway to promote resting-cell infection, Nature 424 (2003) 213–219.

[27] J. Engelmayer, M. Larsson, M. Subklewe, A. Chahroudi, W.I. Cox, R.M. Steinman, N. Bhardwaj, Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion, J. Immunol. 163 (1999) 6762–6768.

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′-caccgattggcagaactacacaccaagagagtgtgtagttctgccaatc-3′, and antisense oligonucleotide, 5′-aaaagattggcagaactacacactctcttggtgtgtagttctgccaatc-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).

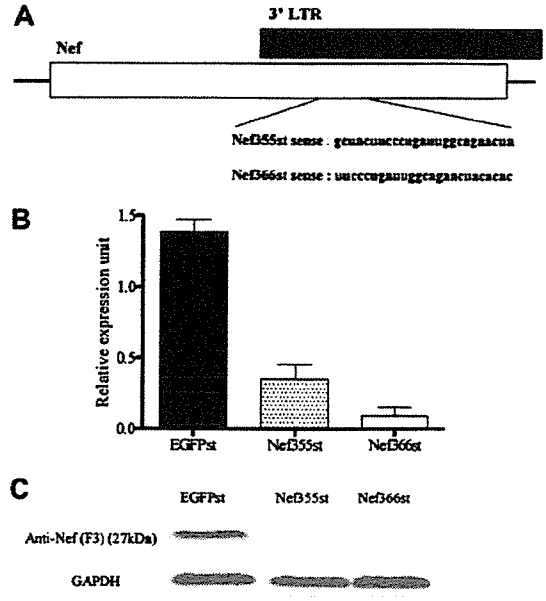

Figure 1. siRNA target sequences in *nef*. (A) Targets of siRNAs against the U3-overlapping region of HIV-1_{NL432} Nef and their sequences. Nef-expressing HeLa CD4 cells were transfected either with 2.5 μM *egfp* siRNAs (control: EGFPst) or *nef* siRNAs (Nef355st or Nef366st). At 48 hours after transfection, 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

4-day intervals and viral production was monitored by HIV of p24 Gag antigen ELISA kit (RETRO TEC; ZeptoMetrix, Buffalo, NY).

Real-time RT-PCR (qRT-PCR) analysis of HIV-1 infection

HIV-1–infected cells were collected and total DNA was prepared 3, 8 and 12 hours after infection. For the detection and quantification of individual forms of HIV-1 DNA, oligonucleotide primer and probe sequences were designed specifically for the *Taq*Man assay as described elsewhere.[19] All probes (Biosearch Technologies, Novato, CA.) were 5′-labeled with the fluorophore FAM as the reporter dye, and 3′-labeled with Black Hole Quencher-1 (BHQ-1) as the quencher dye. The qRT-PCR analysis was performed on an Mx3000P (Stratagene) and the amount of HIV-1–specific DNA per cell was normalized to β-globin gene.

Kinetics of virus production in MDMs and reporter analysis

MDMs (2×10^5/well) were cultured in 48-well tissue-culture plates and infected either with wild-type HIV-1_{NF462} or HIV-$1_{NF462\Delta Nef}$. MDMs were infected with lentivirus at a multiplicity of infection (MOI) of 2 or 10 and washed extensively. The next day, cells were exposed to HIV-1 (5 ng/well) for 2 hours. Cell supernatants were harvested at 3- or 4-day intervals, and viral production was monitored by p24 antigen ELISA.

The cell-culture supernatants at 10 days after HIV infection were examined for infectivity, and 10 days after HIV infection, cell supernatants were collected (termed HIV-1/Lenti cont and HIV-1/Lenti shNef366). CEMx174 CCR5/LTR-EGFP cells were infected with HIV-1/Lenti cont or HIV-1/Lenti shNef366, and the number of HIV-1–infected $EGFP^+$ T cells was determined by fluorescence-activated cell sorter (FACS).

Detection of chemokines

For the detection of chemokine production in MDMs, the cytometric bead array (CBA) kit (BD Bioscience, San Jose, CA) was used, which measured 4 chemokines (IL-8, MIP-1α, MIP-1β, MCP-1) simultaneously.

Restimulation assay of lentivirus-transduced U1 cells

Latent HIV-1–infected U1 cells were transduced with Lenti cont or Lenti shNef366 at an MOI of 1. Two weeks later, $EGFP^+$ cells were sorted and stimulated with 1 ng/mL recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF). Culture supernatants were collected at days 2 and 5, and the level of p24 antigen was measured by ELISA.

Results

siRNA suppresses *nef* mRNA and Nef protein expression

In the HIV-1 genome, *nef* is located at the 3′ end of the viral genome, partially overlapping the 3′ LTR (Figure 1A). Jacque and colleagues demonstrated previously that siRNA targeting of the 5′ region of *nef* (nucleotides 164-185) suppressed HIV replication.[20] Therefore, we selected 3 distinct regions of the HIV-1_{NL432} *nef* sequence using a Web-based program for designing DNA-directed RNAi systems, focusing on the Nef coding region overlapping the 3′ LTR. These were designated as Nef338, 366, and 479 based on the position of the first nucleotide of the siRNA. From initial screening experiments, we found that Nef366 was the most effective target site (data not shown).

The type 1 interferon response is an innate defense mechanism in eukaryote cells against viral infection. It has been shown that some types of siRNA induce type 1 interferon, which in turn mediates the gene-specific effect of RNAi.[21-23] The stealth siRNA system was developed to avoid the interferon response to siRNA in cells (Invitrogen manual). We prepared synthetic stealth siRNAs, designated Nef355st and Nef366st, and a control siRNA designated EGFPst, to determine the effect of RNAi using sequences based on Nef366 (the U3-overlapping region of the Nef-coding region). Neff355st was synthesized based on a Web-based computer program for generating stealth siRNA (Invitrogen), whereas Nef366st represents a slightly modified version of the stealth target site (6-nucleotide difference), so that it conformed to the target sequence as described. These stealth Nef siRNA sequences differed by only 5 nucleotides (Figure 1A).

We established a stable Nef-expressing HeLa-CD4 clonal cell line, designated as HeLa-CD4-Nef. HeLa-CD4-Nef cells were transfected either with 2.5 μM EGFPst or *nef* stealth siRNAs (Nef355st or Nef366st), and harvested 48 hours after transfection. Total RNA was extracted and the level of *nef* mRNA was measured by qRT-PCR. We observed that transfection with Nef366st reduced *nef* mRNA expression more than 90% (Figure 1B), whereas Nef355st suppressed the level of *nef* mRNA approximately 80%, compared with EGFPst controls. When cell lysates of the transfected cells were analyzed by Western blot, we found that both Nef366st and Nef355st suppressed Nef protein levels to below the detection limit of the assay (Figure 1C). Taken together, these results clearly showed that Nef366 is an efficient target sequence for the inhibition of *nef* gene expression by siRNA.

shRNA suppresses *nef* mRNA and Nef protein expression

To assess the effect of endogenous expression of Nef366 siRNA, we constructed expression vectors that encoded shRNAs corresponding to Nef366, or *lacZ* as a control, driven by the human U6 polymerase III promoter, designated as pENTR/shNef366 and pENTR/shLacZ, respectively (Figure 2A). HeLa-CD4-Nef cells were transfected with either pENTR/shNef366 or pENTR/shLacZ by FuGene6 reagent (Roche) and cells were harvested 72 hours after transfection. Total RNA was extracted and analyzed by qRT-PCR. We observed that the level of *nef* mRNA was suppressed by approximately 80% in cells transfected with pENTR/shNef366 (Figure 2B). Western blot analysis confirmed that pENTR/shNef366 strongly suppressed Nef protein levels as well (data not shown). These results indicated that promoter-driven endogenous

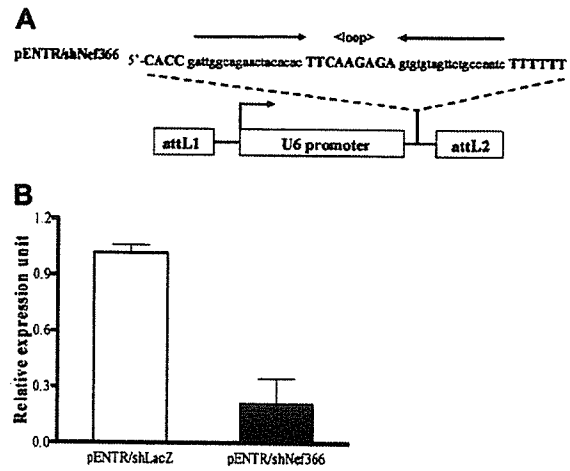

Figure 2. RNAi by transfection with shRNA expression vectors. (A) Schematic of the expression vectors (pENTR/shRNA) encoding shRNAs of Nef366 or *lacZ*, designated as pENTR/shNef366 and pENTR/shLacZ, respectively, in which expression is driven by the human U6 polymerase III promoter. (B) Nef-expressing HeLa-CD4 cells were transfected either with 1.0 μg pENTR/shNef366 or pENTR/shLacZ and cells were harvested 72 hours after transfection. Total RNA was extracted and analyzed by qRT-PCR. The data represent the average ± SD of 3 independent experiments.