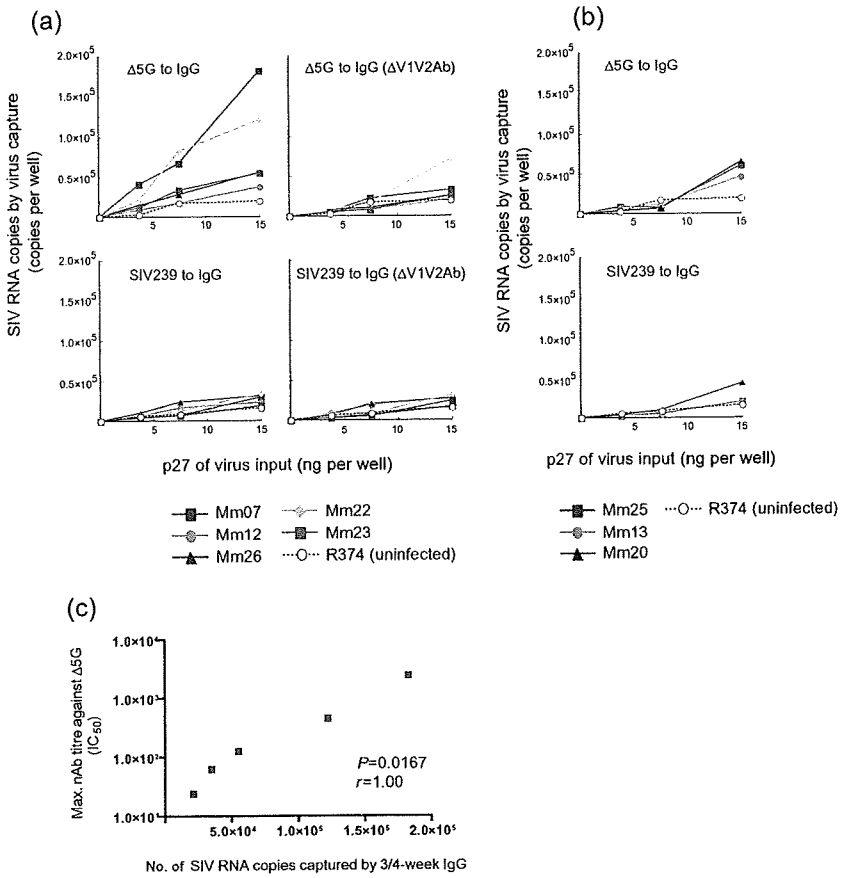

Fig. 7. Virion capture activity of IgG from Δ5G-infected and SIV239-infected animals. Virion capture activity of IgG from the plasma of infected animals at 3 or 4 weeks p.i. was determined by increased captured SIV RNA relative to input (3.75, 7.5 and 15 ng $p27^{gag}$) of Δ5G or SIV239. Plasma samples of Δ5G-infected animals (a) and SIV239-infected animals (b) were used for the assay. IgG (ΔV1V2Ab) indicates IgG depleted of Ab binding to V1V2-9, -10 or -11 peptide. R374 was an uninfected monkey. Correlation between virion capture activity at 3 or 4 weeks p.i. and peak nAb titre in Δ5G-infected animals (Fig. 2b) is shown (c).

Δ5G-infected animals clearly exhibited better nAb responses than SIV239-infected animals, the most stringent nAb assay, based on 90 % inhibition, provided evidence of nAb titres in only two of five Δ5G-infected animals and the appearance of these titres trailed the decline of acute viral loads by almost 4 weeks (Figs 1 and 2). Therefore, we concluded that, although deglycosylation did promote better development of nAbs in Δ5G-infection than SIV239 infection, it was still too late to control acute viraemia.

Zinkernagel and co-workers have categorized viruses into two types: 'acutely cytopathic viruses' and 'poorly or non-cytopathic viruses' (Hangartner *et al.*, 2006b). The former contains viruses such as vesicular stomatitis virus in mice and influenza virus in humans, whose control depends primarily on a rapid and potent nAb response. The latter comprises viruses such as lymphocytic choriomenigitis virus in mice, and hepatitis B and C viruses and HIV in humans, against which a nAb response is apparent only following the reduction of primary viraemia, and which establish persistent chronic infections. Accordingly, although the viral loads in Δ5G infection resembled 'acutely cytopathic virus' infections, the kinetics of nAbs still conformed to the 'non-cytopathic virus' category. As the difference in nAb response between the two types of virus is determined by their surface glycoproteins (Pinschewer *et al.*, 2004), this study suggests that the deglycosylation of Δ5G could not change this intrinsic property of SIV239.

Ab responses to Env peptides in Δ5G-infected animals

Aside from nAb, non-nAb responses to linear epitopes in V1/V2 were specifically induced by 3 weeks p.i. in all Δ5G-infected animals (Figs 4, 5 and 6). The heavy glycosylation of viral spikes clearly prevented access of B-cell receptors to the linear Ab epitopes located within limited regions of gp120 in SIV239, and the reduced glycosylation probably promoted better exposure of these linear epitopes in Δ5G (Fig. 4). Accordingly, the Δ5G-specific epitope in V1/V2 should be closely associated with the deglycosylation mutation at aa 171 in gp120 (Fig. 5). We speculate that this Ab induction might contribute to acute viral suppression in Δ5G infection because of the coincident decrease in peak viraemia (Figs 1 and 6). Non-neutralizing Abs can be divided into those that bind to the intact virion surface and debris-specific Ab. The former non-neutralizing Abs have occasional possibilities for antiviral activities such as antibody-dependent cell-mediated cytotoxicity and complement-mediated virus inactivation (Aasa-Chapman *et al.*, 2005; Ahmad & Menezes, 1996; Forthal *et al.*, 2001; Hangartner *et al.*, 2006a). In fact, readily detectable virion

capture Abs were induced in two of five Δ5G-infected animals (Fig. 7, Mm07 and Mm22). The importance of immediate-early suppression of SIV replication for the long-term containment of infection has been demonstrated by studies of post-exposure anti-retroviral therapy (Lifson *et al.*, 2000; Mori *et al.*, 2000). Thus, the early and complete control of viraemia in Δ5G-infected animals clearly suggests an antiviral mechanism(s) acting as early as 2–4 weeks p.i. Therefore, the early detection of IgG capable of virus capture in Δ5G-infected animals may provide mechanisms capable of contributing to undetectable viral load set points (Fig. 1b). The selective generation of such Ab directed to linear Env epitopes is expected.

Interestingly, deglycosylation in gp120 was also associated with a general reduction in the antigenicity of linear epitopes in gp41: the Ab response against the two epitopes that reside in the regions between the two heptad repeats (aa 601–625) and in the C-terminal heptad repeat (aa 660–685), respectively, was markedly reduced (Fig. 4, Table 1). The former corresponds to the highly conserved immunogenic epitope (Benichou *et al.*, 1993; Gnann *et al.*, 1987; Silvera *et al.*, 1994), and the latter corresponds to an epitope identified in the chronic phase of SIVmac251 infection (Silvera *et al.*, 1994) and corresponds to the nAb epitope of HIV-1 known as 2F5 (Muster *et al.*, 1993), although this linear epitope has not been associated with SIV neutralization (Caffrey *et al.*, 1998). Thus, these epitopes are probably exposed on the surface of viral spikes or their degraded fragments in most SIV and HIV-1 isolates with appropriate glycosylation and correct folding. We believe that the loss of glycosylation might induce a slight conformational change in the gp120 protein backbone, resulting in altered interaction of gp120 and gp41. In fact, the region encompassing the former epitope in gp41 was demonstrated to interact with gp120 (Cao *et al.*, 1993; Maerz *et al.*, 2001; York & Nunberg, 2004). As viral spikes determine virus properties such as viral receptor usage and cell tropism (Kolchinsky *et al.*, 2001; Puffer *et al.*, 2002), different cell populations might be infected in Δ5G-infected animals compared with SIV239 infection. More specifically, because of the distinct properties of the virus, vigorous Δ5G replication in the acute phase did not apparently impair immune function and thus established the control of chronic-phase infection and viral replication.

Host factors required for functional Ab responses against SIV infection

This study also demonstrated remarkable differences in humoral response with regard to nAb and virion capture Ab among Δ5G-infected animals. However, gp120-specific-binding Ab and the linear epitope-specific Ab were initially induced similarly in all animals. These findings imply that Abs measured by ELISA assay and Abs exhibiting antiviral activity are elicited by different pathways and that the properties associated with functional Abs depend largely on the host and underscore the importance of its genetic background. Rhesus macaques are present in various geographical locations within the Asian continent and are subdivided into many subspecies morphologically and genetically (Smith & McDonough, 2005). Some of the genetic differences among rhesus monkeys of different geographical origins, and especially those involving major histocompatibility complex (MHC) genotypes, probably influence the corresponding differences in immune responses, especially cellular response (Bontrop *et al.*, 1996; O'Connor *et al.*, 2003; Reimann *et al.*, 2005). Schmitz *et al.* (2005) reported that Mamu-A*01-positive rhesus monkeys elicited a significantly higher cellular response and lower nAb titres than those in Mamu-A*01-negative animals at the time of challenge infection of animals vaccinated with live attenuated SIV. They suggested that both humoral and cellular immune responses contributed to the protection against the challenge infection and that the relative contribution of each of the responses may be genetically determined. We observed a similar relationship between nAb and cellular responses among Δ5G-infected animals: two animals (Mm07 and Mm22) elicited a lower cellular response while the other three animals (Mm12, Mm23 and Mm26) elicited a higher cellular response (data not shown). Notably two animals exhibiting highly functional Ab (Mm07 and Mm22) were the offspring of seed animals imported from Laos, whilst the others (Mm12, Mm23 and Mm26) were of Burmese origin, suggesting the potential association of such different humoral and cellular responses with host genetic factors. In clinical studies, considerable concordance of adaptive cellular and humoral responses and HIV evolution in monozygotic twins, but not in brothers, infected with the same virus has been reported (Draenert *et al.*, 2006). HIV-1-exposed but uninfected status with significantly higher neutralizing IgA was linked to genotypes on chromosome 22 (Kanari *et al.*, 2005). In the mouse Friend leukemia virus model, MHC II alleles were determined as host genetic factors required for effective nAb response (Miyazawa *et al.*, 1992) and the host genetic factor was mapped to chromosome 15, which was associated with the clearance of viraemia by nAb (Hasenkrug *et al.*, 1995; Kanari *et al.*, 2005).

Taken together, we speculate that the functional humoral response is determined by host genetic properties similar to the cellular immune response. Thus, gaining knowledge of the genetic requirements for both humoral and cellular containment of viral infections will clearly be of primary importance for vaccine development and therapeutics against HIV and other infectious agents.

NOTE ADDED IN PROOF

A discrepancy in the SIV239-infected animals Mm13 and Mm20 was noted between the result shown in Fig. 2 and that in a previous report Mori *et al.*, 2001. The nAb

response against SIV239 in Mm20 was confirmed at multiple time points in the present study.

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Comparison Between Sendai Virus and Adenovirus Vectors to Transduce HIV-1 Genes Into Human Dendritic Cells

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Immuno-genetherapy using dendritic cells (DCs) can be applied to human immunodeficiency virus type 1 (HIV-1) infection. Sendai virus (SeV) has unique features such as cytoplasmic replication and high protein expression as a vector for genetic manipulation. In this study, we compared the efficiency of inducing green fluorescent protein (GFP) and HIV-1 gene expression in human monocyte-derived DCs between SeV and adenovirus (AdV). Human monocyte-derived DCs infected with SeV showed the maximum gene expression 24 hr after infection at a multiplicity of infection (MOI) of 2. Although SeV vector showed higher cytopathic effect on DCs than AdV, SeV vector induced maximum gene expression earlier and at much lower MOI. In terms of cell surface phenotype, both SeV and AdV vectors induced DC maturation. DCs infected with SeV as well as AdV elicited HIV-1 specific T-cell responses detected by interferon γ (IFN-γ) enzyme-linked immunospot (Elispot). Our data suggest that SeV could be one of the reliable vectors for immuno-genetherapy for HIV-1 infected patients. ***J. Med. Virol. 80:373–382, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: viral vector; immuno-genetherapy; AIDS

INTRODUCTION

Introduction of highly active anti-retroviral therapy (HAART) has improved the clinical course of patients infected with human immunodeficiency virus type 1 (HIV-1) dramatically. However, there are many obstacles to the long-term administration of anti-retroviral drugs, such as metabolic disorders, emergence of drug resistant viruses, and high medical expenses. The combination of therapeutic vaccines and HAART could not only reduce the adverse effects of HAART but also decrease the medical expenses especially in developing countries.

Cellular immune responses play a crucial role in controlling the replication of HIV-1 [Yang et al., 1997;

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Matano et al., 1998; Brander and Walker, 1999; Jin et al., 1999; McMichael and Rowland-Jones, 2001]. One of the possible strategies for treating HIV-1 infection is to enhance the cellular anti-viral capacity. Since dendritic cells (DCs) have high ability of antigen presentation, they have been used as stimulators of T-cell responses by inserting HIV-1 antigens [Engelmayer et al., 2001; Stubbs et al., 2001; Tsunetsugu-Yokota et al., 2003; Mwau et al., 2004].

Sendai virus (SeV), one of the members of *Paramyxoviridae*, is an enveloped virus with a nonsegmented negative-strand RNA genome. It causes severe respiratory disease in mice but is nonpathogenic for humans. SeV vector has been developed and shown to have high gene transduction efficiency and protein expression in different cell lineages [Kato et al., 1996; Kawana-Tachikawa et al., 2002]. In addition, the cytoplasmic replication of SeV precludes the integration of its genetic information into cellular genome. Recently, the second generation SeV vector deficient in F gene (dF-SeV) has been developed [Li et al., 2000]. The dF-SeV has been proved not to cause secondary infection. SeV and dF-SeV vectors could be promising systems to introduce HIV-1 genes into DCs for stimulating HIV-1 specific T-cell responses in primates and humans [Kano et al., 2002; Takeda et al., 2003; Kato et al., 2005].

Adenovirus (AdV), vaccinia virus, and retrovirus have been used as viral vectors for gene transfer into DCs [Engelmayer et al., 1999; Rea et al., 1999; Bonini et al., 2001; Rouas et al., 2002]. In this study, we compared the efficiency of inducing green fluorescent protein (GFP) and HIV-1 gene expression in human monocyte-derived DCs between AdV vectors and new viral vectors based on SeV. We showed that SeV vector transduced DCs efficiently and elicited HIV-1 specific T-cell responses. Our results suggest the potential use of SeV vector for immuno-genetherapy for HIV-1 infected patients.

MATERIALS AND METHODS

Viral Vectors

SeV carrying GFP (SeVGFP) [Agungpriyono et al., 2000], HIV-1 NL4-3 Gag (SeVGag), and HIV-1 SF2 Env (SeVEnv) [Yu et al., 1997] were propagated in 10-day-old embryonated chicken eggs. The fusion protein (F)-defective SeV (dF-SeV) [Li et al., 2000] was propagated in the monkey kidney cell line expressing Sendai virus F gene product (LLC-MK2/F7) [Li et al., 2000] because the replication capacity of dF-SeV was incompetent. SeV particles were purified by 50% sucrose (w/v)/10 mM Tris–HCl and 30% sucrose (w/v)/10 mM Tris–HCl density centrifugation, dialyzed against Dulbecco's phosphate buffered saline (PBS) (Sigma, St. Louis, MO), and stored at −80°C. The titers of the vector stocks, determined on LLC-MK2 [Kato et al., 1996], were as follows; SeVGag: 5.2×10^8 CIU/ml, SeVEnv: 6.4×10^7 CIU/ml, SeVGFP: 5.2×10^8 CIU/ml, GFP/dF-SeV: 3.1×10^9 CIU/ml, SeV without inserts: 5.2×10^8 CIU/ml.

Recombinant AdV used in this experiment was derived from AdV type 5 and was replication-deficient with deletion of E1 and E3 genes. The AdV carrying HIV-1 genes (*gag-pol*, *env*, *rev*, and RRE) was generated with AdV Expression Kit (TakaraBio, Shiga, Japan) according to the manufacturer's protocol. Each HIV-1 gene was inserted into the expression cassette of pAxCAwt cosmid vector equipped with CAG promoter and rabbit beta-globin polyadenylation signal. All HIV-1 sequences were derived from SF2 strain [Levy et al., 1986]. 5′ half and 3′ half of *Eco*RI fragments from SF2 provirus were subcloned pUC19, generating pUC19-9B/R7 and pUC19-9B/R6, respectively. HIV-1 *gag-pol* gene was obtained from pUC19-9B/R7 after deleting the sequence upstream of *gag*. *Nar*I site was introduced next to gag initiation codon by PCR using primers *gag/Nar*I-S (5′-CAGGCGGCAAGGAGAGAGATGGGTGC-GAG-3′) and *gag/Apa*I-AS (5′-CCTTTTTCCTAGGGG-CCCTGC-3′) (restriction sites are underlined). PCR-amplified fragment was returned to *Nar*I and *Apa* I-digested pUC19-9B/R7, generating pUC19-GP. The 4.5 kb *Nar*I-*Nde*I fragment containing the HIV-1 *gag-pol* gene was inserted into the *Swa*I site of pAxCAwt cosmid vector to create AdVGP. HIV-1 *rev* responsible element (RRE) was generated from pUC19-9B/R6 by PCR using primers rre/*pfl*MI-S (5′-GCCATAGAATG-GCCAAGGCAAAGAGAAGAGTGG-3′) and rre/*Bam*HI-AS (5′-GGGATCCCAAGGCACAGCAGTGGTTGC-3′). The PCR fragment was inserted between *pfl*MI and *Bam*HI site of pUC19-GP, and thus placed downstream of *gag-pol* gene. The consequent 4.9 kb *Nar*I-*Bam*HI fragment containing the HIV-1 *gag-pol*-RRE sequences was inserted into the *Swa*I site of pAxCAwt cosmid vector to create AdVGPR. HIV-1 *rev* gene was made by two-step PCR from *Stu*I-*Xho*I fragment of pUC19-9B/R6. The 1st-PCR primer set was *rev*/1st-S (5′-CTCA-GGACAGTCAGACTCATCAAGCTTCTCTATCAAAG-CAACCCGCCTCC-3′) and *rev*-AS (5′-GGCTATTCTT-TAGTTCCTGAATCCAATACTGCA-3′), and the 2nd-PCR primer set was *rev*/2nd-S (5′-GGATGGCAGGAA-GAAGCGGAGACAGCGACGAAGAGCTCCTCAGGA-CAG-3′) and *rev*-AS. The PCR fragment was digested with *Sph*I and *Spe*I, and inserted into the *Swa*I site of pAxCAwt to create AdVRev. The absence of PCR errors was confirmed for all PCR-amplified fragments by sequencing. The 2.1 kb *Mlu*I-*Xho*I fragment of pUC19-9B/R6 containing the HIV-1 *env* gene was inserted into the *Swa*I site of pAxCAwt cosmid vector to create AdVEnv. Recombinant AdV, Ax1w1 [Miyake et al., 1996] bearing no insert and AdVGFP [Miyake et al., 1996] expressing GFP, were kindly provided by Dr. Izumu Saito and Yumi Kanegae (University of Tokyo, Tokyo, Japan). AdV was propagated in 293 cells [Graham et al., 1977], purified by two rounds of CsCl density centrifugation [Kanegae et al., 1994], dialyzed against PBS containing 10% glycerol and stored at −80°C. The titer of the viral stocks was determined by a plaque-forming assay on 293 cells. The titers of each virus were as follows; AdVGPR: 7.6×10^9 pfu/ml, AdVEnv: 1.6×10^{11} pfu/ml, AdVRev: 2.7×10^{11} pfu/ml, AdVGFP: 5.9×10^{10} pfu/ml, Ax1w1: 4.7×10^{11} pfu/ml. Vector stocks were tested for the

absence of replication-competent AdV as described [Ishii-Watabe et al., 2003].

Cell Lines and Media

Monkey kidney cell line (LLC-MK2) was cultured in minimal essential medium (MEM) (Sigma) supplemented with 100 U of penicillin/ml, 100 U of streptomycin/ml, and 10% heat-inactivated fetal calf serum (FCS). Two hundred ninety three cells were cultured in Dulbecco's modified eagle medium (DMEM) (Sigma) supplemented with 100 U of penicillin/ml, 100 U of streptomycin/ml, and 10% FCS.

Generation of DCs

Immature myeloid DCs were generated from human peripheral blood mononuclear cells (PBMCs) using previously described methods [Nagayama et al., 2003]. Briefly, PBMCs of healthy adult volunteers were collected in heparinized tubes, subjected to density centrifugation over Ficoll-Paque Plus (Amersham Pharmacia Biotech, Piscataway, NJ), and washed twice with PBS. These PBMCs were plated on 10 cm PRIMARIA tissue culture dish (Becton Dickinson Labware, FranklinLakes, NJ) and kept at 37°C for 30 min to remove nonadherent cells. Floating cells were removed gently by rinsing with 10 ml of PBS three times and the remaining adherent cells were cultured overnight in 6 ml of RPMI 1640 medium at 37°C. Cells were washed three times again on the next day with 10 ml of PBS and the remaining adherent cells were cultured for 7 days in DC medium [6 ml of RPMI 1640 supplemented with 100 U of penicillin/ml, 10 mg of streptomycin/ml, and 10% FCS, 300 ng of recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) (Wako, Osaka, Japan), and 300 ng of recombinant human interleukin-4 (rhIL-4) (Wako)]. After 7 days, DCs were collected with a scraper. Tumor necrosis factor-α (TNF-α) (Wako) was added to the DC medium at a final concentration of 50 ng/ml on day 7 in some experiments and cultured for another 2 days to generate mature DCs. The purity of DCs was >95% based on the expression of CD1a and CD11c and lack of expression of T-cell, B-cell, NK-cell, and monocyte lineage markers by flow cytometry (data not shown).

Infection of DCs With Viral Vectors

5×10^5 DCs were infected with SeV or AdV vectors for 1 hr at 37°C in a final volume of at least 500 μl of serum-free RPMI 1640. After the infection, DCs were washed with serum-free RPMI 1640 medium and cultured in 24 well plates with 1 ml of the DC medium.

SDS-PAGE and Western Blot Analysis

DCs infected with SeV (SeVGag or SeVEnv) at a multiplicity of infection (MOI) of 2 or with AdV (AdVGFR, AdVEnv, AdVRev) at an MOI of 1,000 were harvested after 24 or 48 hr of infection, respectively. After washing with PBS, the cells were resuspended in RIPA buffer (10 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, complete mini as 1× protease inhibitor cocktail [Roche, Basel, Switzerland]), and kept on ice for 10 min. The suspension was spun for 5 min at 9,000*g* to remove cell debris. The amount of protein in the cell lysate was determined by protein assay kit (Bio-Rad Laboratories, Hercules, CA). Cell lysates containing 30 μg of cellular protein were loaded onto a 10–20% Ready Gels J (Bio-Rad Laboratories) and electrophoretically transferred to immobilon polyvinylidene difluoride transfer membrane (Millipore, Billerica, MA). Western blot analysis was performed using Lumi-Light plus Western Blotting Kit (Roche) according to the manufacturer's instructions. Briefly, the membrane was blocked in 1% blocking solution at 4°C overnight. The membrane was incubated with mouse monoclonal antibodies against p24 (Advanced Biotechnologies, Inc., Columbia, MD) and gp120 (Immuno Diagnostics, Inc., Woburn, MA) of HIV-1 for 1 hr. The blots were then washed four times with 1× TBST and incubated with anti-mouse IgG conjugated with horseradish peroxidase (Roche). Proteins were illuminated by Lumilight Plus (Roche) and detected with Lumi Imager (Roche). Quantification was done by densitometric analysis with the Lumi Analyst software (Roche).

Immunostaining and Flow Cytometry

GFP expression and viability of DCs infected with SeVGFP, dF-SeVGFP, or AdVGFP were analyzed by flow cytometry. To determine the viability, 5×10^5 DCs in about 300 μl of media were stained with 10 μl of propidium iodide (PI) (50 μg/ml; SIGMA). Events were acquired on a FACS-Caliber (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson) and Flow Jo software version 4.1 (Tree Star, Asland, OK).

To determine the effects of transduction on the expression of DC surface marker, immature DCs (imDCs) were cultured with the DC medium for 48 hr, mature DCs (mDCs) were cultured with the DC medium plus TNF-α for 48 hr. DCs transduced with SeV or AdV were cultured with the DC medium for 24 or 48 hr, respectively. Those DCs were stained with antibodies at 4°C for 20 min and then washed three times with PBS. Those cells were analyzed by flow cytometry after fixing 1% paraformaldehyde. The antibodies we used were as follows: fluorescein isothiocyanate-anti-Lineage (Lin-FITC) (CD3, CD14, CD16, CD19, CD20, CD56) (Becton Dickinson), phycoerythrin (PE)-anti-CD1a (Immunotech, Marseilles, France), PE-anti-CD83 (Immunotech), PE-anti-HLA-ABC (Dako), Peridinin chlorophyll protein (PerCP) -anti-CD4 (Becton Dickinson), PerCP-anti-HLA-DR (Becton Dickinson), allophycocyanin (APC)-anti-CD14 (Immunotech), APC-anti-CD40 (PharMingen), APC-anti-CD11c (PharMingen), Biotin-conjugated anti-CD86 (Becton Dickinson). Streptavidin-FITC (Becton

Dickinson) was employed as secondary reagents. FITC- (PharMingen, San Diego, CA), PE- (Dako Glostrup, Denmark), Per CP- (Becton Dickinson), APC- (Becton Dickinson) conjugated species- and isotype-matched, mAbs were used to determine the level of background staining.

Elispot Assay

We performed enzyme-linked immunospot (Elispot) assay to know the efficiency of HIV-1 specific T-cell induction by DCs infected with AdV or SeV vector. First, we developed mDCs as described above from PBMCs of two HIV-1-infected patients. On day 7, we infected those mDCs with AdV vector at an MOI of 1,000 or SeV vector at an MOI of 2 for 1 hr, or just added overlapping peptides (*gag*, *env*). We used the overlapping peptides derived from consensus B sequence since both patients were infected with subtype B HIV-1. We did not check the AdV sero-status of these two patients. Both patients were on HAART and have undetectable viral load (<50 copies/ml). CD4 counts of patients 1 and 2 are 408/μl and 336/μl, respectively. We used those mDCs as stimulators in Elispot assay. PBMCs from each patient were used as effectors cells. The protocol of Elispot assay was described previously [Furutsuki et al., 2004].

RESULTS

Sendai Viral Vectors Transduce DCs at Lower MOIs Than Adenoviral Vectors

We infected imDCs with SeVGFP, dF-SeVGFP or AdVGFP at different MOIs (Fig. 1) in order to know which MOI is the best for these three vectors. We stained these cells with PI to evaluate the expression of GFP in viable cells. In SeVGFP, the expression of GFP reached the maximum (32.5%) at an MOI of 2 and the mean fluorescent intensity (MFI) of GFP showed around 1,400 at all MOIs. However, as shown by the fraction of PI-positive cells, SeVGFP killed around 30% of DCs even at an MOI of 0.5. The staining pattern of DCs infected with dF-SeVGFP was similar to that of SeVGFP. In AdVGFP, both the percentage of GFP-positive cells and the MFI of

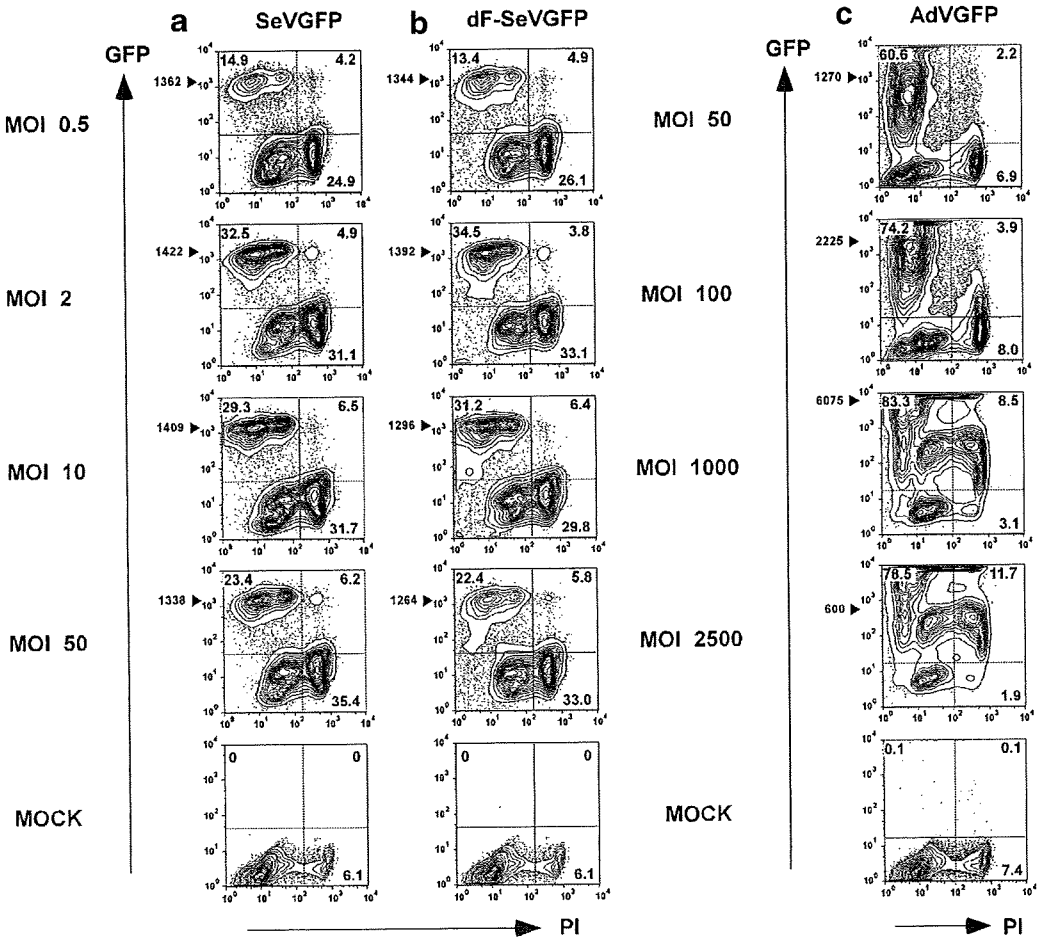

Fig. 1. Comparison of different MOIs for the maximum gene expression by SeV (**a**), dF-SeV (**b**), and AdV (**c**) vectors 48 hr after infection. Cell viability was determined by staining with PI. GFP expression and PI staining were analyzed by flow cytometry. The percentages of GFP- and PI-positive cells are shown on each corner. Arrowheads indicate MFI of GFP-positive cells within PI-negative fraction. The numbers in each panel represent the mean value of three independent experiments.

GFP increased up to an MOI of 1,000. Although PI-positive cells in AdV increased according to MOIs, the percentage of PI-positive cells was less than 14% even at the highest MOI: 2,500. From these results, SeV vector is likely to transduce DCs at much lower MOIs than AdV vector, but kill more DCs than AdV. We chose an MOI of 2 for SeVGFP and dF-SeVGFP, and an MOI of 1,000 for AdVGFP in the subsequent experiments.

Sendai Viral Vectors Showed Maximum Transduction Level Earlier Than Adenoviral Vectors

We next examined the time course of GFP expression (Fig. 2). We detected GFP-positive cells as early as 8 hr after infection in all three vectors. The proportion of GFP-positive cells reached the maximum level (around

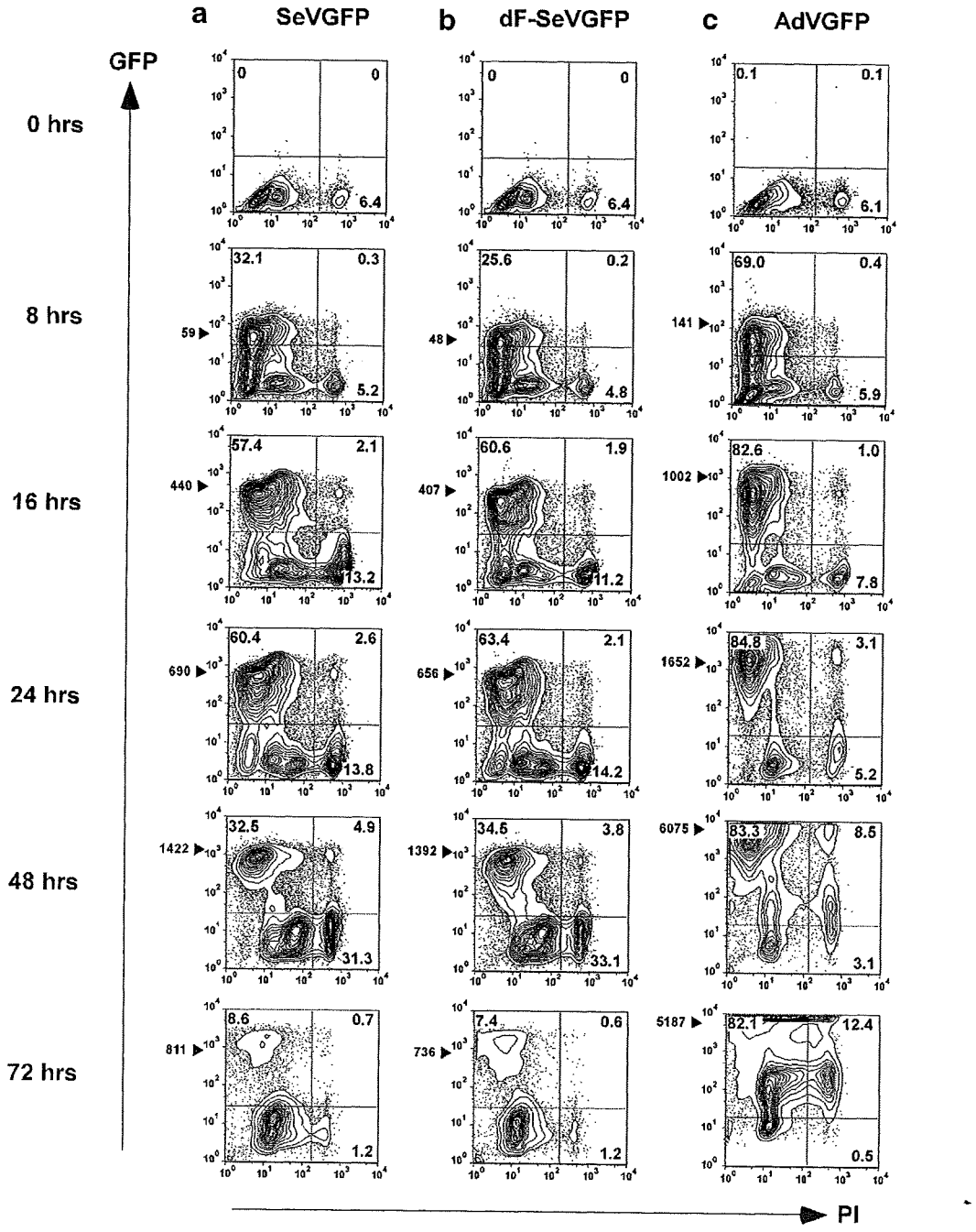

Fig. 2. The time course of the maximum gene expression by SeV (**a**), dF-SeV (**b**), and AdV (**c**) vectors. DCs were infected with SeV and dF-SeV at an MOI of 2 and were infected with AdV at MOI of 1,000 and then cultured for 8–72 hr. Cell viability was determined by staining with PI. GFP expression and PI staining were analyzed by flow cytometry. The percentages of GFP- and PI-positive cells are shown on each corner. Arrowheads indicate MFI of GFP-positive cells within PI-negative fraction. The numbers in each panel represent the mean value of three independent experiments.

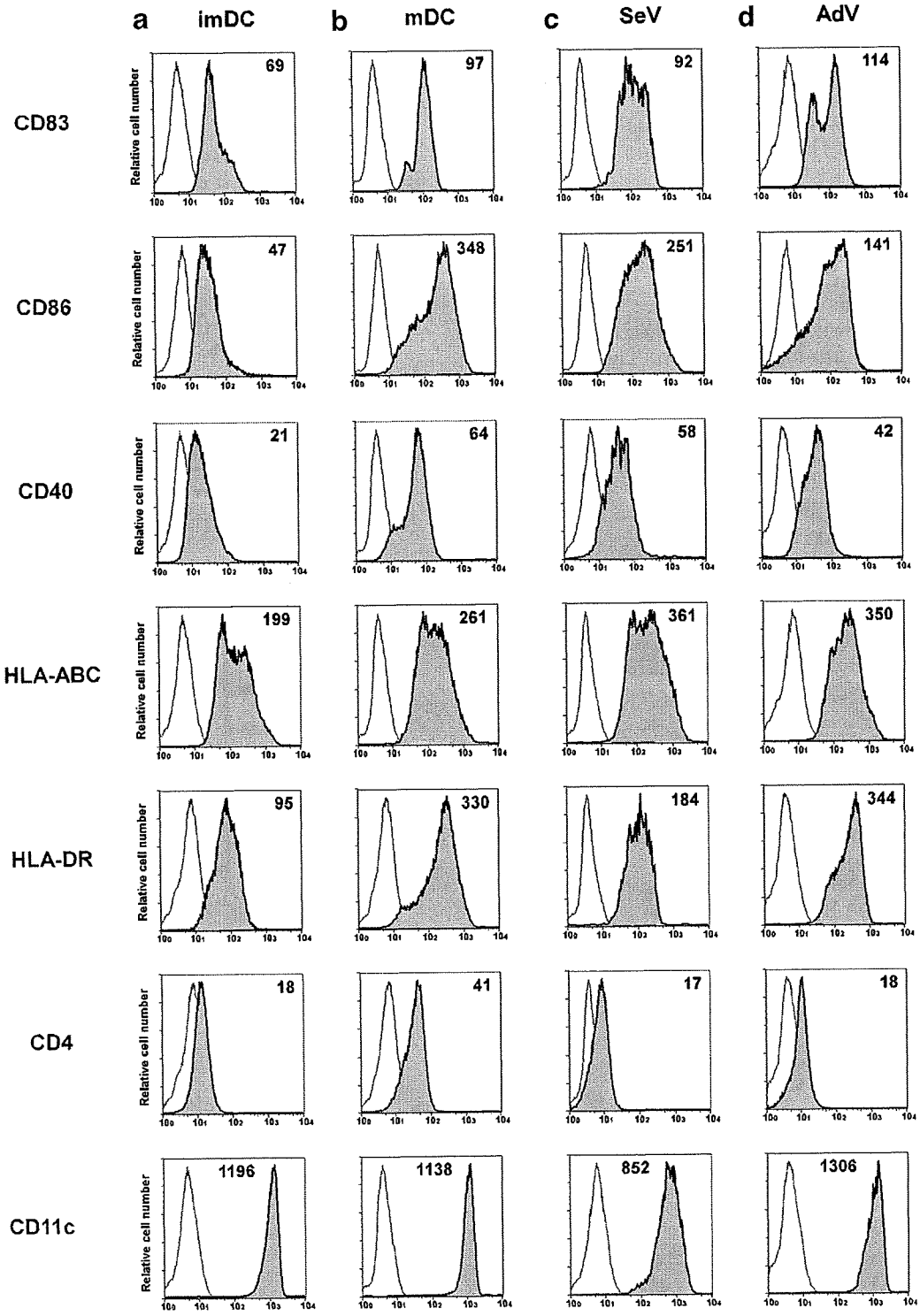

Fig. 3. Infection of DCs with SeV and AdV vectors modified the expression of cell surface markers. DCs infected with SeV vector containing no inserts at an MOI of 2 (**c**) and AdV vector containing no inserts at an MOI of 1,000 (**d**) were maintained in the DC medium for 48 hr (AdV vector) and 24 hr (SeV vector). DCs were incubated with (**b**) or without (**a**) TNF-α for 48 hr, respectively. These DCs were analyzed by flow cytometry with FITC, PE, PerCP, APC-conjugated antibodies for expression of CD86, CD83, HLA-ABC, HLA-DR, CD4, CD40, CD11c. The open profiles represent isotype-matched mAb controls. MFIs are indicated on the right corner in each panel. The background values of all experiments were less than 15. The numbers in each panel represent the mean value of three independent experiments.

60%) 24 hr after infection with SeVGFP or dF-SeVGFP. The proportion of GFP-positive cells decreased to around 30% at 48 hr, although the MFI of GFP showed the maximum at 48 hr. AdVGFP, on the other hand, showed the maximum level of both GFP-positive cells and MFI of GFP 48 hr after infection.

Sendai and Adenoviral Vectors Changed Phenotype of imDCs Following Viral Transduction

In order to determine the effect of transduction on imDCs with these vectors, we examined the surface markers of cells after transduction. The phenotype of imDCs and mDCs are shown in Figure 3a,b, respectively. We infected imDCs with SeV or AdV vectors and cultured them for 24 or 48 hr, respectively (Fig. 3c,d). As compared with the phenotype of uninfected imDCs, DCs infected by SeV and AdV vectors showed up-regulation of a maturation marker CD83, the major histocompatibility complex (MHC) classes I and II molecules (HLA-ABC and HLA-DR), and costimulatory molecules CD40 and CD86. Incubation of DCs in medium and buffers used to prepare vectors did not affect the phenotype of the cells (data not shown). These results indicate that SeV as well as AdV vector infection induced DC maturation in terms of cell surface phenotype.

Both SeV and AdV Vectors Elicited HIV-1 Specific T-Cell Responses

To evaluate protein expressions, we developed five viral vectors carrying HIV-1 structural proteins (Fig. 4a). We infected DCs with these vectors under the optimal conditions we concluded from the results shown above. Gp120 expression by SeV vector was 3.8 times higher than that by AdV vector (Fig. 4b, compare lanes 3–7). Since 3.2 and 5.0 kb are the maximum gene sizes for SeV and AdV vector, respectively [Sakai et al., 1999; the manufacturer's protocol of AdV Expression Kit], we inserted HIV-1 *gag* gene (about 1.5 kb) in SeV and *gag-pol* gene (about 4.9 kb) in AdV vector. Both *cis*-acting RRE sequence and *trans*-acting Rev protein were necessary for Gag protein expression by AdV vector (Fig. 4b, compare lanes 1–2). Rev expression is not required for SeV-mediated Gag or Env expression because SeV replicates in the cytoplasm. In the presence of Rev protein, AdV vector expressed similar levels of Gag protein to SeV vector (Fig. 4b, compare lanes 1–6). Although SeV Gag did not have HIV-1 protease sequence, a band was detected near the size of p24. It was not a nonspecific band derived from SeV because we could not detect the band with other SeV constructs, such as SeV Env (data not shown). Gag might be processed by some proteins of SeV.

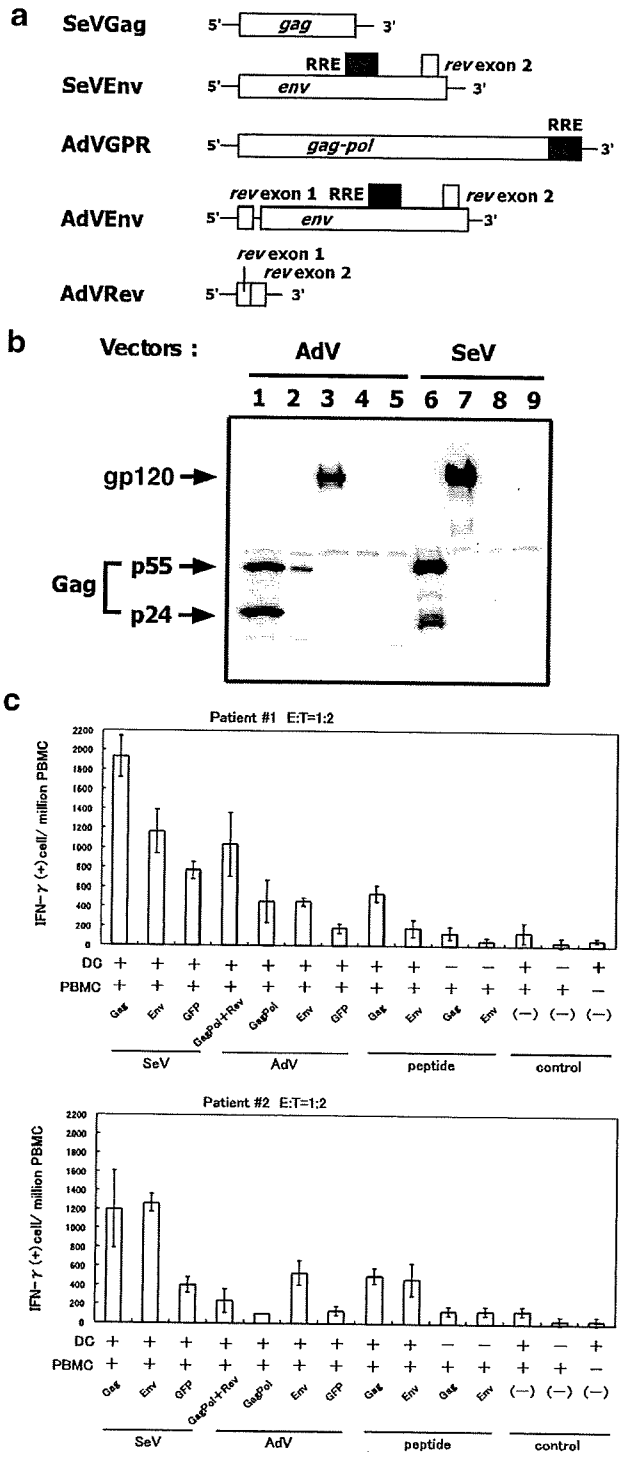

Fig. 4. **a**: Schematic structures of five viral vectors. *gag*, HIV-1 gag gene; *env*, HIV-1 env gene; *gag-pol*, HIV-1 gag and pol genes; *rev*, HIV-1 rev gene; RRE, HIV-1 RRE; GFP, green fluorescent protein. **b**: The expression of HIV-1 structural proteins by AdV and SeV vectors. DCs infected with AdV vector at an MOI of 1,000 and SeV vector at an MOI of 2 were harvested for 48 or 24 hr after infection, respectively. Thirty microgram of lysate was subjected to immunoblot analysis using anti-Gag p24 or anti-Env gp120 mAbs. The other two independent experiments showed similar results. Lane 1, coinfection with AdVGPR and AdVRev; Lane 2, AdVGPR without AdVRev infection; Lane 3, AdVEnv; Lane 4, AdV without inserts; Lane 5, mock; Lane 6, SeVGag; Lane 7, SeVEnv; Lane 8, SeV without inserts; Lane 9, mock. Arrows on the left indicate positions of gp120, Gag p55, and p24. **c**: The results of IFN-γ Elispot assays in two HIV-1 infected patients. Autologous DCs infected with SeV vector or AdV vector, or just added overlapping peptides (*gag*, *env*) were used as stimulators. PBMCs from the same patients were used as effectors cells. Results are shown as mean ± SEM of three independent assays.

After developing mDCs from frozen PBMCs of two HIV-1 infected patients, we infected these mDCs with SeV or AdV vector and used them as stimulators for interferon γ (IFN-γ) Elispot. Both SeV and AdV vectors elicited HIV-1 specific T-cell responses, although some nonspecific responses were also detected (Fig. 4c).

DISCUSSION

DCs are efficient antigen presenting cells that are critical for induction of primary T-cell responses. At present the most useful method for genetic manipulation of DCs is to use viral vectors. As reported previously, AdV vector is efficient at the transduction of DCs [Tan et al., 2005]. SeV is also one of the reliable vectors for immunotherapy and has several unique features, such as cytoplasmic localized replication cycle and brief contact time for cellular uptake. In this study, we analyzed the capacity of SeV as a vector in terms of transducing GFP and HIV-1 genes into human DCs. We showed that SeV vector transduced GFP genes efficiently into monocyte-derived imDCs. DCs infected with SeV and dF-SeV vectors expressed high amount of GFP gene 24 hr after infection at an MOI of 2 (Fig. 2a,b). The expression level of HIV-1 structural gene, *env*, by SeV vector was higher than that by AdV. These results proved the high ability of gene expression by SeV. However, the proportion of GFP positive cells did not increase according to MOI. About 30% of cells were still GFP-negative 48 hr after infection even at an MOI of 50 (Fig. 1a,b). This could be caused by the disruption of sialic acid which is the receptor for SeV.

Both SeV and dF-SeV vectors killed nearly 30% of target DCs at the lowest MOI: 0.5. One of the reasons for this phenomenon is likely to be apoptosis. Several studies reported that SeV is able to induce apoptosis in viral host cells [Tropea et al., 1995; Bitzer et al., 1999]. This cytopathic effect might enhance specific T-cell responses by cross-presentation of DCs. Presentation by DCs derived from virus-infected apoptotic and necrotic cells could activate T-cells efficiently [Arrode et al., 2000; Herr et al., 2000; Larsson et al., 2001; Tabi et al., 2001]. In order to apply SeV in a clinical setting, further studies about cytopathic effect by SeV vector will be required.

AdV vector is known to require high MOI to achieve high transduction rates [Diao et al., 1999]. Our study also demonstrated that much higher MOI was needed in AdV than SeV to transduce DCs. One of the reasons for this phenomenon could be insufficient expression of coxsackievirus and AdV receptor (CAR) [Stockwin et al., 2002] on DCs. CAR is the primary receptor for AdV type 5, and the AdV used in this study was derived from AdV type 5. However, MFI of GFP in AdV vector increased according to MOI. AdV might be able to use other receptors to infect DCs. Several studies have shown that AdV can infect cells through integrins or MHC molecules [Huang et al., 1996; Hong et al., 1997]. Recently, AdV vector containing Ad5/35 chimeric fiber protein was reported as a useful vector for the cells lacking in sufficient CAR expression [Mizuguchi and Hayakawa, 2002]. This chimeric vector would be useful for DCs because the receptor of Ad5/35 vector is CD46, which is expressed on DCs.

When imDCs capture antigens, they maturate while migrating to T-cell areas in the lymph nodes [Banchereau and Steinman, 1998]. DC maturation is critical for strong T-cell binding and stimulation [Lipscomb and Masten, 2002]. Our results showed that SeV vector infection induced DC maturation of human monocyte-derived DCs as well as AdV vector infection. However, the expression levels of CD86 and CD40 were lower as compared to those of mDCs. CD40 expression leads to increased DC survival and stimulates cytokine production [Caux et al., 1994; Wong et al., 1997]. CD86, a ligand for CD28 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), stimulates T-cell proliferation and generation of CTL [Lanier et al., 1995]. In order to achieve further up-regulation of those molecules, the addition of a maturation factor, such as TNF-α, should be considered.

DCs infected with SeV as well as AdV elicited HIV-1 specific T-cell responses detected by IFN-γ Elispot (Fig. 4c). Elispot by SeV GFP showed about 800 SFC/million PBMC which was obtained from patient #1. One possibility of this nonspecific response is antigenic cross-reactivity. SeV belongs to the genus *Respirovirus* of the *Paramyxoviridae* family. *Respirovirus* includes human parainfluenza virus type 1 (hPIV-1) and 3 (hPIV-3). hPIV-1 is the most common cause of pediatric laryngotracheobronchitis (croup), which means many people are infected by hPIV-1 in early life. Previous studies showed SeV and hPIV-1 shared sequence homology and antigenic cross-reactivity [Gorman et al., 1990; Lyn et al., 1991; Smith et al., 1994]. The high nonspecific response by SeV GFP could be caused by cross-reactive immunity induced by previous exposure to human hPIV-1.

In conclusion, our results showed that SeV vector had high ability of gene transduction. SeV vector induced the maturation of DCs in terms of their phenotype and stimulated HIV-1 specific T-cell responses, which is beneficial in vaccination. Though further studies will be required to improve vector design, SeV vector has a potential to be used for immuno-genetherapy.

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Effects of CCR2 and CCR5 Polymorphisms on HIV-1 Infection in Thai Females

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Summary: Polymorphisms in *CCR2* and *CCR5* genes reportedly affect HIV-1 transmission and disease progression in HIV-1–infected individuals. In the study presented here, we examined the effects of *CCR2* and *CCR5* polymorphisms on HIV-1 transmission in 74 Thai females who were exposed to HIV but seronegative (ESN) and in 347 HIV-seropositive females. We found that the combination of 2 non-synonymous substitutions, *CCR2 V64I* and *CCR5 G316A*, tended to occur more frequently in ESN females (2 of 74) than in HIV-1–infected females (1 of 347) (P = 0.08). This suggested that non-synonymous substitution in the *CCR5* gene also affects HIV-1 transmission in an Asian population in which the *CCR5-Δ32* is very rare.

Key Words: CCR2, CCR5, SNPs, HIV-1 infection, Thai

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A considerable number of individuals, such as HIV-seronegative commercial sex workers[1] and HIV-seronegative spouses of HIV-seropositive individuals,[2] have been repeatedly sexually exposed to HIV yet remain negative for anti-HIV antibody. Such individuals have been classified as HIV-exposed but seronegative (ESN). Several studies in Thailand demonstrated that some individuals with HIV-seropositive spouses can remain HIV-seronegative; a recent study of ours (not yet published) showed that the majority of these cases could not be explained by their sexual behavior, such as frequency of unprotected sexual contacts, or by the viral load of their infected spouses.

The association of a number of polymorphisms in host genes that are involved in HIV replication and/or immune regulation with HIV infection and disease progression has been documented.[3] We previously reported that a single nucleotide polymorphism (SNP) in the *DC-SIGNR* gene was more prevalent in Thai female ESNs than in HIV-1–infected individuals.[4] In white individuals, the association of a 32-bp deletion of the C-C chemokine receptor 5 (*CCR5-Δ32*) with HIV infection has been demonstrated.[5,6] *CCR5-Δ32* homozygotes are highly resistant to HIV-1 infection, whereas *CCR5-Δ32* heterozygotes delay progression to AIDS by 2 to 3 years.[7,8] However, the *CCR5Δ32* allele is rarely observed in Asians,[5,8–10] including Thais.[11] Moreover, an additional variant in the upstream regulatory region of *CCR5* (*CCR5 P1*) is associated with more rapid progression to AIDS,[12] and a valine-to-isoleucine substitution in the coding region of another C-C chemokine receptor, CCR2 (*CCR2 V64I*), delays HIV disease progression.[13–15]

In the study presented here, we examined polymorphisms in the *CCR2* and *CCR5* genes of Thai ESNs and HIV-1–infected females and found that a combination of *CCR2 V64I* and one of the *CCR5* coding region polymorphisms tended to be more prevalent in Thai female ESNs than in HIV-1–infected individuals.

METHODS

Clinical Specimens

We used samples obtained from previously described 74 ESNs[4] and 246 HIV-1–infected Thai females[16] who visited the HIV clinic in the Day Care Center of Lampang Hospital in north Thailand between July 6, 2000, and July 12, 2001. We also used samples from an additional 101 HIV-1–infected Thai females who visited the clinic after July 12, 2001. Among 74 HIV-seronegative female spouses, the median (interquartile range [IQR]) duration of marriage before they became aware of the HIV status of their husbands was estimated to be 5 (3, 8) years. Median (IQR) frequency of sexual contact before disclosure of their husbands' HIV status was 5 (3, 8) times per month. Sixty-four females (87.7%) reported that they had never used a condom during those sexual contacts.[4] In October 2003, we examined blood samples of the 74 ESNs again to determine whether they were still HIV seronegative. This study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Thailand. After their written informed consent had been

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obtained, all participants were interviewed by trained study coordinators and clinically examined by 2 designated doctors.

Genotyping

Genomic DNA was extracted and purified from the buffy coat of the samples with the QIAamp mini blood kit (QIAGEN, Hilden, Germany). Each specimen was then analyzed for *CCR2 V64I* and *CCR5 A-2852G* by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP), as described previously.[17] The G allele at position -2852 of the *CCR5* gene in Thais represents the *CCR5* promoter haplotype (*CCR5 P1*), which has been reported to be associated with rapid progression to AIDS in HIV-1–infected individuals.[17] The entire coding region of *CCR5* was amplified and sequenced with a previously described method.[17]

Expression Levels of CCR5

African green monkey kidney CV1 cells were infected with either Sendai virus (SeV) expressing CCR2A 64I or a parental Z strain of SeV. Nine hours after SeV infection, the cells were superinfected with a recombinant vaccinia virus expressing either the wild type CCR5 or CCR5 316A. After incubation for 5 hrs at 37°C, the cells were stained with T227, a rat monoclonal antibody against the N-terminal extracellular portion of CCR5, followed by staining with fluorescein-5-isothiocyanate (FITC)-conjugated goat antibody directed against rat IgG (Cappel, Aurora, OH) and analyzed by FACScalibur (Becton Dickinson, San Jose, CA). For intracellular staining of CCR5, the cells were permeabilized with 0.05% saponin and 0.2% bovine serum albumin in phosphate buffer saline (PBS) before staining.

RESULTS

Sequence analysis of the entire coding region of *CCR5* genes from the 74 ESNs and 140 of the HIV-1–infected females showed that there were only 2 types of polymorphisms of the *CCR5* coding region. A G-to-A substitution at position 316 was found to convert an uncharged glycine residue into a basic arginine residue at position 106 in the third transmembrane domain of CCR5. This SNP, previously detected in Vietnamese and Chinese subjects, was found to affect surface trafficking of the CCR5.[18,19] A G-to-A substitution at position 668 converts a basic arginine into a glutamine at position 223 in the third cytoplasmic region. This SNP was previously identified in almost all ethnic groups examined.[20] We then determined the nucleotide sequences from positions 280 to 880 of the *CCR5* coding region in the remaining 207 HIV-1–infected females for genotyping their *CCR5 G316A* and *CCR5 G668A*.

Table 1 shows the genotype frequency of *CCR2 V64I*, *CCR5 A–2852G*, *CCR5 G316A*, and *CCR5 G668A* alleles in the 74 ESNs and 347 HIV-1–infected females. There was no significant difference between the 2 groups in either genotype distribution or allele frequency of the 4 alleles, although *CCR5* coding region polymorphisms tended to be more prevalent in ESNs than in HIV-1–infected individuals.

Table 2 shows the relationship among the 4 *CCR2-CCR5* polymorphisms. Haplotype construction by means of Arlequin version 2.01 (Genetics and Biometry Laboratory, Geneva, Switzerland) clearly demonstrated that *CCR2 V64I* alleles never occurred together with *CCR5 G–2852A*. This result confirmed our previous observation in Japanese and Thai subjects.[17] We also confirmed that *CCR5 G668A* alleles were in strong linkage disequilibrium with *CCR2 V64I* (D′ = 1).[17] In contrast, *CCR5 G316A* was always associated with *CCR5 A–2852G* but never with *CCR2 V64I*.

The presence of 2 alternatively spliced CCR2 isoforms, CCR2A and CCR2B, has been reported in a freshly isolated human monocyte, THP-1, and in MonoMac 6 leukemia cell lines.[21,22] We previously reported that the CCR2A isoform with V64I substitution could impair cell surface trafficking of CCR5 more strongly than could wild type CCR2A.[23] As mentioned above, *CCR5 G316A* was also found to reduce levels of cell surface CCR5 expression. We therefore further examined the individuals carrying both *CCR2 V64I* and *CCR5 G316A* (Table 1) and found that 2 of the 74 ESNs carried both alleles, whereas only 1 of the 347 HIV-1–infected individuals carried them. There was thus a clear trend for this combination to occur more frequently in ESNs than in HIV-1–infected individuals, although this trend did not reach statistical

TABLE 1. Frequency of *CCR2 V64I*, *CCR5 A-2852G*, *CCR5 G316A*, and *CCR5 G668A* Alleles in 74 Female ESNs and 347 HIV-1–Infected Females at Lampang Hospital

Genotype	ESN	HIV-1 Infected	*P*
CCR2 64I			
Homozygotes	4	13	
Heterozygotes	21	95	
Wild type	49	239	
Allele frequency	0.196	0.174	0.533*
CCR5 G-2852A			
Homozygotes	3	14	
Heterozygotes	25	97	
Wild type	46	236	
Allele frequency	0.209	0.180	0.404*
CCR5 G316A			
Homozygotes	0	0	
Heterozygotes	3	7	
Wild type	71	340	
Allele frequency	0.020	0.010	0.392†
CCR5 G668A			
Homozygotes	0	0	
Heterozygotes	5	12	
Wild type	69	335	
Allele frequency	0.034	0.017	0.199†
CCR2 V64I and *CCR5 G316A*	2	1	
Others	72	346	
Frequency	0.027	0.003	0.081†
Homozygous *CCR5 G-2852A* and *CCR5 G316A*	0	3	
Others	74	344	
Frequency	0	0.009	1†
Homozygous *CCR2 V64I* and *CCR5 G668A*	1	2	
Others	73	345	
Frequency	0.014	0.006	0.441†

*χ^2 test.
†Fisher exact test.

TABLE 2. Linkage Disequilibrium Among *CCR2 V64I, CCR5 A-2852G, CCR5 G316A,* and *CCR5 G668A*

	CCR5 A-2852G			CCR5 G316A			CCR5 G688A		
	Homozygotes	Heterozygotes	Wild Type	Homozygotes	Heterozygotes	Wild Type	Homozygotes	Heterozygotes	Wild Type
CCR2 V64I									
Homozygotes	0	0	17	0	0	17	0	3	14
Heterozygotes	0	22	94	0	3	113	0	14	102
Wild type	17	100	171	0	7	281	0	0	288
CCR5 A-2852G									
Homozygotes	—	—	—	0	3	14	0	0	17
Heterozygotes	—	—	—	0	7	115	0	1	121
Wild type	—	—	—	0	0	282	0	16	266

significance ($P = 0.081$, Fisher exact test). The 2 ESNs were able to maintain their HIV-seronegative status for at least 27 more months after the first blood samples were obtained. In contrast, the combination of disease-accelerating *CCR5 A-2852G* and *CCR5 G316A* (homozygous *CCR5 A-2852G* with *CCR5 G316A*) was observed only in HIV-1–infected individuals and not in ESNs (Table 1). These findings suggest that the combination of *CCR2* and *CCR5* polymorphisms in 2 chromosomes affects heterosexual transmission in Thai females. No such trend was observed with respect to *CCR5 G668A* alleles ($P = 0.441$). It should be noted that neither of the ESNs with *CCR2 V64I* and *CCR5 G316A* carried an A allele at SNP VS2277998 of *DC-SIGNR*, which was more frequently found in the ESNs than in the HIV-1–infected Thai females,[4] indicating that the association of the *CCR2-CCR5* genotype with HIV-1–seronegative status is independent from that of the previously published *DC-SIGNR* genotypes.

Next, we examined whether cell surface expression of the CCR5 316A protein was also impaired by coexpression with the CCR2A 64I protein. CV1 cells were infected with either SeV expressing CCR2A 64I or with a parental Z strain of SeV. Nine hours after SeV infection, the cells were superinfected with a recombinant vaccinia virus expressing either the wild type CCR5 or CCR5 316A. As shown in Figure 1,

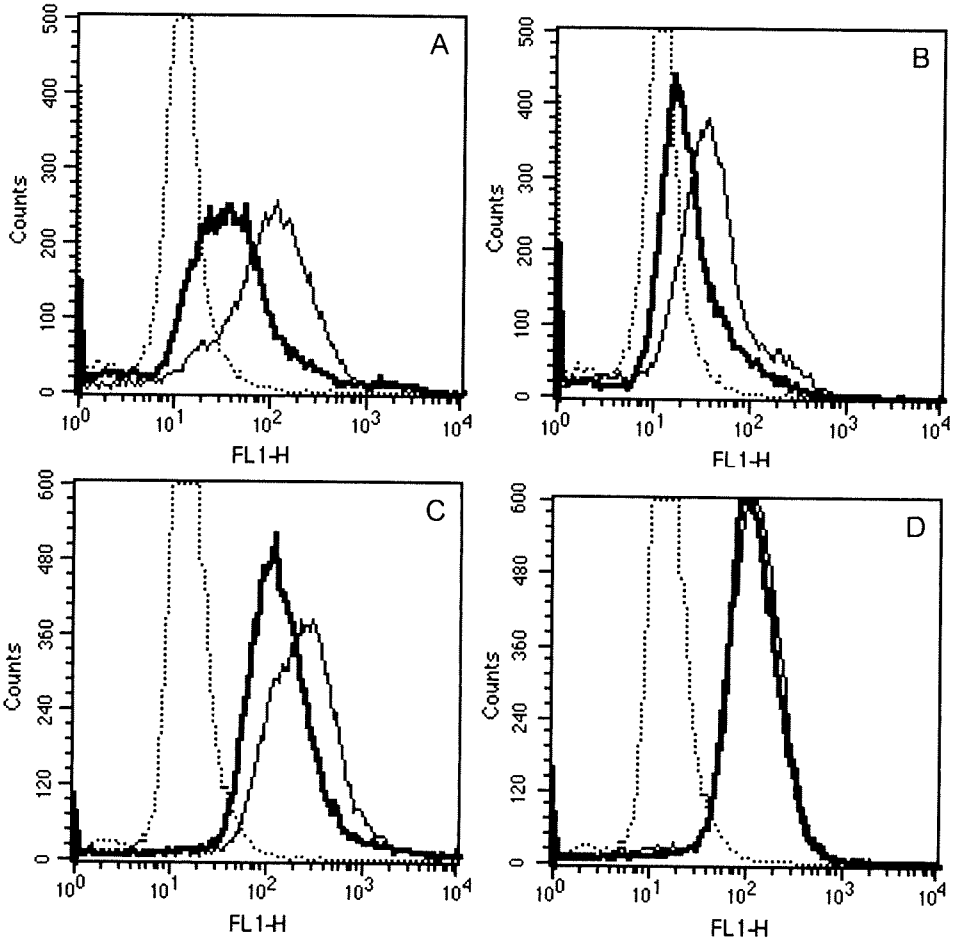

FIGURE 1. Cell surface and intracellular expression of CCR5. African green monkey kidney CV1 cells were infected with the Sendai virus (SeV) expressing CCR2 64I (bold lines) or a parental Z strain of SeV (solid lines). Nine hours after SeV infection, cells were superinfected with a recombinant vaccinia virus expressing either the wild type CCR5 (A and C) or CCR5 316A (B and D). After incubation for 5 hours at 37°C, cells were permeabilized (C and D) or not (A and B) with saponin. The cells were then stained with T227, a rat monoclonal antibody against the N-terminal extracellular portion of CCR5, followed by staining with FITC-conjugated goat antibody against rat IgG and analyzing with a flow cytometer. Dotted lines denote cells stained with the second antibody as negative control. Representative results of 6 independent experiments with similar results are shown.

CCR2A 64I greatly impaired surface expression of the wild type CCR5 (Fig. 1A) and that of CCR5 316A (Fig. 1B). Because the level of cell surface expression of CCR5 316A was very low, coexpression of CCR2A 64I resulted in nearly complete elimination of cell surface CCR5 expression (Fig. 1B). When the cells were permeabilized, however, in cells expressing both CCR5 316A and CCR2A 64I we observed nearly equal levels of CCR5 to those in cells expressing the wild type CCR5 alone (Fig. 1C) or CCR5 316A alone (Fig. 1D). These findings confirmed that the very low levels of surface expression of CCR5 316A in the presence of CCR2A 64I are the result of severe impairment of its cell surface trafficking.

DISCUSSION

Previously, 2 alleles of *CCR5 G316A* were detected in 87 HIV-1–negative Vietnamese, but not in 45 HIV-1–positive Vietnamese, suggesting that this allele could be protective against HIV-1 transmission.[18] In the study of larger numbers of Thais reported here, we detected substantial numbers of this allele in HIV-1–infected Thai females. However, the combination of this allele with the other protective allele, *CCR2 V64I*, tended to occur more frequently in ESNs than in HIV-1–infected individuals. Surface expression of CCR5 316A was almost completely eliminated by coexpression with CCR2A 64I. These findings suggest that *CCR5 G316A* has a protective effect on HIV-1 transmission only in the presence of the other protective allele, *CCR2 V64I*. The lack of protection by *CCR5 G316A* without *CCR2 V64I* against HIV-1 transmission despite its poor surface trafficking may be due to its complete linkage disequilibrium with *CCR5 A-2852G*, which represents the disease-accelerating *CCR5 P1*. However, the combination of *CCR2 V64I* and *CCR5 G316A* was rare even in ESNs, suggesting that factors other than *CCR2* and *CCR5* polymorphisms may contribute to their seronegative status.

Reduced cell surface expression of CCR5 was previously reported on primary CD4 T lymphocytes obtained from heterozygous individuals for *CCR5Δ32*[24] or *CCR5 893(-)*.[25] Although we have clearly shown that surface expression of CCR5 316A was almost completely eliminated by coexpression with CCR2A 64I in our experimental system, it is not clear whether primary cells from individuals with *CCR5 G316A* and *CCR2 V64I* indeed show reduced surface levels of CCR5. It is thus necessary to examine surface expression levels of CCR5 of various cells obtained from those individuals.

We previously reported that all the *CCR2 V64I* alleles and nearly all the *CCR5* coding region polymorphisms in Japanese, and all the *CCR5-Δ32* alleles in the French ALT, IMMUNOCO, and SEROCO cohorts occurred in the CCR5 promoter haplotypes with higher promoter activity.[17] We therefore hypothesized the existence of a certain selective pressure favoring low levels of CCR5 expression during human evolution. Because the disease-accelerating *CCR5 P1* also carries the CCR5 promoter with higher promoter activity, our finding reported here further supports our hypothesis, as both the *CCR5 G316A* and *CCR5 G668A* alleles identified in Thai subjects were also associated with the CCR5 haplotypes with higher promoter activity

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