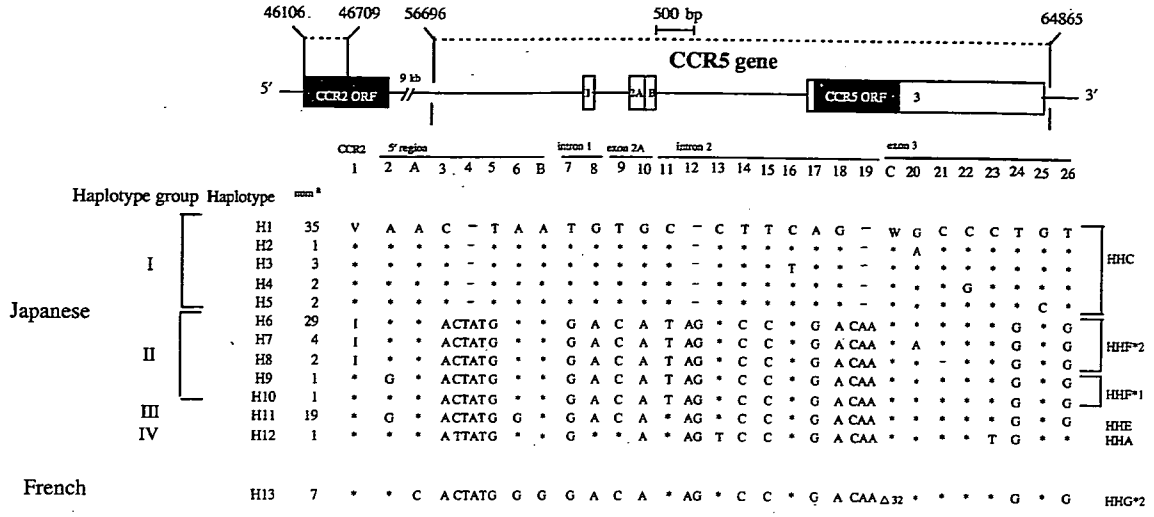

Figure 2. Map of *CCR2, CCR5* and nucleotide polymorphisms. White and black boxes indicate non-coding exons and open reading frames (ORF), respectively. Dotted lines signify the sequenced regions. PCR and sequence screening of eight segments of the *CCR2* and *CCR5* gene identified 29 nucleotide positions with genetic polymorphisms. Twenty-six polymorphic sites observed in Japanese are numbered consecutively across the top. A, B, and C denote polymorphisms specifically observed in the French samples. Combining the 29 polymorphic positions indicated 13 *CCR2-CCR5* haplotype alleles designated H1 through H13, with H1 being exactly the same as the GenBank U95626 sequence. Asterisks indicate nucleotides identical to those of H1; dashes represent deletion sites. W shows *CCR5* coding sequences without 32 bp deletion, while Δ32 indicates 32 bp deletion of the *CCR5* coding region. V and I in position 1 indicate *CCR2 64V* and *CCR2 64I*, respectively. [a]Number in this column represents the actual numbers of haplotypes identified in the 100 sequenced chromosomes. The human haplogroups according to Mummidi *et al.* (2000) are shown on the right.

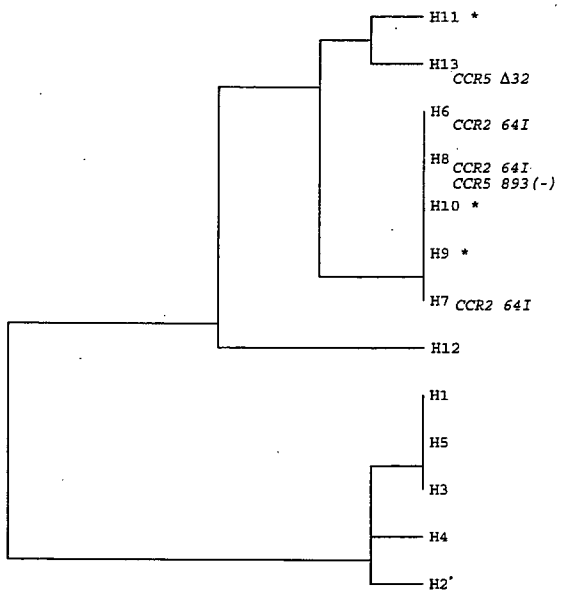

Figure 3. A phylogenetic tree of 13 *CCR2-CCR5* haplotypes defined in Fig. 2. The tree was produced with the UPGMA (unweighted pair-group method with arithmetic averages) method. Haplotypes with *CCR5 Δ32, CCR2 64I, CCR5 893(-)* are shown. Asterisks denote haplotypes with *CCR5 P1 lacking CCR5 Δ32 and CCR2 64I.*

that the *HHC* (haplotype group I) promoter construct demonstrated lower promoter activity than that of *HHF* (haplotype group II) and of *HHE* (haplotype group III). Our study further identified *–2135C* and *–2086A*, which are linked to *–2459A*, as mutations responsible for elevated promoter activity, since Pd-III was shown to express higher luciferase activity than Pd-I (Fig. 4).

To examine the effects of *CCR5 –1835T* on expression level, we generated two series of constructs with part of the intron containing position –1835 and the splicing acceptor site located immediately upstream of *CCR5* exon 4 (LSA and PdSA in Fig. 4). Again, promoters from haplotype groups II and III expressed higher luciferase activity than the promoter from haplotype group I in both the LSA and the PdSA constructs, and since there were no other differences between haplotype groups II and III, this suggests that *CCR5 –1835T* has no effect on CCR5 expression.

Novel method using PCR-restriction fragment length polymorphism for detecting HIV-1 disease-accelerating haplotype

As described above, in Japanese subjects, *–2852G* was associated with 90% of the disease-accelerating haplotype, this is, *CCR5 P1 lacking CCR5Δ32* and *CCR2 64I*, since no *CCR5Δ32* was identified in these individuals. The remaining 10% were accounted for by individuals with *CCR5 –1835T* without *CCR2 64I*. To confirm the strong linkage disequilibrium between *–2852G* and the disease-accelerating allele observed in 50 Japanese, we established a PCR-RFLP method to detect *–2852G* (see Materials and Methods) and used it to genotype 80 additional (38 HIV-1-infected and 42 non-HIV-1-infected) Japanese. *CCR5 C-2135T, G-2086A*, and *C-1835T* were genotyped by direct-sequencing of PCR product R5-4

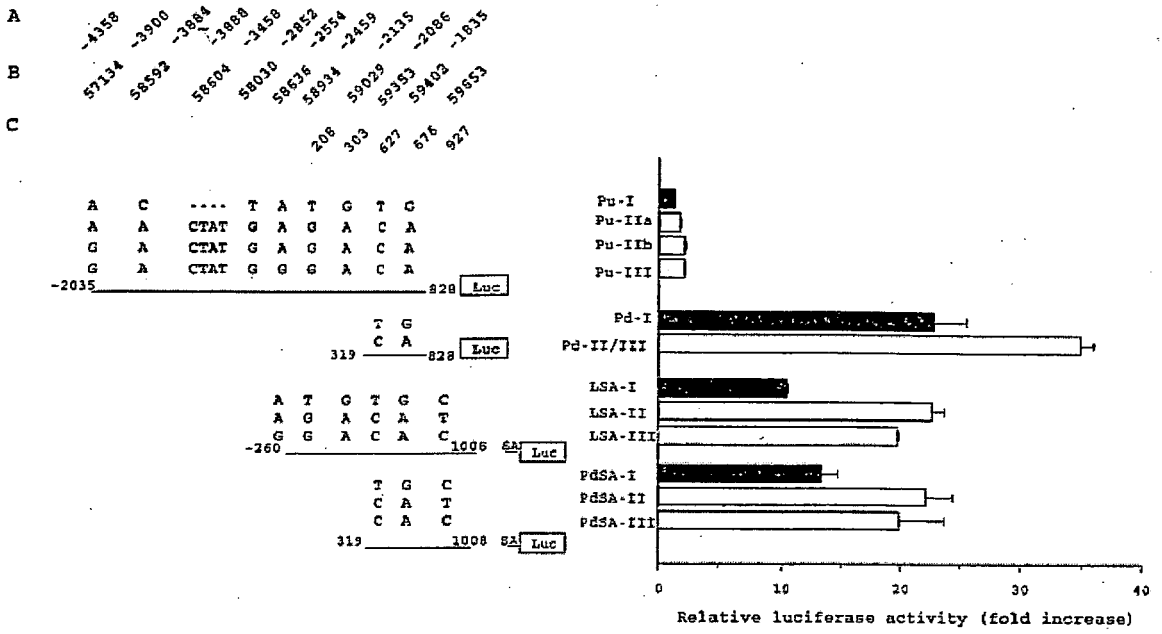

Figure 4. Effect of sequence polymorphism in CCR5 regulatory region on promoter activity. The promoter regions inserted into the pGL3-basic vector are shown by solid lines with the first and last nucleotides enumerated according to numbering system C. SA indicates 0.25 kb of the 5'-non-coding region containing a splice acceptor immediately upstream of the CCR5 open reading frame. Nucleotides at the polymorphic positions are marked. Boxes represent firefly luciferase open reading frames. The relative luciferase activity of each construct is represented by a solid bar (haplotype group I), a grey bar (haplotype group II), and an open bar (haplotype group III). Data shown are representative of five independent experiments with similar results. Error bars indicate fluctuations between measurements of relative luciferase activity in two independent clones of a construct.

Table 4. Linkage disequilibrium between *CCR5 -1835T* and *CCR2 64I* in Japanese and Thai subjects

		CCR2 V64I		
Subjects	*CCR5 C-1835T*	VV	VI	II
Japanese	CC	58	0	0
	CT	2	54	0
	TT	0	1	15
Thais	CC	123	0	0
	CT	0	61	0
	TT	0	0	2

(Table 2), and *CCR2 V64I* was genotyped by PCR-RLFP according to the method described by Smith *et al.* (1997). Frequencies of *CCR2 64I* and *CCR5 -1835T* of 130 Japanese subjects were 33% and 34%, respectively, and were higher than those observed in Caucasians and African-Americans (Table 3). As shown in Table 4 and Table 5, we confirmed the strong linkage disequilibrium between *CCR2 64I* and *CCR5 -1835T* (D′ = 1). We also found the strong linkage disequilibrium between *CCR5 -2852G* and the disease-accelerating haplotype (D′ = 1) since *-2852G* was found in 55 out of 58 (95%) of the disease-accelerating haplotype in 130 Japanese subjects.

In Thai subjects, we previously observed that *CCR2 64I* alleles were 100% associated with *CCR5 -1835T* (unpublished results). We therefore examined whether *-2852G* was associated with the disease-accelerating haplotype in 186 Thai subjects (97 non-HIV-1-infected and 89 HIV-1-infected individuals). Frequencies of *CCR2 64I*, *CCR5 -2852G*, *-2135T*, *-2086 A*, and *-1835T* of 186 Thai subjects were shown in Table 3. As expected, *CCR2 64I* alleles were associated with *CCR5 -1835T* without any exceptions (Table 4). Furthermore, *CCR5 -2852G* was also 100% associated with the disease-accelerating haplotype, *CCR5 P1* lacking *CCR2 64I* and *CCR5Δ32* (Table 5). These results indicated that only one allele (*-2852G*) could be used as a representative of the disease-accelerating haplotype, *CCR5 P1* lacking *CCR2 64I* and *CCR5Δ32* in the Thai population.

Discussion

Polymorphisms in human *CCR5* genes were initially studied for their effects on susceptibility to HIV-1 infection and rate of disease progression to AIDS in HIV-1-infected individuals. Subsequently, these polymorphisms were evaluated for their roles in other human phenotypes such as those accounting for differences in renal transplantation outcomes (Fischereder *et al.*, 2001), myocardial infarction (Gonzalez *et al.*, 2001; Valdes *et al.*, 2002), and autoimmune diseases (Garred *et al.*, 1998; Gomez-Reino *et al.*, 1999; Spagnolo *et al.*, 2005). Our systematic survey of natural sequence variations in an 8.1-kb region of the

Table 5. Linkage disequilibrium between *CCR5 –2852G* and *CCR5* promoter haplotypes in Japanese and Thai subjects

		CCR2 V64I, CCR5 T-2135C, CCR5 G-2086 A, CCR5 C-1835T genotyping					
Subjects	*CCR5 A-2852G*	*V-P1/V-P1*	*V-P1/I-P1*	*V-P1*/Others	*I-P1/I-P1*	*I-P1*/Others	Others/Others
Japanese	AA	0	1	2	15	37	29
	AG	0	17	20	0	0	0
	GG	9	0	0	0	0	0
Thais	AA	0	0	0	2	46	78
	AG	0	15	39	0	0	0
	GG	6	0	0	0	0	0

V-P1: haplotype group III, *CCR5 P1* haplotype (*–2135C, –2086 A*, and *–1835C*) lacking *CCR2 64I*.
I-P1: haplotype group II, *CCR5 P1* haplotype (*–2135C, –2086 A*, and *–1835C*) with *CCR2 64I*.
Others: haplotype group I (*–2135T, –2086G*, and *–1835C*) and haplotype group IV (*–2135T, –2086 A*, and *–1835C*).

entire *CCR5* gene as well as *CCR2V64I* in 50 Japanese individuals revealed several Asian-specific characteristics of the *CCR2-CCR5* haplotype structure that were not seen in those of Caucasians and African-Americans. In particular, we were able to show that the G allele at position –2852 from the *CCR5* open reading frame may represent the *CCR5* promoter haplotype with a higher promoter activity (*CCR5 P1*) without *CCR2 64I* in a *CCR5Δ32*-negative Thai population. For other ethnic groups, the method for *CCR5* promoter haplotype determination is complicated since single-nucleotide polymorphisms (SNPs) at several different positions need to be determined (Martin *et al.*, 1998; McDermott *et al.*, 1998; Mummidi *et al.*, 2000). It would therefore be useful to be able to analyse only one SNP especially in resource-constrained setting.

Our results for *CCR5* promoter activity also confirmed the previously reported observation that polymorphisms of *CCR5* affect its promoter activity (McDermott *et al.*, 1998; Mummidi *et al.*, 2000). Other studies have shown that the SNP at position –2459 affects the binding of certain factor(s) to the *CCR5* promoter region (Bream *et al.*, 1999; Mummidi *et al.*, 2000). However, our study demonstrated that constructs lacking position –2459 still showed differences in promoter activity and that *–2135C* and *–2086 A* were responsible for elevated promoter activity. Further studies will be needed, however, to identify and clarify the exact molecular mechanisms underlying the effects of those SNPs on *CCR5* promoter activity.

On the other hand, we could not detect any effects on luciferase expression level by *CCR5 –1835T*, which is in strong linkage disequilibrium with disease-delaying *CCR2 64I*. Recently, we reported that *CCR2 64I* substitution increases the stability of CCR2A, which is a splicing variant of CCR2B (Nakayama *et al.*, 2004). CCR2A was shown to bind CCR5 while most of CCR2A molecules are retained in cytoplasm. Therefore, cell surface expression levels of CCR5 co-expressed with CCR2A (64I) were lower than those of CCR5 co-expressed with CCR2A (64 V) (Nakayama *et al.*, 2004). Our results reported here also supported the notion that *CCR2 64I* but not *CCR5 –1835T* is directly responsible for the effects of those SNPs on HIV-1 disease progression (Mummidi *et al.*, 1998).

Phylogenetic analysis of human *CCR5* haplotypes clearly demonstrated that all the protective alleles, *CCR2 64I* (*CCR5 –1835T*), *CCR5Δ32* and *CCR5 893(–)*, belong to haplotype groups with higher promoter activity. These results suggest that a certain selective pressure existed that favoured low levels of CCR5 expression during human evolution. CCR5 has been shown to be involved in several inflammatory diseases (Garred *et al.*, 1998; Gomez-Reino *et al.*, 1999), and *CCR5Δ32* to be associated with a reduction in the risks for patients with multiple sclerosis (Barcellos *et al.*, 2000; Kantor *et al.*, 2003) and asthma (Hall *et al.*, 1999). It is therefore possible that impaired function or low levels of CCR5 expression may be beneficial for the survival of individuals with autoimmune or inflammatory diseases.

It is noteworthy that four of the five *CCR5 G668A* (*R223Q*) alleles were found in the *H7* haplotype, which also contains *CCR2 64I* (Fig. 2). Previously, *CCR5 668 A* was reported to be associated with slower disease progression in HIV-1-infected individuals (Zheng *et al.*, 2002). However, since *CCR5 668 A* is associated with another disease-delaying allele, *CCR2 64I*, an epidemiological study is needed to compare individuals with *CCR5 668 A* with those who carry *CCR2 64I* without *CCR5 668 A* in order to evaluate the effects of *CCR5 668 A* on HIV-1 disease progression.

Our study also suggested that not only the frequency but also the degree of linkage disequilibrium of SNPs varies between Japanese and Thai subjects, both Asian. Among the Japanese, 58 of 260 chromosomes were found to carry CCR5 –1835T, and 55 of these 58 chromosomes also carried *CCR2 64I*. No haplotype carrying *CCR2 64I – CCR5 –1835C* was found among the 260 Japanese chromosomes. Studies of *CCR5* and *CCR2* polymorphisms of Caucasian individuals revealed that both *CCR2 64I – CCR5 –1835C* and *CCR2 64 V – CCR5 –1835T* did exist in Caucasians (Mummidi *et al.*, 1998). On the other hand, *CCR2 64I* was completely associated with *CCR5 –1835T* in 372 Thai chromosomes. It is likely that the *CCR2 64I* mutation only occurred on a *CCR5 –1835T* bearing chromosome and that the *CCR2 64I – CCR5 –1835C* and *CCR2 64 V – CCR5 –1835T* haplotypes were generated as the results of a recombination event between

the *CCR2 64I-CCR5 –1835T* and the *CCR2 64 V – CCR5 –1835C* haplotypes. In Thais, however, that kind of recombination never occurred or only at a very low frequency. At the very least, our results presented here point to the need for detailed linkage studies of genetic polymorphisms, such as the HapMap project, in all ethnic groups, since degree of linkage disequilibrium can vary even among Asian groups.

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Letter to the Editor

Early-Onset Pulmonary Complication Showing Organizing Pneumonia Pattern following Cord Blood Transplantation in Adults

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Bronchiolitis obliterans organizing pneumonia (BOOP) is a well-known pulmonary complication after hematopoietic stem cell transplantation (SCT) [1-4]. BOOP generally occurs approximately 100 days or later after SCT. We describe 4 patients who developed a pulmonary disorder with a histologic pattern of OP in the early period after cord blood transplantation (CBT).

Patient 1 was a 32-year-old man with acute myelogenous leukemia. In October 2003, he received 2 antigen–mismatched CB grafts that contained 2.11×10^7/kg total nucleated cells (TNCs) before freezing. The conditioning regimen included 12 Gy total body irradiation (TBI), 120 mg/kg cyclophosphamide, and 12 g/m^2 cytarabine, along with granulocyte colony-stimulating factor [5]. Graft-versus-host disease (GVHD) prophylaxis consisted of cyclosporine and methotrexate. A neutrophil count consistently greater than 500/μL (neutrophil engraftment) was achieved on day +21. Grade II acute GVHD involving the skin occurred from day +23. On day +32, the patient presented with cough, dyspnea, and fever. An arterial blood gas analysis showed a PO_2 of 57.3 mm Hg and a PCO_2 of 37.2 mm Hg. Chest computed tomography (CT) scans showed a diffuse ground-glass opacity in the lungs (Figure 1A). No causative infectious agents were identified in bronchoalveolar lavage fluid (BALF). Transbronchial lung biopsy (TBLB) specimens obtained on day +33 showed a histologic pattern of OP (Figure 2A). On day +34, we initiated prednisolone therapy (1 mg/kg per day), which led to rapid improvement of the symptoms. CT scans on day +57 showed almost complete resolution of the lesions. The patient is currently well without pulmonary symptoms.

Patient 2 was a 35-year-old man with myelodysplastic syndrome. In August 2004, he received 2 antigen–mismatched CB grafts containing 2.39×10^7/kg TNCs. Conditioning and GVHD prophylaxis were the same as for patient 1. Neutrophil engraftment was achieved on day +30. Grade I acute GVHD involving the skin occurred but spontaneously resolved. On day +60, the patient presented with fever without cough and dyspnea. Arterial blood PO_2 and PCO_2 values were 73.6 mm Hg and 39.3 mm Hg, respectively. CT scans on day +62 showed patchy consolidation in the lungs (Figure 1B). No causative infectious agents were identified in the BALF. TBLB specimens taken on day +63 showed the OP pattern (Figure 2B). On day +64, prednisolone therapy (0.5 mg/kg per day) was initiated. CT scans on day +69 showed that the lung lesions were tending to resolve. Because of leukemia relapse, we discontinued cyclosporine administration and reduced the prednisolone dosage on day +82. The consolidation in the lungs did not completely resolve. The patient died of relapse on day +195.

Patient 3 was a 46-year-old man with myelodysplastic syndrome. In June 2005, he received 2 antigen–mismatched CB grafts containing 2.36×10^7/kg TNCs. The patient also had pulmonary alveolar proteinosis, as reported previously [6].

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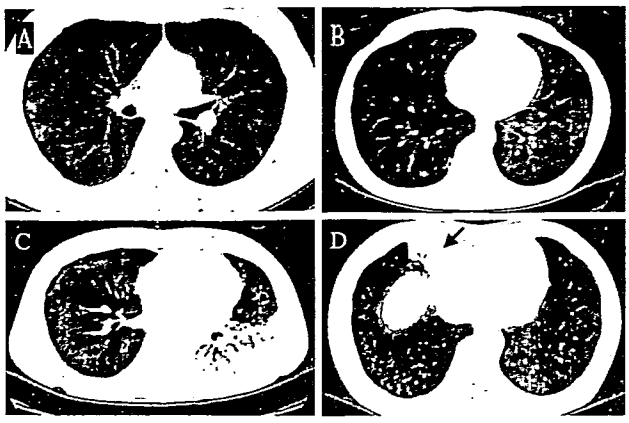

Figure 1. Chest computed tomography (CT) scans at the onset of organizing pneumonia. A, Chest CT scan on day +32 in patient 1. Diffuse ground-glass opacity in the lungs is shown. B, Chest CT scan on day +62 in patient 2. Patchy consolidation in the lungs is evident. C, Chest CT scan on day +50 in patient 3. Extensive consolidation is shown with air bronchograms of the left lung. D, Chest CT scan on day +49 in patient 4. Extensive consolidation is evident with air bronchograms of the right lung.

Conditioning and GVHD prophylaxis were the same as described above. Neutrophil engraftment was achieved on day +34. Grade II acute GVHD involving the skin occurred on day +18 but spontaneously resolved. On day +49, the patient presented with dyspnea and fever without cough. Arterial blood PO_2 and PCO_2 values were 60.3 mm Hg and 31.2 mm Hg, respectively. CT scans on day +50 showed extensive consolidation with air bronchograms of the left lung (Figure 1C). Cytomegalovirus DNA was detected in BALF at 600 copies/mL (normal range, <200 copies/mL), but other infectious agents were not identified. TBLB specimens examined on day +51 showed an OP pattern (Figure 2C). Specific staining did not suggest cytomegalovirus infection. On day +53, we initiated prednisolone therapy (2 mg/kg per day), which led to remarkable improvement of the symptoms. CT scans on day +81 showed almost complete resolution of the consolidation. The patient is currently well without pulmonary symptoms.

Patient 4 was a 38-year-old man with acute lymphoblastic leukemia. In August 2006, he received 2 antigen–mismatched CB grafts containing 1.87×10^7/kg TNCs. The conditioning regimen included 12 Gy TBI, 120 mg/kg cyclophosphamide, and 12 g/m^2 cytarabine. GVHD prophylaxis was the same as described above. Neutrophil engraftment was achieved on day +24. Grade II acute GVHD involving the skin occurred on day +31 but spontaneously resolved. On day +45, the patient presented with cough and fever. Arterial blood PO_2 and PCO_2 values were 75.4 mm Hg and 38.9 mm Hg, respectively. CT scans on day +49 showed extensive consolidation with air bronchograms of the right lung (Figure 1D). Cytomegalovirus DNA was detected in BALF at 200 copies/mL, but other infectious agents were not identified. TBLB specimens taken on day +53 showed a typical OP pattern, as manifested by fibrous-plug formation (Figure 2D). Specific staining did not suggest cytomegalovirus infection. We initiated prednisolone therapy (1 mg/kg per day) on day +54, which led to improvement of the symptoms. CT scans on day +77 showed substantial resolution of the consolidation. The patient is well without pulmonary symptoms.

BOOP is a clinicopathologic syndrome [7]. BOOP without identifiable causes is also termed cryptogenic OP (COP) [8]. The characteristic histologic feature is the presence of buds of fibrous granulation tissue in the distal airspaces [7-9]. TBLB specimens from our patients showed an OP pattern. The typical histologic feature of BOOP or COP was observed in patient 4; however, the degrees of organization in the alveoli were mild in patients 2 and 3, suggesting that OP in the patients might be in the early or immature stages.

Previous studies showed an association between chronic GVHD and BOOP after SCT [2-4]. In addition, Freudenberger et al indicated that prior occurrence of acute GVHD was associated with the subsequent development of BOOP [2]. In our study, OP in patient 1 occurred concomitantly with the presence of acute GVHD, but OP in patients 2 to 4 occurred after the resolution of acute GVHD. Later, limited-type chronic GVHD occurred in patients 1 and 4, and extensive-type chronic GVHD occurred in patient 3. Although the role of alloimmunity in the development of OP was not determined in our patients, steroid therapy resolved the pulmonary lesions in all of the patients to varying degrees.

BOOP is generally recognized as a late complication in SCT patients [1,2]. Of 112 adult patients in our institution who underwent CBT following a conditioning regimen containing

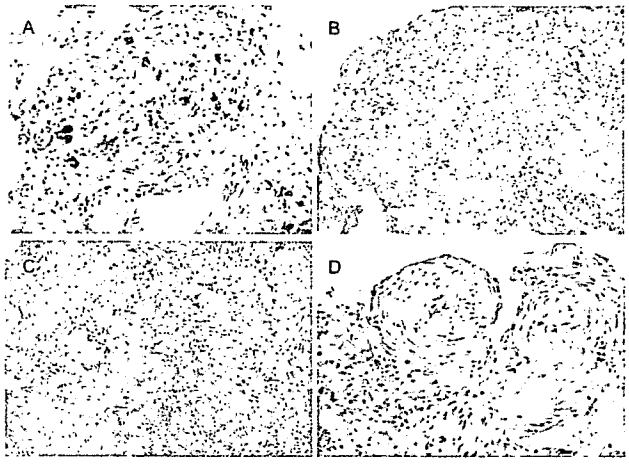

Figure 2. Microscopical features of organizing pneumonia (OP) in transbronchial lung biopsy (TBLB) specimens. A, TBLB specimen obtained from patient 1 on day +33 (hematoxylin and eosin [H&E], original magnification ×200). The TBLB specimen shows the accumulation of foamy macrophages and granulation tissue with fibroblasts in alveolar spaces. B, TBLB specimen obtained from patient 2 on day +63 (H&E, original magnification ×100). The OP pattern observed in patient 1 is evident. C, TBLB specimen obtained from patient 3 on day +51 (H&E, original magnification ×100). The OP pattern observed in patients 1 and 2 is evident. D, TBLB specimen obtained from patient 4 on day +53 (H&E, original magnification ×200). The TBLB specimen shows an intraluminal fibrous plug typical of OP.

12 Gy TBI, OP was histologically diagnosed in 7 patients. The 4 patients in the present study showed an OP pattern on days +33, +51, +53, and +63 after CBT. The remaining 3 patients developed OP on days +257, +432, and +636 after CBT. At the onset of late OP, 1 patient had limited-type chronic GVHD after the occurrence of grade I acute GVHD, and 2 patients had extensive-type chronic GVHD after the occurrence of grade II acute GVHD. We identified no obvious differences in clinical features between early-onset and late-onset OP in our patients after CBT. This study has shown that OP can occur during very early periods after CBT. The features of BOOP after CBT, including the association of GVHD, should be investigated further in a large number of patients.

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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

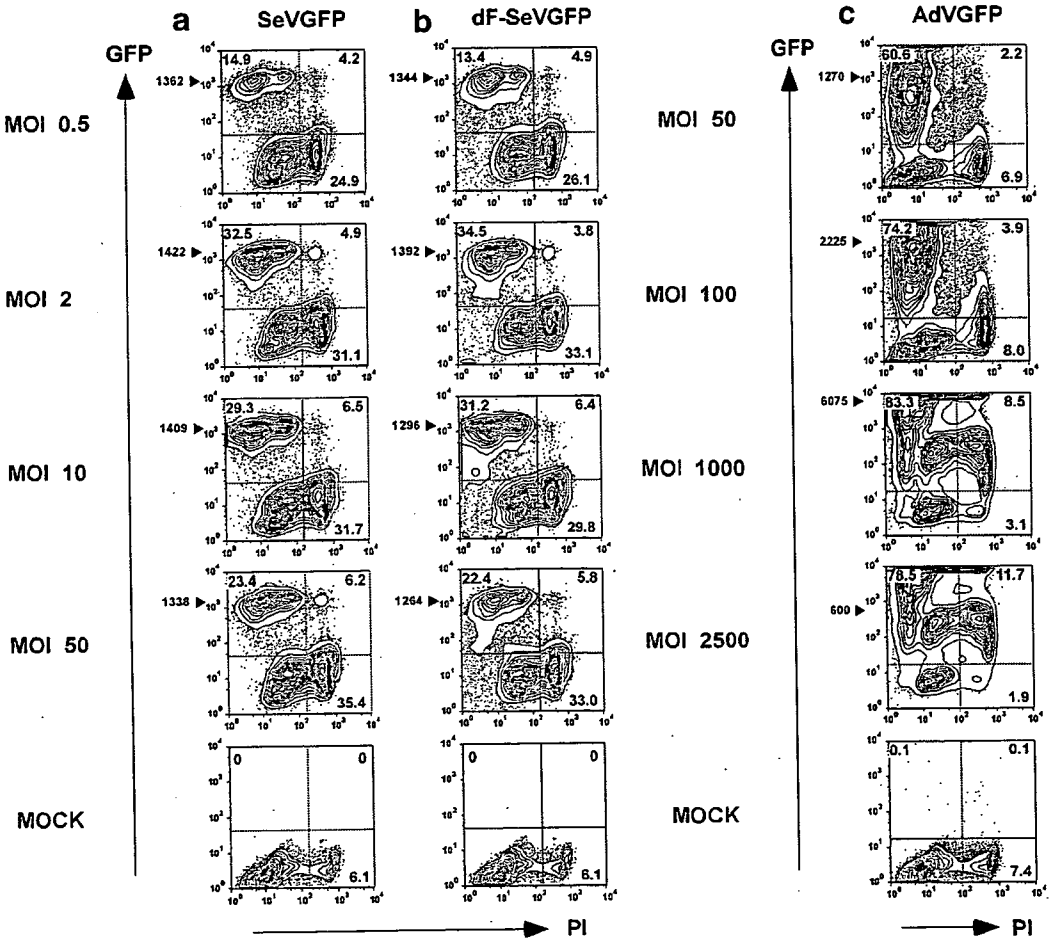

Fig. 1. Comparison of different MOIs for the maximum gene expression by SeV (a), dF-SeV (b), and AdV (c) vectors 48 hr after infection. Cell viability was determined by staining with PI. GFP expression and PI staining were analyzed by flow cytometry. The percentages of GFP- and PI-positive cells are 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

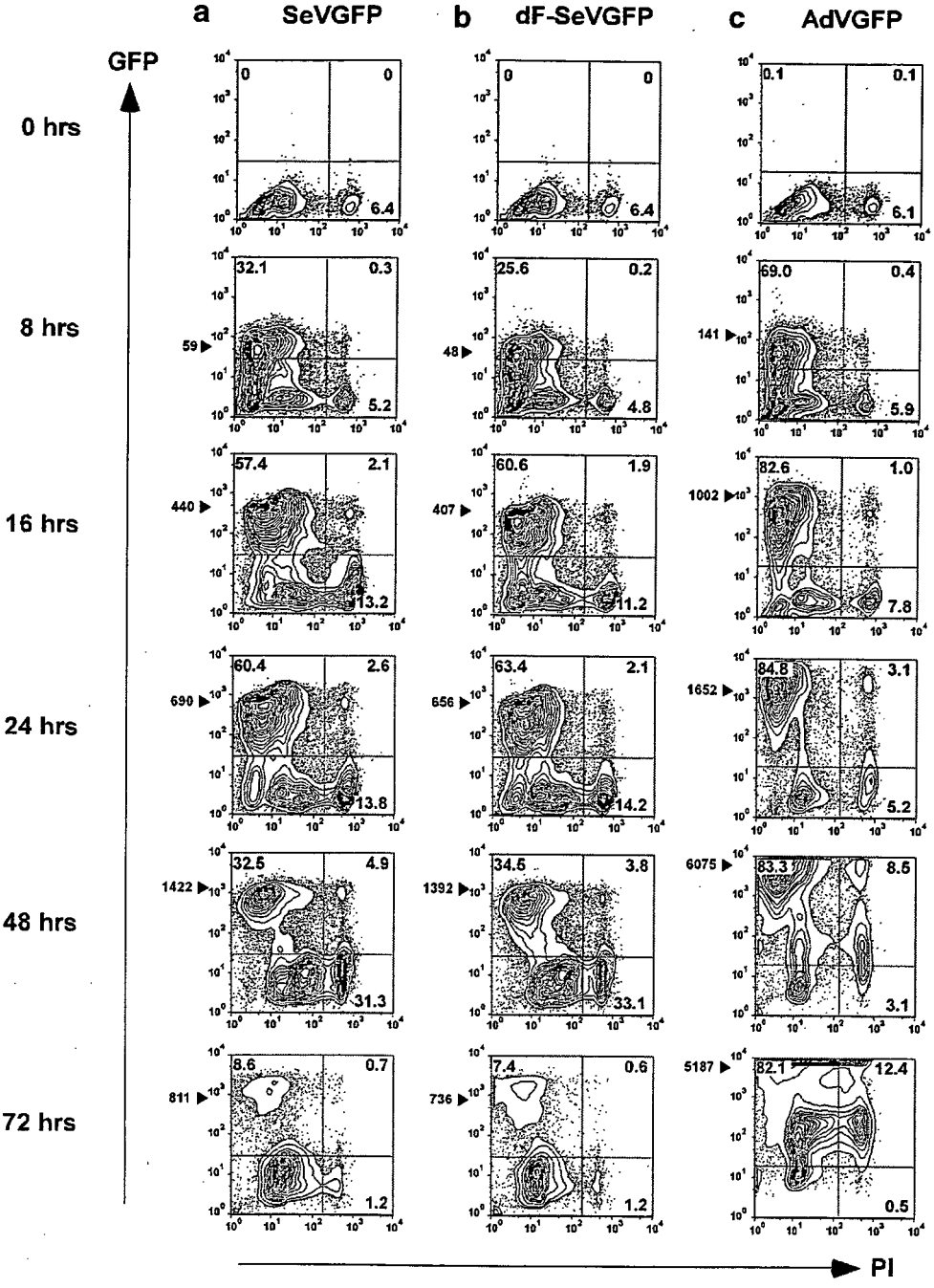

Fig. 2. The time course of the maximum gene expression by SeV (**a**), dF-SeV (**b**), and AdV (**c**) vectors. DCs were infected with SeV and dF-SeV at an MOI of 2 and were infected with AdV at MOI of 1,000 and then cultured for 8–72 hr. Cell viability was determined by staining with PI. GFP expression 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.

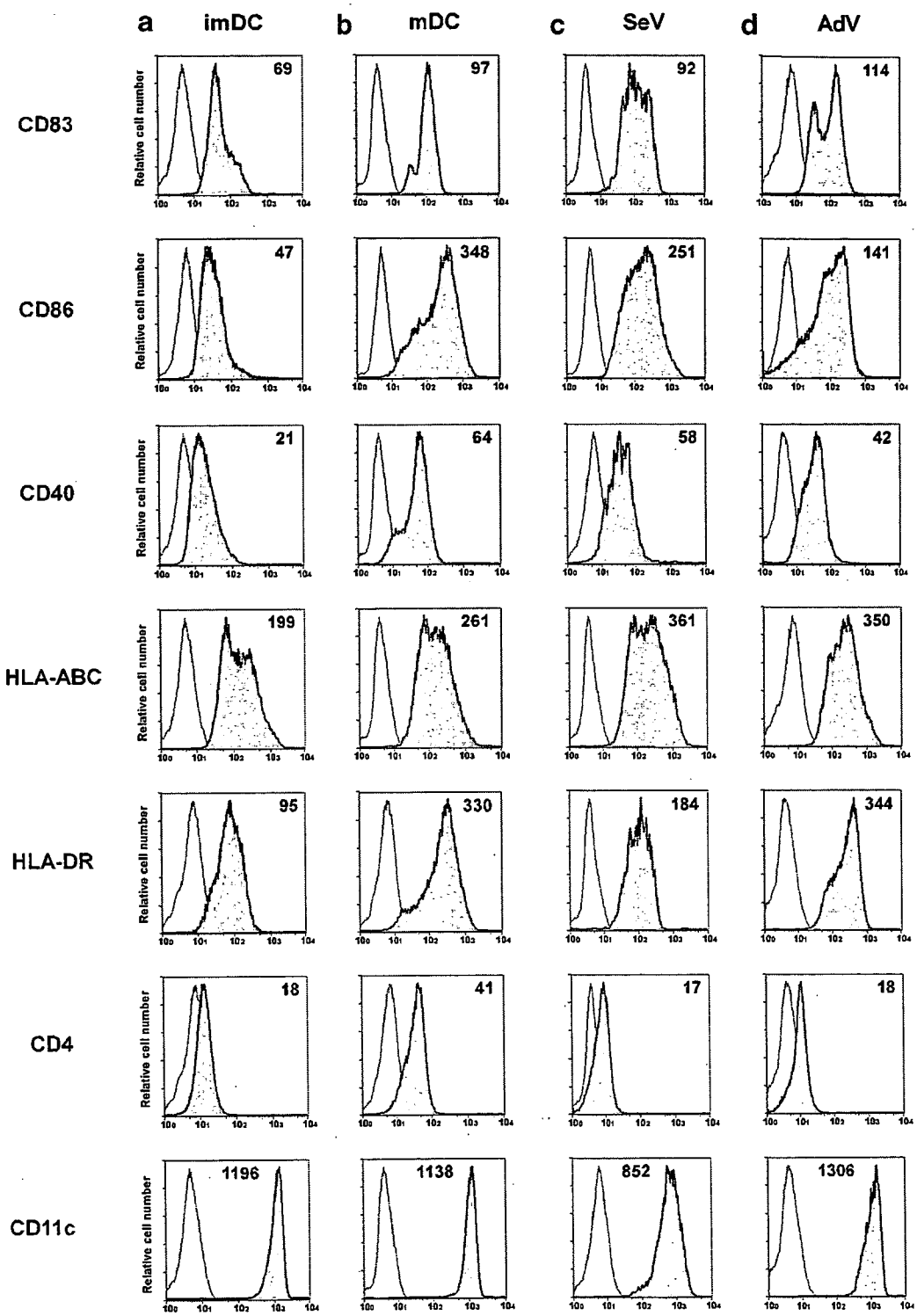

Fig. 3. Infection of DCs with SeV and AdV vectors modified the expression of cell surface markers. DCs infected with SeV vector containing no inserts at an MOI of 2 (**c**) and AdV vector containing no inserts at an MOI of 1,000 (**d**) were maintained in the DC medium for 48 hr (AdV vector) and 24 hr (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.

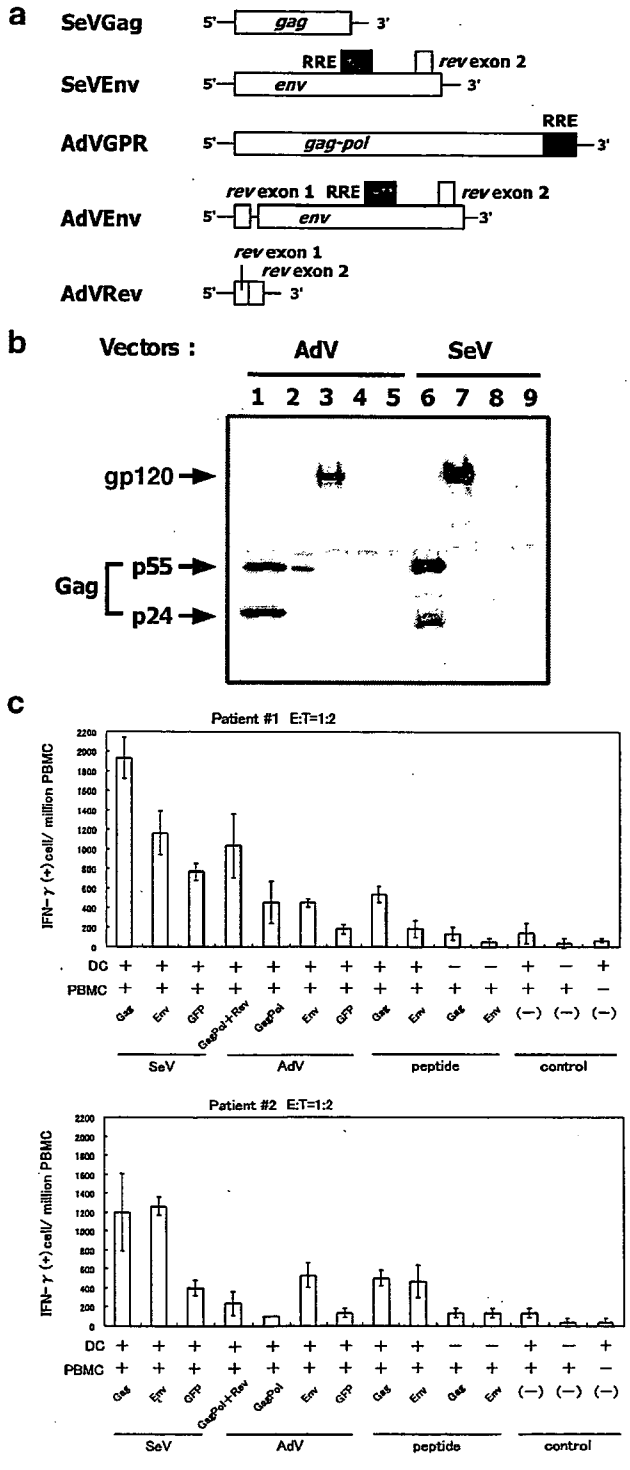

Fig. 4. **a**: Schematic structures of five viral vectors. *gag*, HIV-1 gag gene; *env*, HIV-1 env gene; *gag-pol*, HIV-1 gag and pol genes; *rev*, HIV-1 rev gene; RRE, HIV-1 RRE; GFP, green fluorescent protein. **b**: The expression of HIV-1 structural proteins by AdV and SeV vectors. DCs infected with 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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A three-base-deletion polymorphism in the upstream non-coding region of human interleukin 7 (IL-7) gene could enhance levels of IL-7 expression

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Summary

Interleukin 7 (IL-7) is a key factor in the survival, development and proliferation of B and T lymphocytes. Elevation of plasma IL-7 has been reported in several lymphopenia cases such as HIV-1 patients. After patients started to receive antiretroviral drugs and their $CD4^+$ cell counts had recovered, IL-7 in plasma decreased to normal levels. There are considerable variations in the levels of plasma IL-7 as well as the rate of $CD4^+$ T-cell restoration. Although pre-treatment plasma IL-7 levels have been shown to be prognostic for the rate of post-treatment $CD4^+$ T-cell restoration, the mechanisms responsible for the variations in plasma IL-7 and rate of $CD4^+$ T-cell restoration are still completely unknown. In the study here, we searched for genetic polymorphisms that might affect levels of IL-7 gene expression. For this purpose, we used 1658-bp PCR-amplified fragments of the IL-7 gene containing 1470 bp of the upstream non-coding region obtained from 151 Japanese and 234 Thai subjects. We found two novel human genetic polymorphisms in the upstream non-coding region of the IL-7 gene. The luciferase reporter assay demonstrated that one of those polymorphisms could increase the gene expression of IL-7. We speculate that this polymorphism, a three base ATC deletion just upstream of an out-of-frame ATG codon in the upstream non-coding region of the IL-7 gene, reduces the efficiency of translation from the upstream, out-of-frame ATG, resulting in increased translation efficiency from the authentic ATG of IL-7. Although the frequency of this allele is very low, it would be interesting to analyse this polymorphism in HIV-1-infected individuals with different rates of immune reconstitution after treatment with a highly active antiretroviral therapy.

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Introduction

Human interleukin 7 (IL-7) is a cytokine produced by stromal cells of the thymus and bone marrow (Wolf & Cohen, 1992; Heufler *et al.*, 1993; Sudo *et al.*, 1993) and has the capacity to induce growth of immature B lymphocytes (Namen *et al.*, 1988). Similarly, IL-7 contributes to the development, proliferation and homeostatic maintenance of T cells (Grabstein *et al.*, 1990; Plum *et al.*, 1996; Schluns *et al.*, 2000; Fry *et al.*, 2001). Human IL-7 gene located on chromosome 8q12–13, has six exons that distributed to more than 33-Kb of genomic DNA (Lupton *et al.*, 1990; Fry & Mackall, 2002). It is known that the IL-7 gene has no canonical core promoter sequence in the 5′ upstream region (Lupton *et al.*, 1990; Oshima *et al.*, 2004). Recently, it has been reported, however, that transcription start sites of the IL-7 gene are clustered within two distinct regions that are approximately 515 bp to 600 bp and 130 bp to 217 bp upstream from the translation initiation ATG codon (Oshima *et al.*, 2004). Moreover, the region −282 to −251 upstream from the initiation ATG codon contains an interferon regulatory factor element (IRF-E) and could thus up-regulate the transcription of the IL-7 gene upon stimulation with gamma interferon (IFN-γ) in human intestinal epithelial cells (Oshima *et al.*, 2004). This study also revealed the presence of several out-of-frame ATG codons with unknown function in the upstream non-coding region of the IL-7 gene (Oshima *et al.*, 2004).

With respect to HIV-1 infection, there is a reverse correlation between $CD4^+$ T-cell numbers and IL-7 plasma levels in HIV-1-infected patients (Llano *et al.*, 2001; Beq *et al.*, 2004; Kopka *et al.*, 2005). After these patients started to receive antiretroviral drugs and their $CD4^+$ T-cell counts had recovered, the elevated IL-7 in the plasma decreased to normal levels (Llano *et al.*, 2001). Furthermore, it is well known that there are considerable variations in the levels of plasma IL-7 as well as the rate of CD4 T-cell restoration after HIV-1 patients started to

receive antiretroviral drugs, and pre-treatment plasma IL-7 levels have been shown to be prognostic for the rate of post-treatment CD4 T-cell restoration (Beq *et al.*, 2004). However, knowledge of the molecular mechanisms controlling IL-7 gene expression remains very limited, and the mechanisms responsible for the variations in plasma IL-7 levels and rate of CD4 T-cell restoration among individuals are still completely unknown.

Human genetic polymorphisms have recently been shown to affect expression of the corresponding genes and to consequently modify the clinical course of several human diseases such as HIV-1 infection (Dean *et al.*, 1996; Michael *et al.*, 1997; Kostrikis *et al.*, 1998; Liu *et al.*, 1999; Nakayama *et al.*, 2000). We aimed to know the molecular mechanisms controlling variations in IL-7 gene expression among individuals. For this purpose, we searched for genetic polymorphisms that might affect levels of IL-7 gene expression in 1658-bp PCR-amplified fragments of the IL-7 gene containing 1470 bp of the upstream non-coding region, 9 bp of the first coding exon and 179 bp of the downstream intron, although there was no previous report on human genetic polymorphisms that alter the levels of IL-7 gene expression. We found two novel human genetic polymorphisms in the upstream non-coding region of the IL-7 gene, one of which could enhance IL-7 expression probably by reducing the efficiency of translation from an upstream, out-of-frame ATG that would result in diminished efficiency of translation from the downstream initiation ATG.

Materials and methods

Genotyping of IL-7 gene

Human genomic DNA was obtained from peripheral blood mononuclear cells of 52 unrelated non-HIV-1-infected and 99 HIV-1-infected Japanese, as well as 122 non-HIV-1-infected and 112 HIV-infected Thais, who provided written informed consent. Genomic regions of 1658 nucleotides containing 1470 nucleotides of the upstream non-coding region and the first exon and part of the intron of IL-7 were amplified by using the primer pair P1: 5′-TCCCTCCTCTTCCTTGTTC-3′ and P2: 5′-GGTTCAAGTGGCTATGTGC-3′. Polymerase chain reaction (PCR) was run for 40 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 2 min. Fluorescence-based automated cycle sequencing of the PCR products was then carried out by an ABI 3100 using P1, P2 (mentioned previously), P3: 5′-TGCTGCATTTGGGCTGTAGA-3′, P4: 5′-TGGTTTTTCCTGCGGTGAT-3′ and P5: 5′-GGTCTGCAGGTTCAATCT-3′ as sequencing primers.

Luciferase reporter gene assays

NheI and NcoI-tagged DNA fragments, corresponding to the sequences spanning positions −632 to +3, −632 to −67 and −297 to +3 from the initiation ATG of the IL-7 gene, were inserted into the corresponding restriction enzyme cleavage sites of the pGL3-Basic Vector in order to fuse ATGs in the IL-7 gene directly to the firefly luciferase open reading frame (Promega, Madison, WI). Constructs carrying an ATC deletion at position −29 to −27 from the initiation ATG of IL-7 were generated by PCR-based in vitro mutagenesis using P6: 5′-GGCTAGCAGACGACTTGGCATCGTCC-3′ and P8: 5′-TGGACCATGGTCTGCGGGAGGCGGGCGTAGTCATGACCGC-3′ or P7: 5′-GGCTAGCAGATTGAACCTGCAGACCA-3′ and P8 (mentioned previously) as the respective primer pairs for the −632 to +3 or −297 to +3 upstream region of the IL-7 gene with ATC deletion. All constructs were verified for sequence authenticity. Four micrograms of the resultant constructs was transfected with DMRIE-C (Gibco/BRL, Gaithersburg, MD) into Jurkat ($CD4^+$ T-lymphocyte cell line) and U937 cells (monocytic cell line). Transfection efficiency was normalized by cotransfection with 0.2 μg of pRL-CMV vector, which expresses *Renilla* luciferase under the control of the cytomegalovirus immediate early promoter. When necessary, INF-γ (Peprotech, Rocky Hill, NJ) was added to the transfected cell culture at a final concentration of 50 ng mL^{-1} 5 h after transfection. The cells were harvested 40 h after transfection, and firefly and *Renilla* luciferase activities were determined according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega) with a Luminometer Centro LB960 (Berthold, Bad Wildbad, Germany). Relative luciferase expression (fold increase) was calculated with the following equation: fold increase = (firefly luciferase activity of upstream region of IL-7 gene construct/*Renilla* luciferase activity) / (firefly luciferase activity of promoterless vector pGL3-Basic/*Renilla* luiferase activity).

Statistical analysis

The unpaired *t*-test was used.

Results

Polymorphisms in the upstream non-coding region of the IL-7 gene

We sequenced a 1658-bp PCR-amplified fragment of the IL-7 gene containing 1470 bp of the upstream non-coding region, 9 bp of the first coding exon and 179 bp of the downstream intron. Samples were obtained from 52 unrelated non-HIV-1-infected and 99 HIV-1-infected Japanese, as well as from 122 non-HIV-1-infected and 112 HIV-infected Thais. Polymorphisms were identified at two positions: an A to G substitution at position −485 and an ATC deletion at a position from −29 to −27 upstream from the open frame ATG codon of the IL-7 gene (Fig. 1). Frequencies of these two polymorphisms are summarized in Table 1. As for the A to G mutation at position −485, there was no difference in frequency of the G allele between HIV-1-infected and non-HIV-1-infected individuals. For the allele of the ATC deletion, two of the 99 HIV-1-infected Japanese carried this allele, but none of the Thais. There was no linkage disequilibrium between these two mutations.

