helper T cells reduced CTL susceptibility to AICD through a cell contact-dependent mechanism [29]. Thus, it is possible that activation of 4O/1C8 by RT1AlSCTax180L may fail to induce the protective signal from AICD in the CTLs. Since a syngeneic CD4+ T cell line, FPM1.BP, was able to induce the expansion of 4O1/C8 in spite of the apparent AICD induction, it is also possible that MOLT-4/RT1AlSCTax180L cells failed to trigger the signal(s) which 4O1/C8 was able to activate for the induction of CTL proliferation. Actually, as shown in Figure 3D, we have detected the enhanced production of IL-2 in the mixed culture of 4O1/C8 with FPM1.BP, suggesting the involvement of the IL-2 signal transduction pathway in the proliferation of 4O1/C8. Further analysis is required to clarify the activation mechanisms of CTLs in the rat system for inducing better immune response by SCTs. Nevertheless, it may be still possible to apply the pEF/RT1AlSCTax180L vector for inducing Tax-specific CTL response in rats, since similar SCT complex expressing human papillomavirus-16 E6 antigen was shown to induce protective immunity against the virus in a mouse system in vivo [30]. Thus, it will also be necessary to assess the in vivo effect of the rat SCTs for the evaluation of the system as a therapeutic tool in HTLV-I infection.

Previous reports suggested that insufficient T cell response against HTLV-I is a potential risk factor for ATL. Among HTLV-I infected individuals, the infrequency of HTLV-I-specific CTL induction in vitro has been reported in ATL patients [12,13,31]. Moreover, a recent study using various tetramers clearly demonstrated the reduction of the frequency and diversity of anti-Tax CTLs in ATL patients [32]. The importance of HTLV-I-specific T cell immunity in anti-tumor surveillance was also supported by a previous report showing that Tax-specific CTL response was strongly activated in ATL patients who obtained complete remission after HSCT [16]. These observations suggested the importance of Tax-specific CTLs for prevention and therapy of ATL and should be further verified using suitable animal models. Rats have been used for a number of studies on HTLV-I infection, because they are susceptible to the virus and because the virus-transformed T cell lines can be established in vitro [33,34]. It has previously shown in a rat model that HTLV-I specific T cells were important to inhibit the growth of virus-infected cells in vivo [17]. Moreover, the association of elevated proviral load with insufficient T cell immunity has been also observed in a rat model of oral HTLV-I infection [35]. In this model, it has further demonstrated that re-immunization of orally HTLV-I-infected rats resulted in a reduction of the proviral load [36]. Although these results further support the importance of Tax-specific CTLs for the prophylaxis and treatment of ATL, detailed analysis to understand the interplay between epitope-specific CTLs and HTLV-I infected cells in vivo has not been performed yet. This is mainly due to the lack of tools to identify epitope specific CTLs in rats. In this study, we have demonstrated that the SCT-EGFP system was able to detect Tax180-188 specific CTLs in splenocytes derived from an HTLV-I-infected rat and that the detection of the epitope-specific CTLs by SCT-EGFP system was comparable to the measurement of peptide-induced IFN-γ production. Thus, the activation and detection system established in this study should be useful for further verifying the strategies to fight against HTLV-I.

Conclusion

In this study, we have generated a SCT of rat MHC-I linked to Tax epitope peptide, which can be applicable for the induction of Tax-specific CTLs in rat model systems. We have also established a detection system of Tax-specific CTLs by using cells expressing SCTs fused with EGFP. These systems will be useful tools in understanding the role of HTLV-I specific CTLs in HTLV-I pathogenesis.

Methods

Cell lines

An HTLV-I-immortalized cell line, FPM1.BP, was established previously from an F344/N Jcl-rnu/+ rat [37]. The cells were maintained in RPMI 1640 with 10% heat-inactivated FCS (Biosource, Rockville, MD), penicillin, and streptomycin. A CD8+ Tax-specific CTL line, 4O1/C8, and an IL-2-dependent HTLV-I-negative CD8+ cell line, G14, were also established previously from F344/N Jcl-rnu/+ rats [19]. These cells were maintained in RPMI 1640 medium with 10% FCS and 20 U/ml of IL-2 (PEPROTECH, London, UK). For the maintenance of 4O1/C8 cells, periodical stimulation with formalin-fixed FPM1.BP cells is also required, because their growth is dependent on RT1.A^{l}-restricted presentation of Tax180-188 epitope [37]. Human 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS and MOLT-4 cells were cultured in RPMI 1640 medium with 10% FCS.

Plasmid DNA construction

Plasmid constructs were generated using standard techniques and were confirmed DNA sequence analysis. Briefly, Rat MHC-I (RT1.A^{l}) and β_2m cDNAs were amplified by PCR using G14 cell-derived cDNAs as templates. The PCR products of RT1.A^{l} and rat β_2m were cloned to the pCR2.1 vector using TA cloning kit (Invitrogen, Carlsbad, CA) and were designated as pCR2/RT1Al and pCR2/rβ_2M, respectively. The DNA encoding the epitope peptide-β_2m-RT1.A^{l} fusion protein was synthesized by a multistep PCR using pCR2/rβ_2M as a template. The first PCR was performed to add the L2 sequence at the 3' end of β_2m gene. The following 2 or 3 steps of PCRs were performed to add NotI site-containing region of RT1.A^{l} signal sequence and the epitope sequence fused with the L1

linker at the 5' end of β_2m gene and the AvaI site-containing region of RT1.A^l α_1 domain at the 3' end. The primers used for these stepwise reactions were summarized in Table 1. The third or forth PCR product was digested with NotI and AvaI, and then cloned between NotI and AvaI sites of pCR2/RT1Al to construct pCR2 vectors containing peptide-β_2m-RT1.A^l fusion sequence. The obtained constructs were further amplified by PCR to add BamH1 and Bsp1407I sites at the 5' and 3' end of the fusion constructs, respectively and were ligated between BamH1 and Bsp1407I sites of pEFGFP vector [27]. In this study, we constructed 4 expression vectors with 2 different epitopes and 2 different lengths of linkers. The diagram of SCT expression vectors established in this study was shown in Figure 1B. The short or long linkers consist of 10 or 15 residues of L1 and 15 or 20 residues of L2, respectively. Tax180-188 epitope was previously identified by epitope mapping in a Tax-specific CTL line [20]. A putative epitope in the envelope of HIV-1 NL4-3 strain, NLEnv371-379 was determined by epitope prediction data via http://www.syfpeithi.de[24]. We also constructed the pEF/RT1Al plasmid, which expresses RT1.A^l protein.

For the generation of SCT-EGFP expression vectors, RT1AlSCTax180L or RT1AlSCNLEnv371L cDNAs were further amplified by PCR to delete a stop codon and to add KpnI and BamHI sites at the 5'- and 3'- termini, respectively. The pEF/RT1AlSCTax180L-EGFP and pEF/RT1AlSCNLEnv371L-EGFP vectors were generated by insertion of the corresponding PCR products between KpnI and BamHI sites of pEFGFP vector.

To confirm the accuracy of vectors used in this study, all established constructs were subjected to sequence analysis using ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer's instruction.

Cytokine production assay

An HTLV-I Tax-specific CTL line, 4O1/C8 (2×10^5/well), was mixed with various stimulator cells (2×10^5/well). In some experiments, stimulator cells were fixed with 1% formalin in PBS or pulsed with 10 μM of Tax180-188 or NLEnv371-379 peptide (MBL, Nagoya, Japan) before incubation with the CTL. For the stimulation of primary splenocytes with peptides, 5×10^4 of splenocytes were incubated with 10 μM of Tax180-188 or NLEnv371-379 peptides. After the indicated period of mixed culture, supernatants were harvested and were subjected to rat IFN-γ (eBioscience Inc., San Diego, CA), TNF-α ELISA (eBioscience Inc.), or IL-2 (R&D Systems Inc., Minneapolis, MN) in accordance with the manufacturer's instructions.

Flow cytometric analysis

For the assessment of SCT expression, MOLT-4 cells transfected with various SCT expression vectors were stained with an anti-rat MHC-I antibody (BD Bioscience, San Jose, CA) for 30 min on ice, washed three times with 1% FCS in PBS, and then stained with FITC-conjugated goat anti-mouse IgG+IgM. After being washed, the cells were fixed with 1% formalin in PBS prior to analysis on a FACScalibur (BD Bioscience). For the detection of Tax180-188 specific CTLs by the mixed culture with SCT-EGFP expressing cells, 4O1/C8 or splenocytes were incubated with 293T cells expressing SCT-EGFP fusion proteins for 1 hour. Cells in the mixed cultures were stained with phycoerythrin (PE)-conjugated anti-rat CD8 (clone OX-8; BD Bioscience) for 30 min on ice, washed three times with 1% FCS in PBS, fixed with 1% formalin in PBS, and then subjected to FACS analysis.

Cell growth assay

FPM1.BP or MOLT-4 cells with SCTs were fixed with 1% formalin in PBS for 20 min and then washed four times with RPMI 1640 medium. These formalin-fixed cells (1×10^5/well) were incubated with 4O1/C8 (1×10^5/well) in each well of 96-well round-bottom microtiter plates for 3 days at 37°C. The number of growing cells was determined by using a Cell Counting Kit-8 (Dojinndo Laboratories, Kumamoto, Japan) in accordance with the manufacturer's instructions.

Apoptosis analysis

Formalin-fixed MOLT-4 or FPM1.BP cells (1×10^5/well) were incubated with 4O1/C8 (1×10^5/well) in each well of 96-well round-bottom microtiter plates for 24 hours at 37°C. The percentage of 4O1/C8 cells undergoing apoptosis was determined by FACS analysis using the Annexin V-FITC Apoptosis Detection Kit (MBL) in combination with a PE-conjugated anti-rat CD8 antibody (BD Bioscience).

Immunofluorescence staining

SCT-EGFP expressing 293T cells were cultured with 4O1/C8 in each well of 96-well round-bottom microtiter plates for 1 hour at 37°C. Cells in the mixed cultures were attached on slide glasses (Matsunami Glass Ind., Japan) by centrifugation, fixed with 4% paraformaldehyde in PBS and then stained with an anti-rat CD8 antibody (BD Bioscience) in combination with Cy3-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA). Images were examined with a confocal microscope system (FluoView; Olympus, Tokyo, Japan).

Preparation of immune splenocytes

Female F344/Jcl rats were purchased from Clea Japan, Inc. (Tokyo, Japan). Four-week-old F344/Jcl rats were intra-

Table 1: Primers to construct SCTs of RT1.A^l

Constructs	Sense primer	First PCR	Second PCR*	Third PCR*	Forth PCR
RT1AlSCTax1805	ATTCAGAAAACTC	GGCCGCCCTGGCCCCGAC	CCTGCTGCTGGCGGCC	Not applicable
	CCCAAATTCAAG	CCAGACC	GCCCTGGCC	
	TGTAC	CGCGCGG**GGGGGCCTTCCT**	CCGAC	
		CACCAATG		
		TTCCCTACGGAGGTGGCG		
		GGTCCGG		
		AGGTGGCGGGTCCATTCAG		
		AAAACT		
		CCCCAAATTCAAGTGTACT		
		CTCGCC		
		ATCCA		
	Reverse primer				
	CGCCACCTCCCAT	CCGAGGCCGGGCCGGGAC	CCGGGGCTCCCCGAG	Not applicable
	GTCTCGGTCCCA	ACGGCGA	GCCGGGCCGGGACAC	
	GGTGA	TGTCGAAATACCGCATCGA	GGCGATGTC	
		GTGAGA		
		GCCGGACCCGCCACCTCC		
		GGACCCG		
		CCACCTCCGGACCCGCCA		
		CCTCCCA		
		TGTCTC		
RT1AlSCNLEnv371S	Sense primer				
	ATTCAGAAAACTC	GGCCGCCCTGGCCCCGAC	CCTGCTGCTGGCGGCC	Not applicable
	CCCAAATTCAAG	CCAGACC	GCCCTGGCCCCGAC	
	TGTAC	CGCGCG**CACAGTTTTAA**		
		TTGTGGAG		
		GGGAATTTGGAGGTGGC		
		GGGTCCGG		
		AGGTGGCGGGTCCATTCAG		
		AAAACT		
		CCCCAAATTCAAGTGTACT		
		CTCGCC		
		ATCCA		
	Reverse primer				
	CGCCACCTCCCAT	CCGAGGCCGGGCCGGGAC	CCGGGGCTCCCCGAG	Not applicable
	GTCTCGGTCCCA	ACGGCGA	GCCGGGCCGGGACAC	
	GGTGA	TGTCGAAATACCGCATCGA	GGCGATGTC	
		GTGAGA		
		GCCGGACCCGCCACCTCC		
		GGACCCG		
		CCACCTCCGGACCCGCCA		
		CCTCCCA		
		TGTCTC		

Table 1: Primers to construct SCTs of RT1.A[1] *(Continued)*

RT1AISCTax180L	Sense primer	ATTCAGAAAACTC CCCAAATTCAAG TGTAC	GGAGGTGGCGGGTCCATTC AGAAAA CTCCCCAAATTCAAG	GGCCGCCCTGGCCCCG ACCCAGACCCGCGCG **GGGGCCTTCCTCACC AATGTTCCCTAC**GGA GGTGGCGGGTCCGGA GGTGGCGGGTCCGGA GGTGGCGGGTCC	CCTGCTGCTGGCGGC CGCCCTGGCCCCGAC
	Reverse Primer	CGCCACCTCCCAT GTCTCGGTCCCA GGTGA	GGCCGGGACACGGCGATG TCGAAAT ACCGCATCGAGTGAGAGCC GGACCC GCCACCTCCGGACCCGCC ACCTCCG GACCCGCCACCTCCGGAC CCGCCAC CTCCCATGTCTC	CCGGGGCTCCCCGAG GCCGGGCCGGGACAC GGCGATGTC	GCTCCCCGAGGCCGG GCCGGGACACGGCGA
RT1AISCNLEnv371L	Sense Primer	ATTCAGAAAACTC CCCAAATTCAAG TGTAC	GGAGGTGGCGGGTCCATTC AGAAAA CTCCCCAAATTCAAG	GGCCGCCCTGGCCCCG ACCCAGACCCGCGCG **CACAGTTTTAATTGT GGAGGGGAATTT**GGA GGTGGCGGGTCCGGA GGTGGCGGGTCCGGA GGTGGCGGGTCC	CCTGCTGCTGGCGGC CGCCCTGGCCCCGAC
	Reverse Primer	CGCCACCTCCCAT GTCTCGGTCCCA GGTGA	GGCCGGGACACGGCGATG TCGAAAT ACCGCATCGAGTGAGAGCC GGACCC GCCACCTCCGGACCCGCC ACCTCCG GACCCGCCACCTCCGGAC CCGCCAC CTCCCATGTCTC	CCGGGGCTCCCCGAG GCCGGGCCGG GACACGGCGATGTC	GCTCCCCGAGGCCGG GCCGGGACACGGCGA

* Bold cases indicate sequences encoding Tax180-188 or NLEnv371-379 epitope peptide.

peritoneally inoculated with 1×10^7 FPM1.BP cells. The rats received two boost inoculations with the same dose at 2 and 10 weeks after initial inoculation. One week after the last inoculation, splenocytes were isolated, purified by centrifugation on a density separation medium (Lympholyte-Rat; Cedarlane, Ontario, Canada) and subjected to the analysis for the detection of Tax180-188-specific CTLs or the quantification of IFN-γ production. All rats were maintained at the P3 level animal facilities in Laboratory of Animal Experiment, Institute for Genetic Medicine, Hokkaido University. The experimental protocol was approved by the Animal Ethics Review Committee of our University.

Statistical analysis

Comparisons between individual data points were made using a Student's *t*-test. Two-sided P values < 0.05 were considered statistically significant.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

TO designed the study, performed all the experiments and the analysis, and wrote the manuscript. MN performed microscopic examinations. HO made contributions to design the study and participated in flow cytometric analysis. RT participated in flow cytometric analysis and HTLV-I infection experiments. HS made contributions to design the study and drafted the manuscript.

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References

1. Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, Kinoshita KI, Shirakawa S, Miyoshi I: **Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera.** *Proc Natl Acad Sci USA* 1981, **78**:6476-6480.
2. Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC: **Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma.** *Proc Natl Acad Sci USA* 1980, **77**:7415-7419.
3. Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A, de The G: **Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis.** *Lancet* 1985, **2**:407-410.
4. Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A, Matsumoto M, Tara M: **HTLV-I associated myelopathy, a new clinical entity.** *Lancet* 1986, **1**:1031-1032.
5. Hall WW, Liu CR, Schneewind O, Takahashi H, Kaplan MH, Roupe G, Vahlne A: **Deleted HTLV-I provirus in blood and cutaneous lesions of patients with mycosis fungoides.** *Science* 1991, **253**:317-320.
6. LaGrenade L, Hanchard B, Fletcher V, Cranston B, Blattner W: **Infective dermatitis of Jamaican children: a marker for HTLV-I infection.** *Lancet* 1990, **336**:1345-1347.
7. Mann DL, DeSantis P, Mark G, Pfeifer A, Newman M, Gibbs N, Popovic M, Sarngadharan MG, Gallo RC, Clark J, Blattner W: **HTLV-I-associated B-cell CLL: indirect role for retrovirus in leukemogenesis.** *Science* 1987, **236**:1103-1106.
8. Nishioka K, Maruyama I, Sato K, Kitajima I, Nakajima Y, Osame M: **Chronic inflammatory arthropathy associated with HTLV-I.** *Lancet* 1989, **1**:441.
9. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H: **Adult T-cell leukemia: clinical and hematologic features of 16 cases.** *Blood* 1977, **50**:481-492.
10. Seiki M, Hikikoshi A, Taniguchi T, Yoshida M: **Expression of the pX gene of HTLV-I: general splicing mechanism in the HTLV family.** *Science* 1985, **228**:1532-1534.
11. Yoshida M: **Discovery of HTLV-1, the first human retrovirus, its unique regulatory mechanisms, and insights into pathogenesis.** *Oncogene* 2005, **24**:5931-5937.
12. Jacobson S, Shida H, McFarlin DE, Fauci AS, Koenig S: **Circulating CD8+ cytotoxic T lymphocytes specific for HTLV-I pX in patients with HTLV-I associated neurological disease.** *Nature* 1990, **348**:245-248.
13. Kannagi M, Sugamura K, Kinoshita K, Uchino H, Hinuma Y: **Specific cytolysis of fresh tumor cells by an autologous killer T cell line derived from an adult T cell leukemia/lymphoma patient.** *J Immunol* 1984, **133**:1037-1041.
14. Kannagi M, Sugamura K, Sato H, Okochi K, Uchino H, Hinuma Y: **Establishment of human cytotoxic T cell lines specific for human adult T cell leukemia virus-bearing cells.** *J Immunol* 1983, **130**:2942-2946.
15. Parker CE, Daenke S, Nightingale S, Bangham CR: **Activated, HTLV-I-specific cytotoxic T-lymphocytes are found in healthy seropositives as well as in patients with tropical spastic paraparesis.** *Virology* 1992, **188**:628-636.
16. Harashima N, Kurihara K, Utsunomiya A, Tanosaki R, Hanabuchi S, Masuda M, Ohashi T, Fukui F, Hasegawa A, Masuda T, Takaue Y, Okamura J, Kannagi M: **Graft-versus-Tax response in adult T-cell leukemia patients after hematopoietic stem cell transplantation.** *Cancer Res* 2004, **64**:391-399.
17. Ohashi T, Hanabuchi S, Kato H, Koya Y, Takemura F, Hirokawa K, Yoshiki T, Tanaka Y, Fujii M, Kannagi M: **Induction of adult T-cell leukemia-like lymphoproliferative disease and its inhibition by adoptive immunotherapy in T-cell-deficient nude rats inoculated with syngeneic human T-cell leukemia virus type I-immortalized cells.** *J Virol* 1999, **73**:6031-6040.
18. Hanabuchi S, Ohashi T, Koya Y, Kato H, Takemura F, Hirokawa K, Yoshiki T, Yagita H, Okumura K, Kannagi M: **Development of human T-cell leukemia virus type I-transformed tumors in rats following suppression of T-cell immunity by CD80 and CD86 blockade.** *J Virol* 2000, **74**:428-435.
19. Ohashi T, Hanabuchi S, Kato H, Tateno H, Takemura F, Tsukahara T, Koya Y, Hasegawa A, Masuda T, Kannagi M: **Prevention of adult T-cell leukemia-like lymphoproliferative disease in rats by adoptively transferred T cells from a donor immunized with human T-cell leukemia virus type I Tax-coding DNA vaccine.** *J Virol* 2000, **74**:9610-9616.
20. Hanabuchi S, Ohashi T, Koya Y, Kato H, Hasegawa A, Takemura F, Masuda T, Kannagi M: **Regression of human T-cell leukemia virus type I (HTLV-I)-associated lymphomas in a rat model: peptide-induced T-cell immunity.** *J Natl Cancer Inst* 2001, **93**:1775-1783.
21. Yu YY, Netuschil N, Lybarger L, Connolly JM, Hansen TH: **Cutting edge: single-chain trimers of MHC class I molecules form stable structures that potently stimulate antigen-specific T cells and B cells.** *J Immunol* 2002, **168**:3145-3149.
22. Greten TF, Korangy F, Neumann G, Wedemeyer H, Schlote K, Heller A, Scheffer S, Pardoll DM, Garbe AI, Schneck JP, Manns MP: **Peptide-beta2-microglobulin-MHC fusion molecules bind antigen-specific T cells and can be used for multivalent MHC-Ig complexes.** *J Immunol Methods* 2002, **271**:125-135.
23. Tomaru U, Yamano Y, Nagai M, Maric D, Kaumaya PT, Biddison W, Jacobson S: **Detection of virus-specific T cells and CD8+ T-cell epitopes by acquisition of peptide-HLA-GFP complexes: analysis of T-cell phenotype and function in chronic viral infections.** *Nat Med* 2003, **9**:469-476.
24. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S: **SYFPEITHI: database for MHC ligands and peptide motifs.** *Immunogenetics* 1999, **50**:213-219.
25. Oved K, Lev A, Noy R, Segal D, Reiter Y: **Antibody-mediated targeting of human single-chain class I MHC with covalently**

linked peptides induces efficient killing of tumor cells by tumor or viral-specific cytotoxic T lymphocytes. *Cancer Immunol Immunother* 2005, **54**:867-879.
26. Hansen TH, Lybarger L: **Exciting applications of single chain trimers of MHC-I molecules.** *Cancer Immunol Immunother* 2006, **55**:235-236.
27. Ohashi T, Hanabuchi S, Suzuki R, Kato H, Masuda T, Kannagi M: **Correlation of major histocompatibility complex class I down-regulation with resistance of human T-cell leukemia virus type I-infected T cells to cytotoxic T-lymphocyte killing in a rat model.** *J Virol* 2002, **76**:7010-7019.
28. Satou Y, Yasunaga J, Yoshida M, Matsuoka M: **HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells.** *Proc Natl Acad Sci USA* 2006, **103**:720-725.
29. Kennedy R, Celis E: **T helper lymphocytes rescue CTL from activation-induced cell death.** *J Immunol* 2006, **177**:2862-2872.
30. Huang CH, Peng S, He L, Tsai YC, Boyd DA, Hansen TH, Wu TC, Hung CF: **Cancer immunotherapy using a DNA vaccine encoding a single-chain trimer of MHC class I linked to an HPV-16 E6 immunodominant CTL epitope.** *Gene Ther* 2005, **12**:1180-1186.
31. Arnulf B, Thorel M, Poirot Y, Tamouza R, Boulanger E, Jaccard A, Oksenhendler E, Hermine O, Pique C: **Loss of the ex vivo but not the reinducible CD8+ T-cell response to Tax in human T-cell leukemia virus type I-infected patients with adult T-cell leukemia/lymphoma.** *Leukemia* 2004, **18**:126-132.
32. Kozako T, Arima N, Toji S, Masamoto I, Akimoto M, Hamada H, Che XF, Fujiwara H, Matsushita K, Tokunaga M, Haraguchi K, Uozumi K, Suzuki S, Takezaki T, Sonoda S: **Reduced frequency, diversity, and function of human T cell leukemia virus type I-specific CD8+ T cell in adult T cell leukemia patients.** *J Immunol* 2006, **177**:5718-5726.
33. Tateno M, Kondo N, Itoh T, Chubachi T, Togashi T, Yoshiki T: **Rat lymphoid cell lines with human T cell leukemia virus production. I. Biological and serological characterization.** *J Exp Med* 1984, **159**:1105-1116.
34. Ishiguro N, Abe M, Seto K, Sakurai H, Ikeda H, Wakisaka A, Togashi T, Tateno M, Yoshiki T: **A rat model of human T lymphocyte virus type I (HTLV-I) infection. I. Humoral antibody response, provirus integration, and HTLV-I-associated myelopathy/tropical spastic paraparesis-like myelopathy in seronegative HTLV-I carrier rats.** *J Exp Med* 1992, **176**:981-989.
35. Hasegawa A, Ohashi T, Hanabuchi S, Kato H, Takemura F, Masuda T, Kannagi M: **Expansion of Human T-Cell Leukemia Virus Type I (HTLV-I) Reservoir in Orally Infected Rats: Inverse Correlation with HTLV-I-Specific Cellular Immune Response.** *J Virol* 2003, **77**:2956-2963.
36. Komori K, Hasegawa A, Kurihara K, Honda T, Yokozeki H, Masuda T, Kannagi M: **Reduction of human T-cell leukemia virus type I (HTLV-I) proviral loads in rats orally infected with HTLV-I by reimmunization with HTLV-I-infected cells.** *J Virol* 2006, **80**:7375-7381.
37. Nomura M, Ohashi T, Nishikawa K, Nishitsuji H, Kurihara K, Hasegawa A, Furuta RA, Fujisawa J, Tanaka Y, Hanabuchi S, Harashima N, Masuda T, Kannagi M: **Repression of tax expression is associated both with resistance of human T-cell leukemia virus type I-infected T cells to killing by tax-specific cytotoxic T lymphocytes and with impaired tumorigenicity in a rat model.** *J Virol* 2004, **78**:3827-3836.

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Immunogenicity of newly constructed attenuated vaccinia strain LC16m8Δ that expresses SIV Gag protein

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ABSTRACT

We developed the method to efficiently construct recombinant vaccinia viruses based on LC16m8Δ strain that can replicate in mammalian cells but is still safe in human. Immunization in a prime-boost strategy using DNA and LC16m8Δ expressing SIV Gag elicited 7–30-fold more IFN-γ-producing T cells in mice than that using DNA and non-replicating vaccinia DIs recombinant strain. As the previous study on the DNA-prime and recombinant DIs-boost anti-SIV vaccine showed protective efficacy in the macaque model [Someya K, Ami Y, Nakasone T, Izumi Y, Matsuo K, Horibata S, et al. Induction of positive cellular and humoral responses by a prime-boost vaccine encoded with simian immunodeficiency virus gag/pol. J Immunol 2006;176(3):1784–95], LC16m8Δ would have potential as a better recombinant viral vector for HIV vaccine.

1. Introduction

As vehicles for delivering antigens of HIV-1, replication-defective viral vectors have been extensively studied because of their safety. For example adenovirus and vaccinia virus-based vectors expressing Gag, Nef, and other components of HIV-1 have been evaluated in monkeys [1,3] and human trials [2,4,5]. They, however, generally have not induced sufficient level of immunity nor protected human from HIV-1 infection although they elicited considerable anti HIV/SIV immunities in animal models [6]. Moreover, controversial results have been reported on containment of challenged viruses depending on SIV or SHIV, a hybrid virus between HIV-1 and SIV, in monkey models [1,7]. Therefore more effective vehicles may be needed for HIV vaccine development.

Replication-competent vaccinia virus that has been proven to be safe in human vaccination against small pox could be a good candidate for a better vehicle. Vaccinia LC16m8 strain has been shot to 100,000 people without any serious adverse effects [8]. The LC16m8, however, has been found to be genetically unstable and to generate spontaneously more virulent revertants from stock of LC16m8 viruses. To improve LC16m8, we identified the B5R gene responsible for the reversion, and constructed genetically stable LC16m8Δ, which is essentially as same as LC16m8 in antigenicity and safety in mice, and approximately 1000-fold more immunogenic than non-replicating vaccinia, MVA strain. In particular, LC16m8Δ never elicited any symptoms in severe combined immunodeficiency disease mice even at 10^7 pfu dose [9]. Therefore LC16m8Δ could be a better vehicle for vaccines against HIV and other human diseases.

Gag proteins of HIV-1 and SIV are major antigens to elicit cytotoxic T lymphocyte (CTL) responses. Activity of anti Gag CTL in HIV-1-infected people inversely correlates with their viral loads [10]. In some monkey experiments of SIV infection, the strength of anti Gag CTL has been reported to correlate with the containment of SIV [11]. Therefore, we constructed LC16m8Δ that expresses the *gag* gene of SIVmac239 to compare its ability to elicit anti Gag immunity with replication-defective vaccinia virus DIs strain, which has been reported to be immunogenically similar to MVA [12], and to evaluate its potential as a recombinant vector for HIV vaccine development.

During the course of constructing LC16m8Δ-based recombinant viruses, we encountered a drawback, such as inefficient incorporation of the foreign gene by conventional method in which an *in vivo* recombination process is involved. Therefore, in this paper we firstly describe our new device including construction of a new strain m8Δvnc110, which prompts construction of m8Δ express-

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ing the *gag* gene of SIV by *in vitro* ligation of the vaccinia genome with foreign DNA.

2. Materials and methods

2.1. Cells and viruses

Rabbit RK13 cells were cultured in RPMI1640 supplemented with 10% FCS. Human HeLa, mouse L929, NIH3T3, hamster BHK, and primary chicken embryo fibroblast (CEF) cells were maintained in DMEM supplemented with 10% FCS. Canarypox virus (a kind gift of National Institute of Animal Health) [13], and LC16m8Δ [9] and DIs [14] strains of vaccinia virus were used. Viral titers were calculated on the basis of the number of plaques on CEF. The titer of LC16m8Δ was similar when titrated on RK13 and CEF monolayers.

2.2. Construction of pJWSIVgag

To construct the plasmid that expresses the SIV *gag* gene under the cytomegalovirus promoter, the *gag* coding region was amplified with a pair of primer SIVGAGF1 (GCCAAGCTTGCCACC-ATGGGCGTGAGAAACTCCGTCTTGTCAGG; the underlined sequence is HindIII site) and SIVGAGR1 (CGCGCCCGGGCTACTGGT CTCCTC-CAAAGAGAGAATTGAGGTGCAGC; the underlined sequence is XmaI site) using pSIVmac239 [15] as a template under the condition: 2 min at 94 °C, 20 cycles of 30 s at 94 °C, 60 s at 60 °C, 2 min at 72 °C, and a final extension for 5 min at 72 °C. The *gag* fragment generated was digested with HindIII and XmaI, and then ligated with the enzyme-digested pJW322, which harbors the cytomegalovirus promoter derived from pJW4303 [16] (a kind gift of Dr. Y. Takebe).

2.3. Construction of m8Δvnc110

To generate a transfer plasmid pVNC110, the vnc/KE sequence (5′-GGTACCCGCCCGGGCCGGACCGGCCGGCCGAATTC-3′) containing four restriction enzyme sites (SrfI, SfiI, RsrII, and FseI), which are not present in the vaccina virus genomes, was inserted between KpnI and EcoRI sites of pSFJ1-10, which harbors a strong composite promoter consisting of the cowpox A-type inclusion body (ATI) and multiple mutated-p7.5 promoters (PSFJ1-10 promoter), which are sandwiched with the segments of the vaccinia hemagglutinin (*HA*) gene [17,18]. pVNC110 resultant was verified by sequencing to harbor these sites downstream of PSFJ1-10 promoter, which is sandwiched by the segments of the vaccinia *HA* gene.

Next, we transfected 1.5 μg of pVNC110 to 1×10^5 BHK cells, which had been infected with LC16m8Δ at 0.05 moi, to construct m8Δvnc110. After the culture at 33 °C for 2 days, the progeny viruses were harvested and their plaques were formed on RK13 cell monolayer. The candidate viruses were selected on the basis of HA^- phenotype [19]. To ascertain whether the virus contains the expected sequences of pVNC110 in the *HA* gene, the virus-enriched fraction was prepared by disruption of the infected cells by repeated freeze and thaw followed by clarification by low speed centrifugation and concentration by centrifugation at 15,000 rpm for 30 min in microcentrifuge at 4 °C. Then, it was used as a template for PCR with a pair of the primers vvHA867s (GGATC-TACACATTCACCAGA) and vvHA1009as (CTAGTGTATGTGACGGTGT), the sequences of which were present in the *HA* gene, under the condition: 2 min at 94 °C, 30 cycles of 30 s at 94 °C, 60 s at 54 °C, 60 s at 72 °C, and a final extension for 5 min at 72 °C. Virus containing the sequence of VNC110 produced a 1 kb fragment of PCR product.

2.4. Construction of m8ΔSIVgag

Viral particles of m8ΔVNC110 were purified by method including banding in sucrose gradient centrifugation [20], and then viral DNA was isolated by phenol extraction method. The vaccinia DNA was digested with CpoI and FseI followed by purification with phenol extraction and ethanol precipitation. The *gag* region in SIVmac239 genome was amplified by PCR using pSIVmac239 [15] as a template with a pair of the primers CPO-SIV gag f2 (TTTCGGACCGCCACCATGGGCGTGAGAAACTCCGTCTTG; underlined sequence is CpoI site) and FSE-SIV gag r1 (TATGGCCGGCCTAC-TGGTCTCCTCCAAAGAGAGA; underlined sequence is FseI site) under the condition: 2 min at 94 °C, 20 cycles of 30 s at 94 °C, 60 s at 60 °C, 2 min at 72 °C, and a final extension for 10 min at 72 °C. The gag fragment was digested with CpoI and FseI followed by purification with PCR purification kit (Qiagen, Hilden, Germany). The digested vaccinia genome (5 μg) and gag fragment (0.3 μg) were ligated using a ligation kit (Takara, Otsu, Japan) according to the manual, purified by phenol extraction and concentrated by ethanol precipitation. The ligated DNA was transfected with lipofectamine LTX (Invitrogen, Carlsbad, USA) to 3.5×10^5 BHK cells that had been infected at 10 moi with canarypox virus. Usage of avipox viruses as a helper virus has been well established [21]. After 2 days culture at 33 °C, the progeny viruses were harvested by repeated freeze and thaw and titrated on the monolayer of RK13 cells. m8ΔSIVgag was cloned from single plaque and its homogeneity was evaluated by staining the plaques with sera of monkey infected with SIVmac239 and alkaline phosphatase-conjugated anti monkey IgG antibody followed by NBT/BCIP coloring reaction. All plaques were positively stained.

2.5. Construction of rDIs/PSFJ/SIVgag

To construct a complementary transfer vector for the deleted region of DIs, we used a pDIsgptmH5 plasmid (a kind gift of Dr. K. Ishii) that possesses both the modified H5 promoter and the *E. coli* guanine phosphoribosyltransferase (gpt) gene driven by a P7.5 promoter, which are sandwiched with the DIs fragments adjacent to the deleted region [22]. A vaccinia synthetic PSFJ1-10 promoter sequence [17] was amplified by PCR at 52 °C of the annealing temperature using a pair of the primers: PSFJ1-10s (ACATGCATGCATGAAGTTGAAGATGATG: underlined sequence is SphI site) and PSFJ1-10r (GATATCCTCGAGCA-GCACACCGTGCAATAAATT: underlined sequence is EcoRV and XhoI sites). To substitute the PSFJ1-10 promoter for the mH5 promoter, the PCR product was inserted into the SphI and EcoRV sites of pDIsgptmH5, generating pUC/DIs/PSFJ that could express the foreign antigen gene under the control of the PSFJ1-10 promoter. A DNA fragment encoding the full-length gag gene of SIVmac239 was amplified by PCR at 55 °C of the annealing temperature using a pair of the primers: gag-s (CCCCCGGGATGGGCGTGAGAAACTCC: underlined sequence is SmaI site) and gag-r (CCGGAGCTCCTACTGGTCTCCTCCAAAGAG: underlined sequence is SacI site), and inserted into the SmaI and SacI sites of pUC/DIs/PSFJ to generate the transfer vector, named pUC/DIs/PSFJ/SIVgag. This plasmid (10 μg) was transfected by Gene-Pulser (Bio-Rad Laboratories, Inc. Hercules, USA) to CEF infected with DIs at 1.0 moi. Recombinant DIs clones expressing the SIV *gag* gene were selected in the presence of gpt [23].

2.6. Western blotting

m8ΔSIVgag and rDIs/PSFJ/SIVgag were infected to various cells at 3 or 5 moi and cultured for 24 h at 33 °C. Then the infected cells and culture medium were collected and their protein amounts were quantified by BCA assay. Appropriate amounts of the cell lysates and medium fraction indicated in the figure legends were subjected to 12% SDS-PAGE and immunologically detected using 500-fold-diluted sera from SIVmac239-infected monkey and alkaline phosphatase-conjugated anti monkey IgG antibody (Promega, Madison, USA) followed by NBT/BCIP coloring reaction.

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2.7. Immunization

Seven-week-old female C57/BL6 mice were purchased from CLEA Co. Ltd. (Tokyo, Japan). Fifty microgram of pJWSIVgag was intramuscularly injected into the right and left quadriceps, and 1×10^6 pfu of vaccinia viruses were inoculated intradermally according to the schedule indicated in Figs. 3A and 4A. All mice were maintained according to the institutional animal care and the guidelines of Hokkaido University. The study was conducted in a biosafety level 2 facility under the approval of an institutional committee for biosafety and in accordance with the requirements of the World Health Organization.

2.8. Assay of cellular immune response by IFN-γ ELISPOT

SIV Gag specific IFN-γ producing cells were quantified 2 or 3 weeks after the final immunization using an ELISPOT kit for mouse IFN-γ (R&D Systems, Minneapolis, USA). The excised spleens were disrupted with a syringe plunger and passed through a cell strainer (Becton Dickinson, Franklin Lakes, USA). Isolated spleen cells were suspended at 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin. Aliquots (100 μl) were plated into wells that were coated with anti-mouse IFN-γ antibody and stimulated with SIV Gag-specific 15 mer overlapping peptide pools (0.5 μg of peptides/10^5 cells) (A gift of AIDS Research and Reference Reagent program. Catalog #6204). Cells mock-stimulated with medium alone served as a negative control while cells treated with 50 ng/ml of phorbol 12-myristate (PMA) and 0.5 μg/ml of calcium ionomycin were used as a positive control to ascertain the number of viable T cells. After 24 h incubation, IFN-γ secreting cells were detected according to the manufacturer's instructions. Numbers of spot forming cells (SFC) were determined using the ImmunoScan Plate Reader with ImmunoSpot software (Cellular Technology Limited, Cleveland, USA).

2.9. Proliferation assays

Lymphocyte proliferation was measured by incorporation of BrdU into the stimulated-lymphocytes using cell proliferation ELISA BrdU kit (Roche Applied Science, Mannheim, Germany). Isolated spleen cells (1×10^5) were cultured in a 96-well assay plate (BD Falcon, Franklin Lakes, USA) in the presence or absence of recombinant SIV Gag protein (SIVmac251 p27; Advanced Biotechnologies, Inc., Columbia, USA) at 5.0 μg/ml for antigen-specific stimulation. The plates were incubated for 2 days at 37 °C, and then another 24 h in the presence of BrdU (100 μM). Uptake of BrdU was determined using luminometer (Wallac 1420; PerkinElmer, Branchburg, USA). The results were expressed as the stimulation index (SI), which was calculated as a ratio of relative light unit per second in the presence to that in the absence of the antigen.

2.10. Statistical analysis

Data were expressed as arithmetic mean ± standard error of means (mean ± S.E.M.). The data analysis was carried out by using Student's *t*-test (EXCEL version 11.5, Microsoft). A *P*-value of <0.05 was considered significant.

3. Results

3.1. Construction of m8ΔSIVgag

Fig. 1 illustrates the outline for construction of m8ΔSIVgag. Firstly we constructed m8ΔVNC110 strain by usual method in which pVNC110 was transfected to BHK cells that had been infected with LC16m8Δ. Resultant m8ΔVNC110 harbors PSFJ1-10 promoter followed by the multi-cloning sites containing the restriction enzyme sites which are not present in the vaccinia genome. To construct m8ΔSIVgag, the genomic DNA extracted from ΔVNC110 virions was digested with CpoI and FseI, which do not cut the other part of the vaccinia genome, and ligated with SIV *gag* fragment *in vitro*. Then the ligation mixture was transfected to BHK cells that had been infected with canarypox virus, which cannot replicate in mammalian cells. A clone, named m8ΔSIVgag3, that was isolated from one among six plaques formed by the progeny viruses produced SIV Gag protein judged by staining the plaques with sera derived from a SIV-infected monkey.

3.2. Comparison of Gag production by LC16m8Δ and DIs-based recombinants

Previously Honda's group constructed replication-deficient vaccinia DIs strain-based recombinant, which had immunogenicity similar to MVA-based recombinant [12]. We now compared by

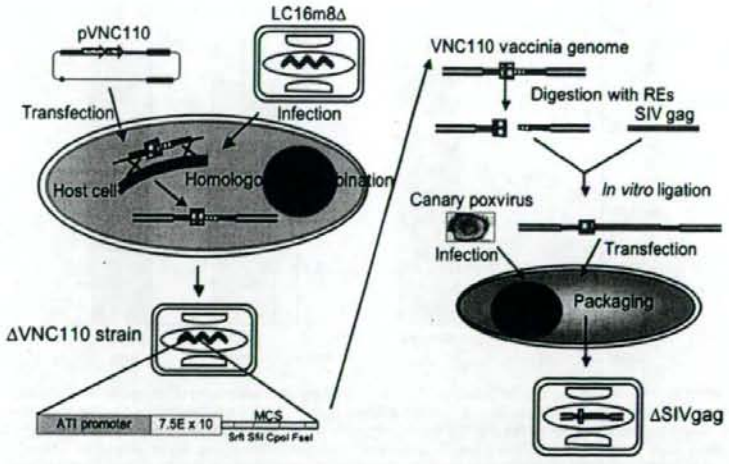

Fig. 1. Schematic presentation for construction of m8ΔSIVgag.

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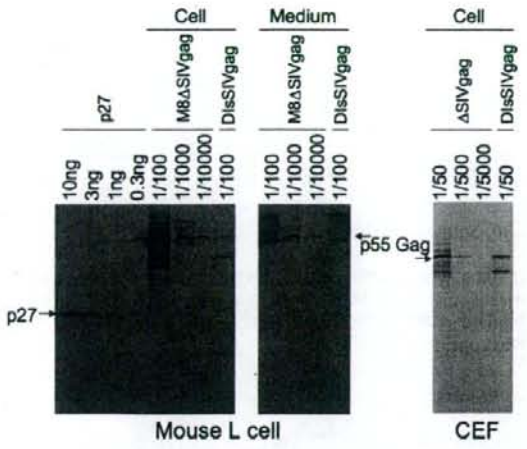

Fig. 2. Western blotting for p55 Gag produced by vaccinia recombinants. Appropriate fractions (1/50–1/10,000) of the cell lysates and medium prepared from m8ΔSIVgag- or rDIs/PSFJ/SIVgag-infected cells were subjected to Western blotting. One hundredth of the cell lysates contains approximately 1 μg of proteins.

Western blotting the amount of Gag protein produced by LC16m8Δ and DIs, both of which used the same promoter for expression of the foreign gene (Fig. 2). In mouse L cells, where DIs is not able to replicate, approximately 100-fold more amount of Gag protein was detected in both medium and cells infected at high multiplicity with m8ΔSIVgag than that in the cells infected with rDIs/PSFJ/SIVgag, whereas m8ΔSIVgag produced Gag protein just several fold more than rDIs/PSFJ/SIVgag in CEF in which both viruses replicate. In human HeLa, mouse NIH3T3, and rabbit RK13 cells m8ΔSIVgag again produced Gag protein 100-fold more than rDIs/PSFJ/SIVgag (data not shown). These results suggest that production of Gag is affected by not only the promoter just upstream of the foreign gene but also the replication capability of the vector virus.

3.3. Immunogenicity of LC16m8Δ and DIs-based recombinants

Next, we evaluated the immunogenicities of these recombinant viruses in mice by priming with plasmid pJWSIVgag expressing the *gag* gene followed by boosting with these recombinant viruses. Considering the preceding reports that viral vectors failed to elicit enough immunities in human although they were nicely antigenic in mice under optimal immunization schedule [4,24], we compared their immunogenicities under the suboptimal condition that includes two or three priming with pJWSIVgag followed by boosting once with 1×10^6 pfu of the recombinant viruses. We have assessed the number of IFN-γ producing cells by ELISPOT assay 2 weeks after a shot of the recombinant viruses, and found that sevenfold more cells were induced by prime-boost vaccination with pJWSIVgag and SIVm8Δgag than that of rDIs/PSFJ/SIVgag (Fig. 3). We also evaluated the induction of Gag specific IFN-γ producing cells by single immunization with pJWSIVgag, SIVm8Δgag or rDIs/PSFJ/SIVgag. In contrast to prime-boost regimen, significant positive spots were not detected by ELISPOT assay (data not shown). When assayed 3 weeks after final immunization with the viruses, differences were more prominent in that only one among four mice immunized with rDIs/PSFJ/SIVgag were ELISPOT positive com-

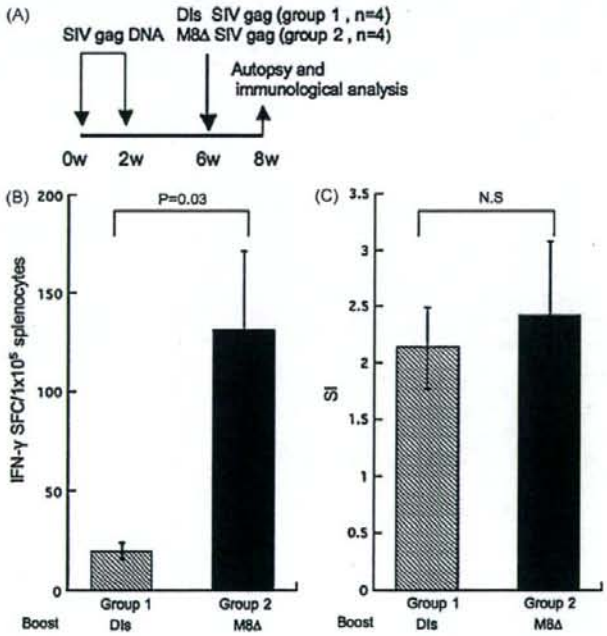

Fig. 3. Comparison of booster effect by m8ΔSIVgag and rDIs/PSFJ/SIVgag. (A) Schematic drawing of experimental design for immunization. Mice were immunized twice with SIVgag DNA followed by one boost with rDIs/PSFJ/SIVgag (group 1) or m8ΔSIVgag (group 2). (B) Frequency of SIV Gag-specific IFN-γ-producing cells in immunized mice. Spleen cells were stimulated with pooled SIV Gag peptides, and IFN-γ-producing cells were detected by IFN-γ-specific ELISPOT assays. Data are expressed as the mean number of SFC per 10^5 splenocytes ± S.E.M. (C) Induction of SIV Gag-specific lymphocyte proliferatives response. Spleen cells were cultured in the presence or absence of SIV p27 antigen, and incorporation of BrdU was measured as described in Section 2. Proliferative responses were presented as the value of the stimulation index (SI). Data are mean ± S.E.M. N.S. means not significant.

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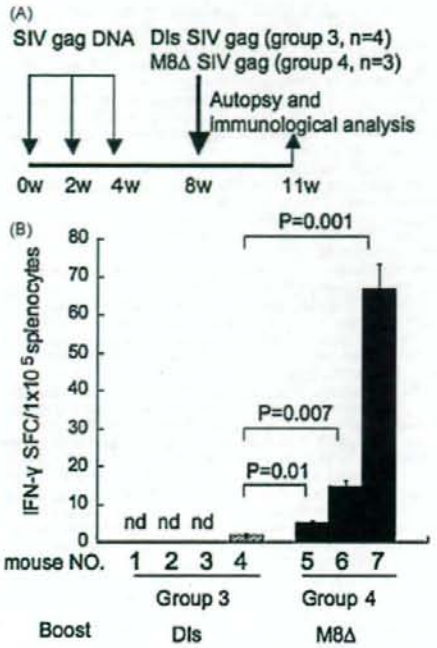

Fig. 4. Comparison of immunogenicities of m8ΔSIVgag and rDIs/PSFJ/SIVgag. (A) Schematic drawing of experimental design for immunization. Mice were immunized three times with SIVgag DNA followed by one boost with rDIs/PSFJ/SIVgag (group 3) or m8ΔSIVgag (group 4). (B) Frequencies of SIV Gag-specific IFN-γ-producing cells in individual immunized mice were presented as the number of SFC per 10^5 splenocytes. SFC of individual mouse was counted in triplicate and presented as the means ± S.E.M.

pared with all positive mice with m8Δgag and average of ELSPOT was approximately 30-fold more in mice immunized with m8Δgag than rDIs/PSFJ/SIVgag (Fig. 4). To monitor the sensitivity of ELISPOT assays, we always included positive controls that were splenocytes stimulated with PMA and ionomycin, and ascertained that they produced 300–500 spots/10^5 splenocytes in every experiment (data not shown).

Proliferation capacities of the lymphocytes derived from the immunized mice were also compared based on BrdU incorporation. Splenocytes from both immunized groups showed low levels of T-cell proliferation in response to stimulation with SIV Gag protein (Fig. 3). But we did not find significant difference between the mice immunized by either virus in contrast to the results of ELISPOT assay described above.

4. Discussion

In this study, we devised a new method involving *in vitro* ligation to efficiently construct recombinant vaccinia viruses expressing the foreign genes. We could construct SIV Gag expressing m8Δ only by this technique but not by the conventional method, which involves *in vivo* ligation. Moreover, we have successfully constructed two additional recombinant viruses expressing the chimeric genes, which contain rat MHC class I with an epitope sequence fused with β2 microglobulin. Approximately 60% of the progeny viruses expressed the transgenes even when no methods were used to enrich the recombinants (They will be published elsewhere.), suggesting that this new technique is generally applicable to construct m8Δ-based recombinant viruses.

Here, using the same promoter in both recombinant DIs and LC16m8Δ strains of vaccinia, we have demonstrated a much more efficient expression of SIV Gag transgene by the latter in several mammalian cells, which were infected at a high dose of inoculum. In contrast, less difference was observed in the level of Gag protein expression in CEF probably because both recombinant viruses propagated at comparable level in this cell type. These results suggest that vaccinia viruses, which propagate better, could provide more efficacious expression of immunogens of interest.

The propagation capability and related efficacy of Gag production by these recombinant vaccinia viruses may reflect their immunogenicity. IFN-γ producing T cells evaluated by ELISPOT were more efficiently elicited by m8ΔSIVGag and lasted longer than those by rDIs/PSFJ/SIVgag. Since several non-replicating vaccinia virus vectors including DIs, MVA, and NYVAC have been shown to be similarly immunogenic in mice [12,25], replication-competent vector such as LC16m8Δ may be more immunogenic than general non-replicating vectors. Since anti-SIV vaccination comprising the DNA-prime and recombinant DIs-boost has been reported to elicit protective immunity in the macaque model [26], it may be expected that m8ΔSIVGag would confer better protection against SIV challenge.

Our results are in contrast with reports by Hirsch et al., that showed similar level of immunogenicity between SIV Gag recombinant MVA and a replication-competent vaccinia vectors when a very high dose of vaccine was applied [27]. However, it should be important to evaluate the immunogenicities of vaccinia recombinants under the suboptimal immunization schedule, including a single boost with a low dose of vaccinia recombinants, which was adopted in this study, given that unsuccessful outcome of the human trials by the vaccines [2,4] that had been appreciated based on protective immune responses elicited by optimal immunization schedule in model animals [24,28].

Mucosal immunity has been suggested to be important for protection against HIV, because it sexually transmit in most cases. Since history of exposure of replicating virus in mucosal tissues has been reported to prime the mucosal immune system and lead to the induction of secretary IgA [29], it is expected that LC16m8Δ vaccination via the mucosal route may induce effective mucosal immunity. Moreover, replicating adenovirus vector has also been reported to be more effective than non-replicating one [30].

In contrast to the more efficient induction of IFN-γ producing T cells by m8ΔSIVGag, the Gag-specific lymphocyte proliferation responses were similarly elicited by both recombinants. Vaccinia viruses produce various kinds of immunomodulatory factors, which may tend to elicit uneven immunities [31]. Therefore, it is conceivable that quantitative and qualitative differences of the factors produced by LC16m8Δ and DIs strains may cause the diverged immune responses.

In summary, we devised an efficient method to construct a recombinant virus based on LC16m8Δ and evaluated it as a vaccine candidate. This replication-competent virus vector showed merits for further development in the viewpoint of its ability to elicit enhanced cell-mediated and hopefully humoral and mucosal immune responses.

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Institute of Animal Health was obtained through Dr. S. Morikawa (NIID).

References

[1] Casimiro DR, Wang F, Schleif WA, Liang X, Zhang Z, Tobery TW, et al. Attenuation of simian immunodeficiency virus SIVmac239 infection by prophylactic immunization with DNA and recombinant adenoviral vaccine vectors expressing Gag. J Virol 2005;79(24):15547–55.
[2] Cox KS, Clair JH, Prokop MT, Sykes KJ, Dubey SA, Shiver JW, et al. DNAgag/Ad5gag and Ad5gag/Ad5gag vaccines induce distinct T cell response profiles. J Virol 2008;82(16):8161–71.
[3] Vogel TU, Reynolds MR, Fuller DH, Vielhuber K, Shipley T, Fuller JT, et al. Multispecific vaccine-induced mucosal cytotoxic T lymphocytes reduce acute-phase viral replication but fail in long-term control of simian immunodeficiency virus SIVmac239. J Virol 2003;77(24):13348–60.
[4] Goonetilleke N, Moore S, Dally L, Winstone N, Cebere I, Mahmoud A, et al. Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8 T-cell epitopes. J Virol 2006;80(10):4717–28.
[5] Harari A, Bart PA, Stöhr W, Tapia G, Garcia M, Medjitna-Rais E, et al. An HIV-1 clade C DNA prime, NYVAC boost vaccine regimen induces reliable, polyfunctional, and long-lasting T cell responses. J Exp Med 2008;205(1):63–77.
[6] Walker BD, Burton DR. Toward and AIDS Vaccine. Science 2008; 320(5877):760–4.
[7] Shiver JW, Fu TM, Chen L, Casimiro DR, Davies ME, Evans RK, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature 2002;415(6869):331–5.
[8] Hashizume S, Yoshizawa H, Morita M, Suzuki K. Properties of attenuated mutant of vaccinia virus, LC16m8, derived from Lister strain. In: Ouinnan GV, editor. In Vaccinia viruses as Vectors for Vaccine Antigens. Amsterdam: Elsevier; 1985. p. 421–8.
[9] Kidokoro M, Tashiro M, Shida H. Genetically stable and fully effective smallpox vaccine strain constructed from highly attenuated vaccinia LC16m8. Proc Natl Acad Sci USA 2005;102(11):4152–7.
[10] Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, Moodley E, et al. CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 2007;13(1):46–53.
[11] Matano T, Kobayashi M, Igarashi H, Takeda A, Nakamura H, Kano M, et al. Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J Exp Med 2004;199(12):1709–18.
[12] Okamura T, Someya K, Matsuo K, Hasegawa A, Yamamoto N, Honda M. Recombinant vaccinia DIs expressing simian immunodeficiency virus gag and pol in mammalian cells induces efficient cellular immunity as a safe immunodeficiency virus vaccine candidate. Microbiol Immunol 2006;50(12):989–1000.
[13] Amano H, Morikawa S, Shimizu H, Shoji I, Kurosawa D, Matsuura Y, et al. Identification of the canarypox virus thymidine kinase gene and insertion of foreign genes. Virology 1999;256(2):280–90.
[14] Ishii K, Ueda Y, Matsuo K, Matsuura Y, Kitamura T, Kato K, et al. Structural analysis of vaccinia virus DIs strain: application as a new replication-deficient viral vector. Virology 2002;302(2):433–44.
[15] Kestler H, Kodama T, Ringler D, Marthas M, Pedersen N, Lackner A, et al. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science 1990;248(4959):1109–12.
[16] Lu S, Manning S, Arthos J. Antigen Engineering in DNA Immunization. Totowa, NJ: Humana Press; 1999. pp. 355–74.
[17] Jin NY, Funahashi S, Shida H. Constructions of vaccinia virus A-type inclusion body protein, tandemly repeated mutant 7.5 kDa protein, and hemagglutinin gene promoters support high levels of expression. Arch Virol 1994;138(3–4):315–30.
[18] Shida H, Tochikura T, Sato T, Konno T, Hirayoshi K, Seki M, et al. Effect of the recombinant vaccinia viruses that express HTLV-I envelope gene on HTLV-I infection. EMBO J 1987;6(11):3379–84.
[19] Shida H, Matsumoto S. Analysis of the hemagglutinin glycoprotein from mutants of vaccinia virus that accumulates on the nuclear envelope. Cell 1983;33(2):423–34.
[20] Joklik WK. The purification of four strains of poxvirus. Virology 1962;18:9–18.
[21] Scheiflinger F, Dorner F, Falkner FG. Construction of chimeric vaccinia viruses by molecular cloning and packaging. Proc Natl Acad Sci USA 1992;89(21):9977–81.
[22] Ishii K, Hasegawa H, Nagata N, Mizutani T, Morikawa S, Suzuki T, et al. Induction of protective immunity against severe acute respiratory syndrome coronavirus (SARS-CoV) infection using highly attenuated recombinant vaccinia virus DIs. Virology 2006;351(2):368–80.
[23] Wolffe EJ, Isaacs SN, Moss B. Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. J Virol 1993;67(8):4732–41.
[24] Hanke T, Blanchard TJ, Schneider J, Hannan CM, Becker M, Gilbert SC, et al. Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. Vaccine 1998;16(5):439–45.
[25] Gómez CE, Nájera JL, Jiménez EP, Jiménez V, Wagner R, Graf M, et al. Head-to-head comparison on the immunogenicity of two HIV/AIDS vaccine candidates based on the attenuated poxvirus strains MVA and NYVAC co-expressing in a single locus the HIV-1BX08 gp120 and HIV-1(IIIB) Gag-Pol-Nef proteins of clade B. Vaccine 2007;25(15):2863–85.
[26] Someya K, Ami Y, Nakasone T, Izumi Y, Matsuo K, Horibata S, et al. Induction of positive cellular and humoral responses by a prime-boost vaccine encoded with simian immunodeficiency virus gag/pol. J Immunol 2006;176(3):1784–95.
[27] Hirsch VM, Fuerst TR, Sutter G, Carroll MW, Yang LC, Goldstein S, et al. Patterns of viral replication correlate with outcome in simian immunodeficiency virus (SIV)-infected macaques: effect of prior immunization with a trivalent SIV vaccine in modified vaccinia virus Ankara. J Virol 1996;70(6):3741–52.
[28] Juillard V, Villefroy P, Godfrin D, Pavirani A, Venet A, Guillet JG. Long-term humoral and cellular immunity induced by a single immunization with replication-defective adenovirus recombinant vector. Eur J Immunol 1995;25(12):3467–73.
[29] Herremans TM, Reimerink JH, Buisman AM, Kimman TG, Koopmans MP. Induction of mucosal immunity by inactivated poliovirus vaccine is dependent on previous mucosal contact with live virus. J Immunol 1999;162(8):5011–8.
[30] Peng B, Wang LR, Gómez-Román VR, Davis-Warren A, Montefiori DC, Kalyanaraman VS, et al. Replicating rather than nonreplicating adenovirus-human immunodeficiency virus recombinant vaccines are better at eliciting potent cellular immunity and priming high-titer antibodies. J Virol 2005;79(16):10200–9.
[31] Seet BT, Johnston JB, Brunetti CR, Barrett JW, Everett H, Cameron C, et al. Poxviruses and immune evasion. Annu Rev Immunol 2003;21:377–423.

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Effects of Immunization with CCR5-Based Cycloimmunogen on Simian/HIV$_{SF162P3}$ Challenge[1]

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A synthetic cycloimmunogen targeting the HIV-1 coreceptor CCR5 was evaluated for its capacity to induce CCR5-specific Abs with anti-HIV-1 activity in cynomolgus macaques. The cyclic closed-chain dodecapeptide (cDDR5) mimicking the conformation-specific domain of human CCR5 was chemically prepared, in which the Gly-Glu dipeptide links the amino and carboxy termini of the decapeptidyl linear chain (Arg^{168} to Thr^{177}) derived from the undecapeptidyl arch (Arg^{168} to Cys^{178}) of extracellular loop-2 in CCR5. The immunization of cynomolgus macaques with the cDDR5-conjugated multiple-Ag peptide (cDDR5-MAP) induced anti-cDDR5 serum production for ~15 wk after the third immunization. The antisera raised against cDDR5-MAP reacted with both human and macaque CCR5s, and potently suppressed infection by the R5 HIV-1 laboratory isolate (HIV$_{JRFL}$), R5 HIV-1 primary isolates (clade A:HIV$_{93RW004}$ and clade C:HIV$_{MJ4}$), and a pathogenic simian/HIV (SHIV$_{SF162P3}$) bulk isolate in vitro. To examine the prophylactic efficacy of anti-CCR5 serum Ab for acute HIV-1 infection, cynomolgus macaques were challenged with SHIV$_{SF162P3}$. The cDDR5-MAP immunization attenuated the acute phase of SHIV$_{SF162P3}$ replication. The geometric mean plasma viral load in the vaccinated macaques was 217.10 times lower than that of the control macaques at 1 wk postchallenge. Taken together, these results suggest that cDDR5-MAP immunization is an effective prophylactic vaccine strategy that suppresses and delays viral propagation during the initial HIV-1 transmission for the containment of HIV-1 replication subsequent to infection. *The Journal of Immunology,* **2006, 176: 463–471.**

Although numerous trials on preventive HIV-1 vaccines are ongoing (International AIDS Vaccine Initiative report, ⟨www.iavi.org/iavireport⟩) or being planned, a major obstacle to the development of an HIV-1 vaccine is unfortunately the marked genetic diversity of HIV-1 (1). To contend with this issue, some attempts have been made to match candidate vaccines with strains prevalent in sites in which phase III efficacy trials are to be conducted (2–4). Other strategies include the use of mixture vaccines containing Ags representative of several genetic subtypes (5, 6), the design of candidate vaccines targeting conserved HIV-1 epitopes (7, 8), and the use of candidate vaccines based on the consensus or ancestor sequences selected to minimize genetic differences between vaccine strains and current isolates (2, 9).

As alternative approaches for developing HIV-1 vaccine, other attempts have also been made to induce anti-CCR5 Abs that can bind native CCR5 and block viral infection because CCR5 is genetically stable, unlike viral targets that may rapidly mutate during the course of infection (10–14), and has been considered important in HIV-1 transmission on the basis of the findings that individuals homozygous for a 32-bp deletion in the CCR5-coding region have a very low susceptibility to HIV-1 infection (15–19). Furthermore, CCR5 is also considered as a redundant molecule in adults because CCR5-defective individuals have normal inflammatory and immune reactions (20). In fact, it is reported that CCR5-specific autoantibodies that strongly block HIV infection are induced in the sera of HIV-seronegative individuals (referred to exposed seronegative (ESN)[4] subjects) despite multiple exposures to HIV-1 (21). Therefore, CCR5 may be an important target for developing a more effective HIV-1 vaccine.

In this study, we developed a CCR5-based cycloimmunogen that can elicit an anti-CCR5 autoantibody to reconstruct the immune response induced in ESN subjects, and examined the in vivo protective effects of vaccination on acute viral infection in cynomolgus macaques, as well as the duration and magnitude of autoantibody induction.

Materials and Methods

Preparation of cyclic closed-chain dodecapeptide (cDDR5)-MAP and biotinylated cDDR5

A CCR5-derived linear dodecapeptide (linear DDR5, H_2N-ERSQKEGL HYTG-COOH), in which all side-chain groups are protected, was synthesized using an automatic peptide synthesizer and cyclized, as previously described (10). The free β-carboxyl group of Glu$_1$ in the protected cDDR5 was

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[4] Abbreviations used in this paper: ESN, exposed seronegative; cDDR5, cyclic closed-chain dodecapeptide; MOE, molecular operating environment; SHIV, simian/HIV; TCID, tissue culture infective dose; UPA, undecapeptidyl arch; wpim, weeks postinitial immunization.

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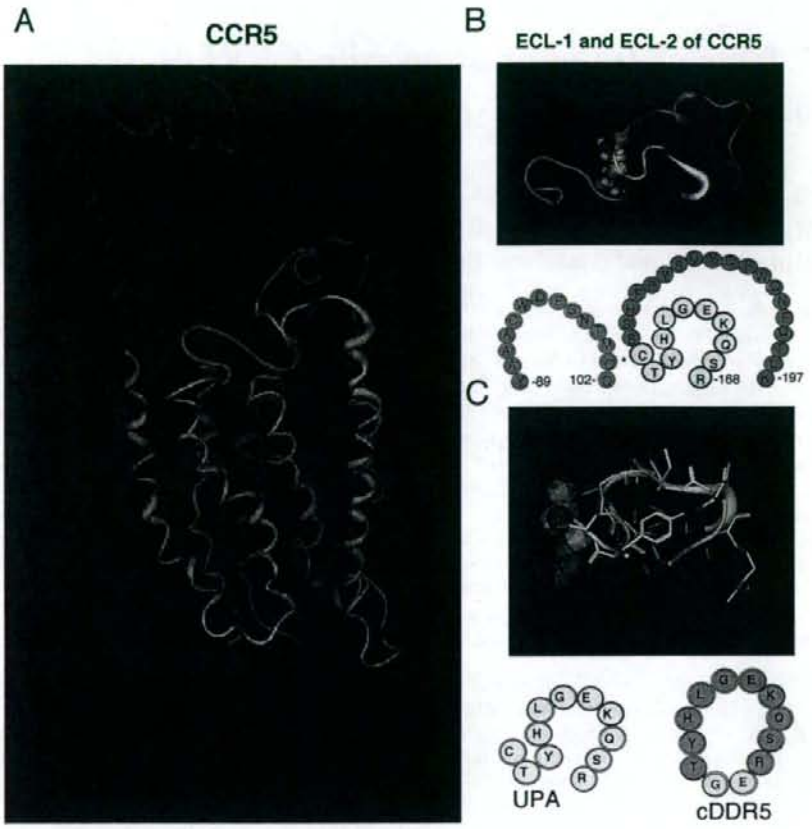

FIGURE 1. Deduced structure of UPA in extracellular loop-2 of CCR5. *A*, The predicted model of CCR5 was constructed using the segmented approach, and MOE was used for actual calculation. The determined structure of rhodopsin was used as the template. Transmembrane helixes are in cyan. The extracellular loops of CCR5 are color coded: N terminus, blue; extracellular loop-1, green; extracellular loop-2, yellow-orange; UPA, yellow; and extracellular loop-3, magenta. The intercellular loops are in gray, and the C terminus is in red. *B*, Predicted model of UPA in CCR5. Because cysteine residues in extracellular loop-1 and extracellular loop-2 form a disulfide bond, the extracellular loop-2 region of CCR5 has a unique arch structure consisting of 11 aa residues (yellow). In this study, UPA was selected as the target of peptide immunogen. *C*, To mimic the deduced conformational epitope of UPA in CCR5, the decapeptide (R_{168}SQKEGLHYT$_{177}$) derived from the UPA sequence was cyclized by inserting the spacer-armed dipeptide (Gly-Glu), and the deduced structure of cDDR5 (in cyan) was adopted as the deduced structural model of UPA in CCR5 using the MOE-Align tool.

separately conjugated to the MAP resin (Applied Biosystems) and 5-[5-(*N*-succinimidyloxycarbonyl)pentylamido]hexyl D-biotinamide through ethylenediamine. cDDR5 derivatives, in which all protected groups were removed using trifluoroacetic acid, were used for the following purposes. cDDR5-MAP was used to immunize cynomolgus macaques. MAP is commercially available (Applied Biosystems) and is composed of a 2-fold bifurcating polylysine core developed as a carrier of a peptide Ag. In contrast, biotinylated cDDR5 was used to confirm the specific binding of Abs to cDDR5 using a BIAcore biosensor. Unless otherwise specified, all of the peptides used were purified by HPLC (Waters), and the molecular masses of the compounds were determined by MALDI-TOF mass spectrometry (Burker Franzen Analytik).

Immunization schedule

All of the cynomolgus macaques were housed in individual cages and maintained according to the rules and guidelines of the National Institute for Infectious Diseases for experimental animal welfare. In the pilot test, a 4-year-old cynomolgus macaque was immunized i.p. at 0 and 1 wk with 300 μg of cDDR5-MAP in CFA and boosted s.c. at 6 wk with 300 μg of cDDR5-MAP in IFA. Furthermore, three 4- to 6-year-old cynomolgus macaques (nos. 11, 13, and 16) were also immunized with cDDR5-MAP according to the same schedule. Another three cynomolgus macaques (nos. 7–9) were immunized with MAP following the same immunization schedule as the controls. Immune sera were obtained at 0, 1, 2, 4, 6, 8, 10, and 21 wk postinitial immunization (wpim), and were then subjected to BIAcore analysis and MAGIC-5 assay.

Real-time biomolecular interaction analysis using surface plasmon resonance

The principle in analyzing Ag-Ab interactions has been described (10). Biotinylated cDDR5 was injected into a streptavidin-coated sensor chip (BIAcore KK). Binding experiments were performed by injecting immune sera diluted 1/9 with PBS (0.02% KH_2PO_4, 0.29% $Na_2HPO_4{\cdot}12H_2O$, 0.8% NaCl, and 0.02% KCl).

Flow cytometry

CEM-CCR5 cells (22) were washed with PBS and then suspended in a cold washing buffer (PBS containing 2% FBS and 0.02% NaN_3) at 1×10^6

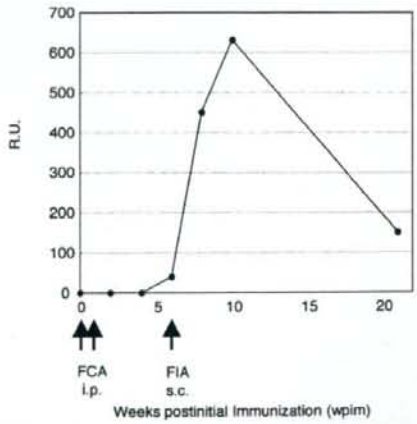

FIGURE 2. Induction of cDDR5-specific Abs after immunization with cDDR5-MAP in cynomolgus macaques. Serum samples obtained before and after immunization with cDDR5-MAP (at 0, 1, 2, 4, 6, 8, 10, and 21 wpim) were examined to investigate whether the antisera against the moiety of cDDR5 can be raised in cynomolgus macaques using real-time biomolecular interaction analysis using surface plasmon resonance with a biotinylated cDDR5-bound BIAcore biosensor.

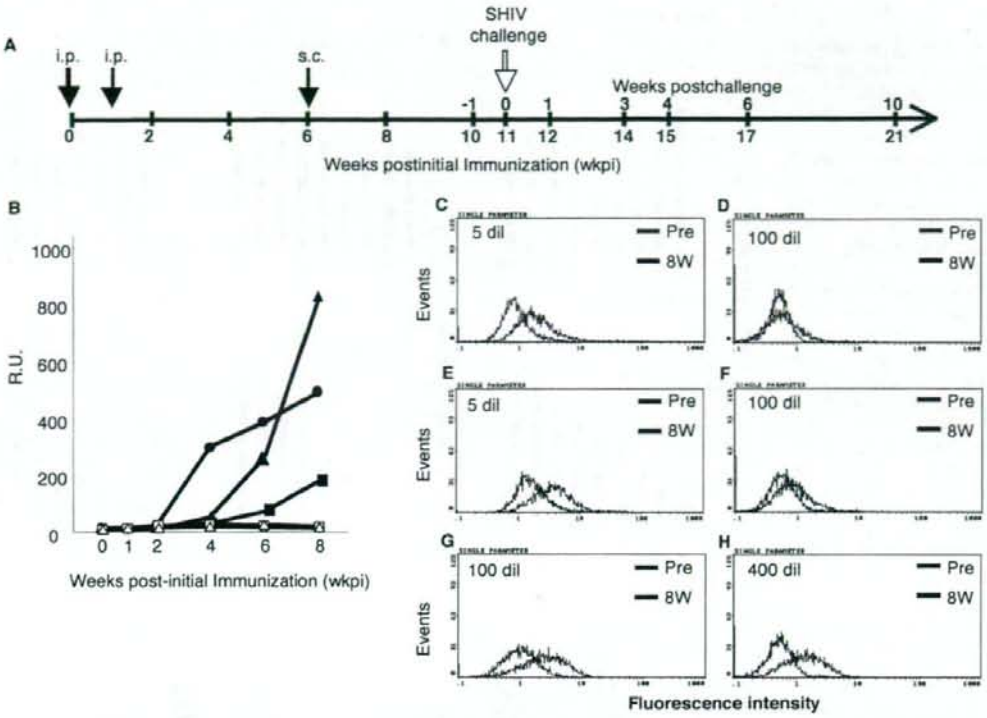

FIGURE 3. Immunization schedule and detection of anti-CCR5 Abs in cynomolgus macaques. *A*, Immunization schedule for cynomolgus macaques. Three cynomolgus macaques (nos. 11, 13, and 16) were immunized i.p. at 0 and 1 wk with 300 μg of cDDX4-MAP, and boosted s.c. at 6 wk with 300 μg of cDDR5-MAP. Another three cynomolgus macaques (nos. 7, 8, and 9) were immunized with MAP as the control. Blood sampling was performed at 0, 1, 2, 4, 6, 8, 10, 12, 14, 15, 17, and 21 wpim. *B*, Detection of anti-cDDR5-MAP serum Abs in cynomolgus macaques. Serum samples obtained before and after immunization with cDDR5-MAP (macaque nos. 11 (○), 13 (■), and 16 (▲) or MAP (macaque nos. 7 (○), 8 (□), and 9 (△) were examined to investigate whether the antisera against the moiety of cDDR5 can be raised in cynomolgus macaques. The anti-cDDR5 macaque Abs from each serum sample were detected by real-time biomolecular interaction analysis using surface plasmon resonance with a biotinylated cDDR5-bound BIAcore biosensor, as shown in Fig. 2. Sensorgrams from the serum samples taken before and after immunization were obtained, and the highest response units (R.Us.) in each sample were plotted. *C–H*, Flow cytometry of serum Abs from cynomolgus macaques (no. 11, *C* and *D*; no. 13, *E* and *F*; no. 16, *G* and *H*) immunized with cDDR5-MAP. CEM-CCR5 cells were subjected to flow cytometry, as described in *Materials and Methods*, in which the cells were separately incubated with the preimmunization (dark blue histogram) and 8 wpim (red histogram) sera diluted 1/5, 1/100, or 1/400 with PBS. The dilution folds are shown as 5, 100, or 400 dil in each figure. Significant binding to a cell was determined positive when the mean fluorescence intensity ratio of 8 wpim serum to 0 wpim serum is >1.5.

cells/ml. The cells were incubated with preimmunization and 8 wpim sera, which were dialyzed using Spectra/Por (cutoff molecular mass, 100,000; Spectrum Laboratories), according to the manufacturer's instructions, and diluted 1/5, 1/100, or 1/400 with PBS. Because immunization with the extracellular linear peptide of CCR5 up-regulates the concentrations of CCL5, CCL3, and CCL4, which are CCR5 ligands (11), the sera were dialyzed and then diluted with PBS for flow cytometry. The cells were resuspended in the washing buffer containing FITC-conjugated anti-monkey Ig (IgG, IgA, and IgM) Abs (H&L) (Rockland). After 30 min of incubation at 4°C, the cells were washed three times and then analyzed using an EPICS XL flow cytometer (Beckman Coulter).

The specificity of anti-cDDR5 serum for native CCR5 expressed on cynomolgus macaque PBMCs or HSC-F (23) was tested by examining the ability of anti-cDDR5 serum to block binding of a CCR5-specific mAb (3A9; BD Pharmingen). A total of 1×10^6 cells was incubated with pre-immunization or 10 wpim serum, which was dialyzed and diluted (1/2) with PBS, from vaccinated macaques no. 16 for 15 min at room temperature. The cells were washed and then stained with FITC-labeled anti-CD95 (BD Pharmingen) and PE-labeled anti-CCR5 (3A9; BD Pharmingen) for 20 min at room temperature. Finally, the cells were washed again and then analyzed using an EPICS XL flow cytometer. Control experiments were conducted without preincubation with preimmunization or 10 wpim serum.

MAGIC-5 assay

The antiviral activity of the sera obtained before and after immunization with cDDR5-MAP was determined using MAGIC-5 cells, as previously described (10). MAGIC-5 cells were plated at 1×10^4 cells/well (96-well plates) and incubated overnight in RPMI 1640 containing 5% FCS (200 μl); the medium was then replaced with the preimmunization and 8 wpim sera (30 μl), which were dialyzed using Spectra/Por, and serially diluted (1/5–1/1280) with PBS. The cells were then separately incubated in suspensions of R5 and X4 clade B viruses, nonclade B primary isolates, or simian/HIV (SHIV) (10 μl: HIV_{LAV-1}, 5077; HIV_{JRFL}, 3749; $HIV_{93RW004}$, 1406; HIV_{MJ4}, 844; $SHIV_{SF162P3}$, 713 tissue culture infective $dose_{50}$ ($TCID_{50}$)/ml in the presence of 20 μg/ml DEAE dextran for 2 h, and then cocultured in the medium (160 μl) for 48 h. The cells were fixed, and HIV-1-infected cells identified by their blue staining were counted by conventional methods.

Productive infection assay using $SHIV_{SF162P3}$

CEM-CCR5 cells (5×10^5) (22) were infected with $SHIV_{SF162P3}$ (3.2 ng/ml as measured using p27 Ag) in the presence of the preimmunization and 8 wpim sera for 18 h. The cells were washed with PBS, and then plated onto 24-well plates and cultured in 3 ml of the RPMI 1640 medium containing 10% FBS. The culture supernatants 24, 48, 72, 96, and 120 h after

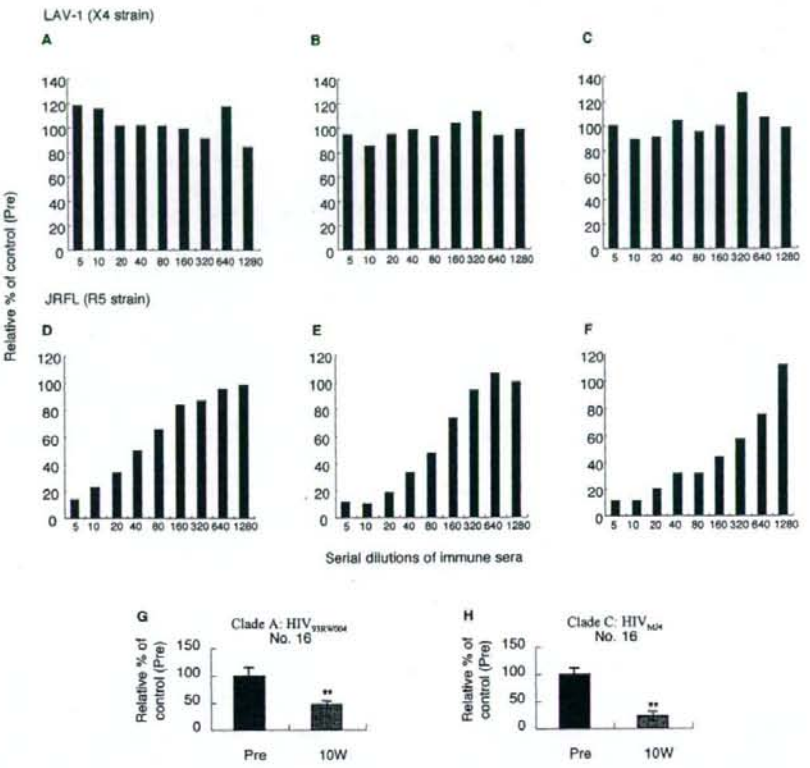

FIGURE 4. Inhibitory effects of pre- and postimmunization sera on HIV-1 R5 and X4 infections. MAGIC-5 cells were separately incubated with serially diluted 8 wpim serum from cDDR5-MAP-immunized macaques (*A* and *D*, no. 11; *B* and *E*, no. 13; *C* and *F*, no. 16). Preimmunization serum was used as control. The cells were further cocultured in a medium containing various HIV-1 strains (*A–C*, LAV-1; *D–F*, JRFL) for 48 h, as described in *Materials and Methods*. The infected cells, which were stained blue, were counted by a conventional method. The number of cells stained blue is expressed as percentage relative to the number of cells in the preimmunization serum (control) stained blue for each macaque. No significant cytotoxicity of the immunization serum-containing medium was observed. Values represent the mean of three determinations. *G* and *H*, MAGIC-5 cells were separately incubated with preimmunization and 10 wpim sera, which were dialyzed and diluted (1/2) with PBS, from vaccinated macaque no. 16. The cells were further cocultured in a medium containing nonclade B HIV-1 strains ($HIV_{93RW004}$, HIV_{MJ4}) for 48 h, as described in *Materials and Methods*.

infection were removed to detect SIV p27 Ag by RETRO-TEK SIV type 1 $p27^{gag}$ Ag ELISA, according to the manufacturer's instructions.

HIV-1 and SHIV strains

Clade B laboratory-adapted strains $HIV\text{-}1_{JRFL}$ and $HIV\text{-}1_{LAV\text{-}1}$ were used. These clade B viruses were propagated in a chronically $HIV\text{-}1_{JRFL}$-infected T cell line (Molt4-CCR5/JRFL) and a chronically $HIV\text{-}1_{LAV\text{-}1}$-infected T cell line (CEM/LAV-1) grown in a complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated, defined FBS (HyClone), penicillin (100 IU/ml), and streptomycin (0.1 mg/ml). The nonclade B strains $HIV_{93RW004}$ and HIV_{MJ4} (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were propagated in 3-day-cultured, PHA-activated human PBMCs. Furthermore, $SHIV\text{-}1_{SF162P3}$ (24, 25) (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) was similarly propagated in 3-day-cultured, PHA-activated cynomolgus macaque PBMCs. Each cell-free virus stock was prepared from tissue culture supernatants harvested from chronically and acutely infected cells. Virus-containing supernatants were pooled, filtered through a 0.45-μm membrane, aliquoted, and frozen to provide a uniform stock of infectious virus.

Macaque challenge

All of the six cynomolgus macaques were i.v. challenged with 1 ml of 10 $TCID_{50}$/ml $SHIV_{SF162P3}$. Blood, serum, and plasma samples were collected from the infected animals at regular intervals after infection.

Determination of SHIV RNA viral load by SYBR green-based quantitative real-time PCR assay

Viral RNA was extracted from plasma using a QIAamp viral RNA minikit (Qiagen), then retrotranscribed using the SuperScript III First-Strand Synthesis system (Invitrogen Life Technologies). cDNA duplicates were amplified by SYBR green real-time PCR assay previously described (26) with some modifications. Briefly, primers that recognize specific and highly conserved sequences on the *gag* region of SIV described by Ui et al. (27) were selected. The sequences of SIV gag primers were 5′-GGAAATTAC CCAGTACAACAAATAGG-3′ and 5′-TCTATCAATTTTACCCAG GCATTTA-3′. The SIV *gag* gene was amplified in 20 μl of a PCR mixture consisting of 10 μl of 2× master mix containing modified DyNAmo hot start DNA polymerase, SYBR green I, optimized PCR buffer, 5 mM $MgCl_2$, a dNTP mix including dUTP (Finnzymes), 2 μl of each primer, and 8 μl of cDNA. PCR was conducted as follows: initial activation of hot start DNA polymerase at 95°C for 15 min; 40 cycles of four steps of 95°C for 10 s, 57°C for 20 s, 72°C for 20 s, and 76°C for 2 s. At the end of the amplification cycle, melting temperature analysis was conducted by gradually increasing the temperature (0.5°C/s) to 95°C. Amplification, data acquisition, and analysis were conducted with the DNA Engine Opticon 2 System (MJ Research) using Opticon Monitor version 2.02 software (MJ Research). The detection limit of this system was 1×10^3 copies/ml.

Results

Design and synthesis of cDDR5

The hypothetical structural model of CCR5 was based on its homology with rhodopsin (28), and was energy minimized using the molecular operating environment (MOE; Chemical Computing Group) (Fig. 1*A*). The extracellular loop-2 region of CCR5 and its structure deduced on the basis of the Cys^{178} residue bound to the Cys^{101} residue of extracellular loop-1 by a disulfide bond using MOE showed a unique arch consisting of 11 aa residues (undecapeptidyl arch (UPA)) (Fig. 1*B*). The cDDR5 moiety designed to mimic the deduced conformational epitope of UPA was generated by the cyclization of a decapeptide (R_{168}SQKEGLHYT$_{177}$) derived from the UPA sequence by inserting a spacer-armed dipeptide (Gly-Glu). The deduced structure of cDDR5 (shown in cyan) was adopted in the construction of the hypothetical structural

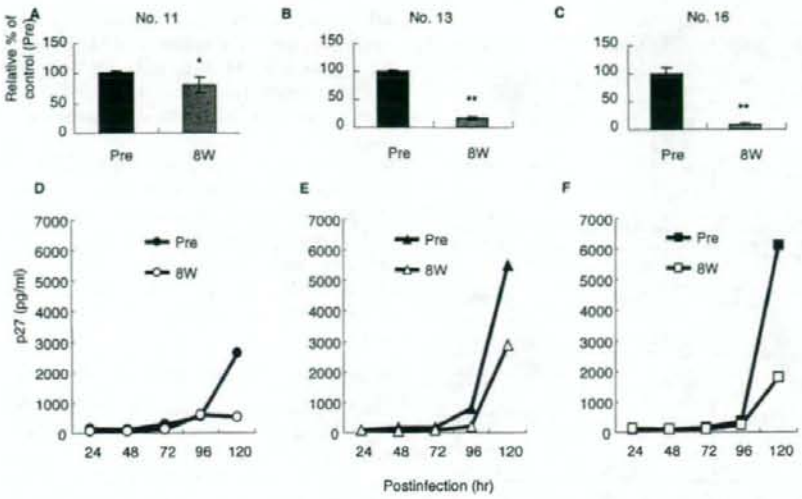

FIGURE 5. Inhibitory effects of pre- and postimmunization sera on CCR5-specific $SHIV_{SF162P3}$ infection. *Upper* and *bottom figures*, Show the results of MAGIC-5 assay (*A*, no. 11; *B*, no. 13; *C*, no. 16) and productive infection assay (*D*, no. 11; *E*, no. 13; *F*, no. 16), respectively. In MAGIC-5 assay, MAGIC-5 cells were separately incubated with preimmunization and 8 wpim sera, which were dialyzed and diluted (1/10) with PBS, and cocultured in the $SHIV_{SF162P3}$-containing medium for 48 h, as described in *Materials and Methods*. The infected cells, which were stained blue, were counted. ■ and ▨, Represent the results from preimmunization and 8 wpim sera, respectively. No significant cytotoxicity of the immunization serum-containing medium was observed. Diluted virus stocks that infected 100–300 cells, as identified by their blue staining, were used. The number of cells stained blue was expressed as percentage relative to the number of cells in the preimmunization sera (control) stained blue. All data represented means obtained from three separate experiments. Statistically significant differences compared with preimmunization serum are indicated by asterisks (*, $p < 0.05$; **, $p < 0.01$). In the productive infection assay, CEM-CCR5 cells (5×10^5) were infected with the $SHIV_{SF162P3}$-containing medium in the presence of preimmunization and 8 wpim sera for 18 h. The cells were washed with PBS, plated onto 24-well plates, and then cultured in the RPMI 1640 medium containing 10% FBS. The p27 Ag in culture supernatants obtained at 24, 48, 72, 96, and 120 h postinfection was detected, as described in *Materials and Methods*. Cynomolgus macaque no. 11, Pre (●) and 8 wk (○); no. 13, Pre (▲) and 8 wk (△); no. 16, Pre (■) and 8 wk (□).

model of UPA in CCR5 (shown in yellow) using the MOE-Align tool (Chemical Computing Group) (Fig. 1*C*).

Induction of cDDR5-specific Abs after immunization with cDDR5-MAP in cynomolgus macaques

We pilot tested whether the immunization of cynomolgus macaques with cDDR5-MAP induces cDDR5-specific Abs. To examine the duration and magnitude of anti-cDDR5 autoantibody induction, a cynomolgus macaque was immunized i.p. at 0 and 1 wk with cDDR5-MAP in CFA and boosted s.c. at 6 wk with cDDR5-MAP in IFA. Ab responses against cDDR5 were measured by real-time biomolecular interaction analysis using surface plasmon resonance. As shown in Fig. 2, the titer of anti-cDDR5 sera measured 4 wk after the third immunization (10 wpim) was the highest in the cynomolgus macaques (titer of ~630 response units). Furthermore, the immunization with cDDR5-MAP induced anti-cDDR5 serum production for ~15 wk after the third immunization, although the titer of anti-cDDR5 sera declined over time until 21 wpim (Fig. 2).

Vaccination of cynomolgus macaques with cDDR5-MAP and induction of CCR5-specific Abs

To verify whether cDDR5-MAP could induce CCR5-specific Abs with anti-HIV-1 activity in nonhuman primates, an experiment was performed using cynomolgus macaques immunized following the time schedule shown in Fig. 3*A*. Three cynomolgus macaques were immunized with cDDR5-MAP in CFA or IFA by i.p. or s.c. injection, as described in Fig. 2. Another three cynomolgus macaques were immunized with MAP as the control. cDDR5-specific Abs were significantly induced in cynomolgus macaque nos. 11, 13, and 16 at 8 wpim (Fig. 3*B*). In contrast, the immunization of cynomolgus macaque nos. 7–9 with MAP did not elicit cDDR5-specific Abs (Fig. 3*B*). Furthermore, the sera from cynomolgus macaque nos. 11, 13, and 16 at 8 wpim with cDDR5-MAP were also examined by flow cytometry to determine whether they recognize intact cell surface-expressed CCR5 on CEM-CCR5 cells. The sera from no. 11 (diluted 1/5 and 1/100), no. 13 (diluted 1/5 and 1/100), and no. 16 (diluted 1/100 and 1/400) macaques showed the immunofluorescence staining of CEM-CCR5 cells, compared with preimmunization sera from these macaques (Fig. 3, *C–H*). In contrast, the immunization of cynomolgus macaque nos. 7–9 with MAP did not induce CCR5-specific Abs (data not shown).

Inhibition of HIV-1 infection

The anti-HIV-1 activities of the immune sera from cynomolgus macaque nos. 11, 13, and 16 were also determined using MAGIC-5 cells expressing CCR5. The cells were separately inoculated with two laboratory strains of clade B HIV-1 (R5 HIV-1, $HIV\text{-}1_{JRFL}$; X4 HIV-1, $HIV\text{-}1_{LAV\text{-}1}$) or R5 nonclade B HIV-1 primary isolates (clade A:$HIV_{93RW004}$ and clade C:HIV_{MJ4}) in the presence or absence of immune sera. As expected, 8 wpim sera from cDDR5-MAP-immunized cynomolgus macaque nos. 11, 13, and 16 markedly suppressed infection by $HIV\text{-}1_{JRFL}$ (R5 HIV-1) in a dose-dependent manner (Fig. 4, *D–F*). Furthermore, 10 wpim sera from macaque no. 16 suppressed infection by R5 nonclade B HIV-1 primary isolates (clade A:$HIV_{93RW004}$ and clade C:HIV_{MJ4}) (Fig. 4, *G* and *H*). In contrast, the immune sera did not prevent $HIV\text{-}1_{LAV\text{-}1}$ (X4 HIV-1) infection as observed in the control experiment (Fig. 4, *A–C*).

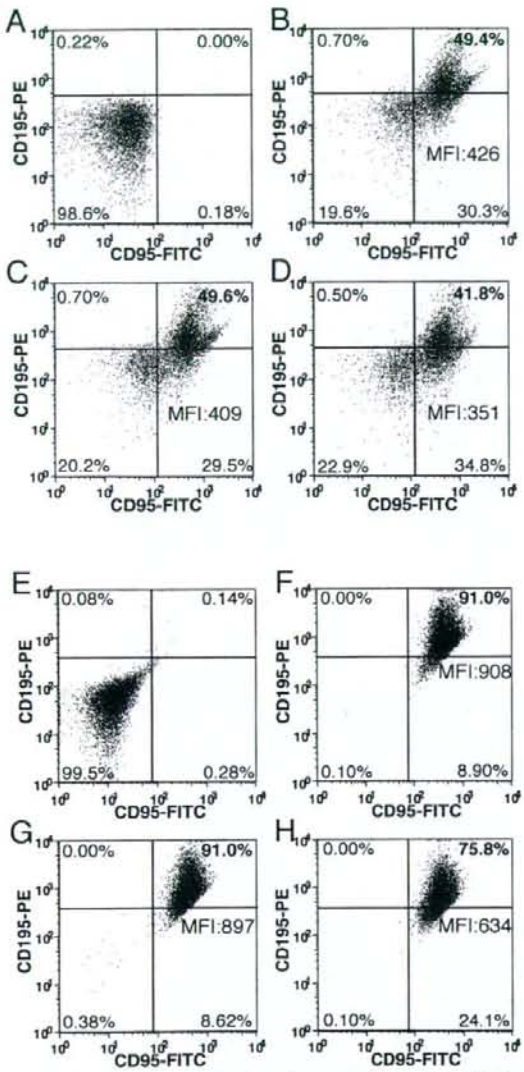

FIGURE 6. Inhibition of binding of a mAb against CCR5 (3A9) to cynomolgus macaque PBMC or HSC-F by anti-cDDR5 serum. Macaque PBMC (*A–D*) or HSC-F (*E–H*) was incubated with FITC-labeled IgG1 and PE-labeled IgG2 (*A* and *E*), FITC-labeled anti-CD95 and PE-labeled anti-human CCR5 mAb (3A9) (*B* and *F*), and FITC-labeled anti-CD95 and PE-labeled anti-human CCR5 mAb (3A9) after preincubation with preimmunization (*C* and *G*) or 10 wpim sera (*D* and *H*). Staining was assessed by flow cytometry, and fluorescence was measured after gating the lymphocyte population. Inhibition of 3A9 binding was determined by two methods: first, by quantitating the percentage of $CD95^+$ cells that were also 3A9-PE positive; second, by determining the mean fluorescence index (MFI) of $CD95^+$ cells that were bound by 3A9. These values are displayed in the quadrant.

Inhibition of CCR5-specific $SHIV_{SF162P3}$ infection

The SHIV-$1_{SF162P3}$ bulk isolate is a pathogenic CCR5-specific SHIV in rhesus macaques (25). The 8 wpim sera from macaque nos. 11, 13, and 16 were examined by infection assay using both MAGIC-5 and CEM-CCR5 cells to determine whether they inhibit SHIV-$1_{SF162P3}$ bulk isolate infection. The 8 wpim sera significantly suppressed the infection of MAGIC-5 cells by the SHIV-$1_{SF162P3}$ bulk isolate, compared with the preimmune sera (Fig. 5, *A–C*). To verify the antiviral activity of immune sera in another experiment, the inhibitory effect of immune sera from cynomolgus macaque nos. 11, 13, and 16 on $SHIV_{SF162P3}$ replication in CEM-CCR5 cells was investigated. CEM-CCR5 cells, a CCR5-transfected human T cell line, were acutely infected with $SHIV_{SF162P3}$ in the presence of preimmunization and 8 wpim sera from cynomolgus macaque nos. 11, 13, and 16, and the spread of infection was then monitored on the basis of the accumulation of p27 Ag in culture supernatants. The 8 wpim sera from macaque nos. 11, 13, and 16 effectively suppressed $SHIV_{SF162P3}$ propagation (Fig. 5, *D–F*).

Ability of macaque anti-cDDR5 sera to bind to macaque CCR5

The UPA sequence in human and macaque CCR5s differs in 1 aa ($Lys^{171}Arg$). Anti-human CCR5 Ab, 2D7, binds to the epitope that includes Lys^{171}, and this binding ability was prevented by the sequence substitution in CCR5 ($Lys^{171}Arg$). To rule out whether anti-cDDR5 serum cross-reacts with native CCR5 as expressed on macaque lymphocytes, we examined the ability of anti-cDDR5 sera to block the binding of a commercially available CCR5-specific mAb (3A9). The 3A9 can bind to macaque CCR5. Cynomolgus macaque PBMC or the lymphocytic cell line HSC-F (23) was incubated with the anti-CD95 Ab with either the preimmunization or 10 wpim serum from cynomolgus macaque no. 16 and then reacted with a limiting amount of PE-labeled 3A9 according to the protocol of Chackerian et al. (13). The ability of anti-cDDR5 serum to block 3A9-PE binding was assessed by flow cytometry, gating lymphocytes, and examining the $CD95^+$ population. The incubation of PBMC or HSC-F with 10 wpim serum reduced the percentage of $CD95^+$ lymphocytes that were 3A9 positive (Fig. 6, *D* and *H*). In addition, the mean fluorescence index of cells that remained 3A9 positive was lower than that of cells that were preincubated with preimmunization serum. The 10 wpim serum did not decrease the binding of a control mAb (anti-CD95-FITC), suggesting that the observed inhibition of 3A9 binding was specific. In addition, the incubation of PBMC or HSC-F with preimmunization serum from macaque no. 16 had no effect on 3A9-PE binding (Fig. 6, *C* and *G*). Taken together, these results support the conclusion that anti-cDDR5 serum cross-reacts with native CCR5 as expressed on macaque lymphocytes.

Macaque challenge with $SHIV_{SF162P3}$

Five weeks after the final boost (11 wpim), all of the MAP- and cDDR5-MAP-immunized macaques were i.v. challenged with 1 ml of 10 $TCID_{50}$/ml $SHIV_{SF162P3}$, which is a chimeric virus that contains the *env*, *tat*, and *rev* genes from HIV_{SF162}, an R5 virus, in the background of SIV_{mac239}. The course of acute viral infection was monitored by measuring plasma viral RNA load and $CD4^+$ T cell number in acutely infected macaques (Fig. 7). All of the three control macaques developed detectable plasma viremia, as demonstrated by viral peaks between 1×10^9 and 3×10^9 viral RNA copies/ml plasma with a moderate decrease in peripheral $CD4^+$ T cell number (%), as previously described (25), and sustained plasma viremia $>10^8$ viral RNA copies/ml plasma for 3 wk (1–4 wk postchallenge). The actual percentage of surviving peripheral $CD4^+$ T cells inversely correlated with viral load (Fig. 7). Furthermore, we compared the geometric mean plasma viral RNA loads of the vaccinated and control groups to monitor the effectiveness of vaccination. The difference between viral loads of the two groups was statistically significant ($p = 0.03$) at one time point (week 1) by 4 wk after infection (Table I), and there were

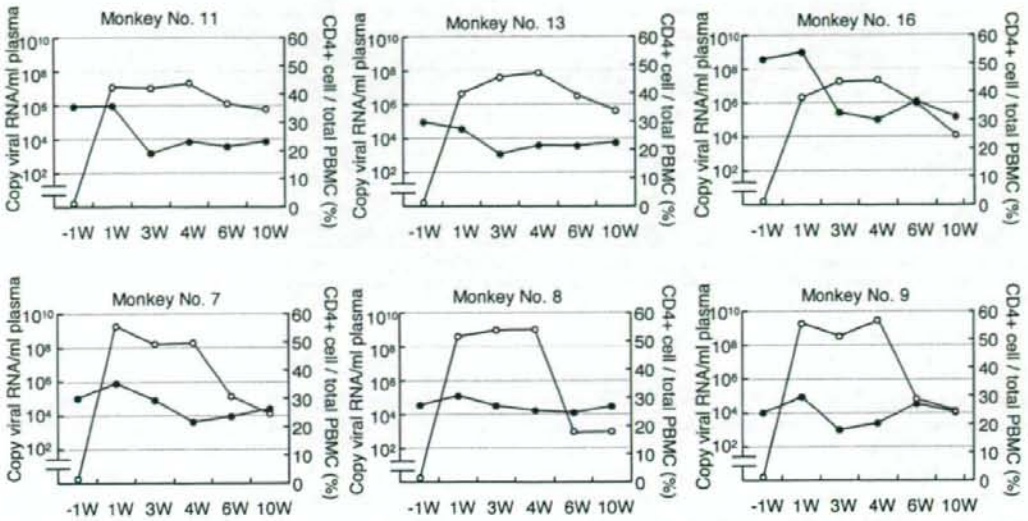

FIGURE 7. Intravenous challenge with $SHIV_{SF162P3}$. Whole-blood samples were collected at −1, 1, 3, 4, 6, and 10 wk postchallenge, and the samples were examined for plasma viremia (○) and peripheral $CD4^+$ T cell count (%) (●). Viral RNA was extracted from macaque plasma and then reverse transcribed. The resulting cDNA duplicates were amplified by quantitative real-time PCR, as described in *Materials and Methods*.

differences of ~19.95- to 217.10-fold in the geometric mean viral loads of the two groups between 1 and 4 wk postchallenge. The vaccinated macaques had a lower ~3-wk delayed peak viremia than the controls, so that the vaccinated macaques sustained plasma viremia between 10^4 and 3×10^6 viral RNA copies/ml plasma at 6 and 10 wk postchallenge in contrast to control macaques (between 10^3 and 10^5 viral RNA copies/ml plasma). Furthermore, we investigated whether the high in vitro anti-HIV and in vitro anti-SHIV activities are associated with low viral loads during the acute phase of SHIV infection. The in vitro anti-HIV and in vitro anti-SHIV activities before challenge were compared with the peak plasma viral RNA load at 1 wk postchallenge (Table II). Macaque no. 16 with the highest anti-HIV and anti-SHIV activities had the lowest viral load among vaccinated macaques at 1 wk postchallenge (2.0×10^6 viral RNA copies/ml plasma), suggesting that the higher in vitro anti-HIV and in vitro anti-SHIV activities in vaccinated macaques were responsible for the low viral loads (Table II). Taken together, these results suggest that viral loads in vaccinated macaques following a challenge with $SHIV_{SF162P3}$ are controlled for a longer time if the anti-CCR5 Ab continues to be strongly induced by vaccination for a longer time.

Table I. *Statistical analysis of geometric mean viral loads*

	Comparison of Geometric Mean Viral Load of Vaccinated Group with That of Control Group	
Postchallenge (wk)	Ratio[a]	*p* Value[b]
1	**217.10**	**0.03**
3	19.95	0.11
4	28.40	0.05
6	0.04	[c]
10	0.02	[c]

[a] The ratio of the geometric mean titers at the indicated time points postchallenge. A value of 217.10 indicates that the geometric mean viral load of the control group is 217.10 times higher than that of the vaccinated group.

[b] Values of *p* were obtained from a two-sample *t* test at the indicated time points. The Mann-Whitney *U* test also provided similar statistical results. Value in boldface is statistically significant ($p < 0.05$).

[c] Value of *p* was not calculated because the geometric mean viral load of the vaccinated group is higher than that of the control group.

Discussion

CCR5 is considered important in HIV-1 transmission on the basis of the findings that individuals homozygous for a 32-bp deletion in the CCR5-coding region have a very low susceptibility to HIV-1 infection (15–18), and CCR5-reactive Abs in ESN subjects downmodulate surface CCR5 in vivo and neutralize the infectivity of R5 strains (21). Therefore, the induction of CCR5-specific autoantibody-based immunity specific to the infection by R5 HIV-1 from various HIV-1 clades may be a strategy for the development of a prophylactic HIV-1 vaccine.

Some vaccination strategies of inducing CCR5-specific autoantibodies have been reported. One of our previous attempts was to induce CCR5-specific autoantibodies with anti-R5 HIV-1 activity by the inoculation of cDDR5-MAP from the UPA (from Arg^{168} to Cys^{178}) of extracellular loop-2 in CCR5 into BALB/c mice (10). Other attempts include the induction of CCR5-specific autoantibodies with anti-R5 HIV-1 activity by the inoculation of recombinant papillomavirus-like particles, which represent an extracellular loop of CCR5, into C57BL/6 mice and pig-tail macaques (12, 13), by the genetic immunization of cynomolgus macaques with the DNA of CCR5 (14), and by the immunization of rhesus macaques with synthetic linear peptides (N-terminal peptide 1–20, first-loop peptide 89–102, and second loop peptide 178–197) derived from the N terminus, first loop, and second loop in CCR5 (11). Results of these previous studies indicate that vaccines aimed at inducing CCR5-specific autoantibodies can be developed, as well as conventional viral protein-based vaccines.

In our present study, a cyclopeptide immunogen strategy was used to induce CCR5-specific autoantibodies in cynomolgus macaques. The advantages of a cyclopeptide immunogen are as follows: 1) it can induce Abs against a very restricted region that includes a biologically active conformational epitope; 2) its immunogenicity can be controlled by polymerization or conjugation with small carrier molecules such as MAP; and 3) its chemical

Table II. *Relationship among anti-cDDR5 Ab response, antiviral activity, and viral load in cynomolgus macaques*

Cynomolgus Macaque	Anti-HIV Activity (IC_{50}, dilution of dialyzed immune sera) at 8 wk Postinitial Immunization[a]	Anti-SHIV Activity (%) at 8 wk Postinitial Immunization[b]	Peak Viral Load at 1 wk Postchallenge[c]
Monkey no. 11	1/40	20	1.0×10^7
Monkey no. 13	1/105	84	4.0×10^6
Monkey no. 16	1/200	91	2.0×10^6
Monkey no. 7	Nd[d]	Nd[d]	2.0×10^9
Monkey no. 8	Nd[d]	Nd[d]	4.0×10^8
Monkey no. 9	Nd[d]	Nd[d]	2.0×10^9

[a] Antiviral activity was determined by MAGIC-5 assay using HIV-1_{JRFL} data shown in Fig. 4, D–F.
[b] Antiviral activity was determined by MAGIC-5 assay using SHIV-$1_{SF162P3}$ data shown in Fig. 5, A–C. Values were calculated using the following equation: 100 − (relative % of control (%)).
[c] Based on data shown in Fig. 7.
[d] Antiviral activity was not detected.

purity can be exactly defined (29, 30). In particular, the conformational stability of a peptide immunogen in vivo is a key factor for generating Abs against a native protein. In general, an Ab induced by a linear peptide can recognize a denatured protein, but not a native protein. In contrast, an Ab that recognizes the conformational epitope of an Ag cannot recognize a denatured protein. Indeed, the commercially available anti-CXCR4 Ab 12G5 can recognize the conformational epitope of cell surface CXCR4, but cannot detect denatured CXCR4. Therefore, cDDR5-MAP was designed on the basis of the deduced conformation of UPA (Arg^{168} to Cys^{178}) in CCR5 to induce Abs that can recognize native CCR5. In addition, the reason for selecting UPA as the target in our study is that the conformational heterogeneity of UPA is unlikely to arise, except for other extracellular domains because cysteine residues (Cys^{101} and Cys^{178}) in extracellular loop-1 and extracellular loop-2 form a rigid disulfide bond. Consequently, cDDR5 was prepared by the cyclization of the decapeptide (R_{168}SQKEGL HYT_{177}) derived from the UPA sequence by the insertion of the spacer-armed dipeptide (Gly-Glu). cDDR5 can induce CCR5-specific autoantibodies capable of significantly inhibiting infection by the R5 laboratory-adapted strain (HIV-1_{JRFL}), R5 HIV-1 primary isolates (clade A:$HIV_{93RW004}$ and clade C:HIV_{MJ4}), and $SHIV_{SF162P3}$ bulk isolates (Figs. 4 and 5). These results suggest that UPA in CCR5 is a good target for preventing CCR5-dependent viral infection. Thompson et al. (31) found that in contrast to clade B isolates, a cluster of residues in the second extracellular loop of CCR5 significantly affects the fusion and entry of all the nonclade B isolates tested. Therefore, cDDR5-MAP derived from UPA may be a more useful immunogen than that derived from other domains of CCR5, except UPA as a HIV-1 coreceptor-based vaccine candidate for use in worldwide AIDS epidemics.

To assess vaccine efficiency, we infected the control and cDDR5-MAP-immunized macaques with an R5-tropic $SHIV_{SF162P3}$. All of the three control macaques developed detectable plasma viremia, as demonstrated by viral peaks between 1×10^9 and 3×10^9 viral RNA copies/ml plasma with a moderate decrease in peripheral $CD4^+$ T cell number (%) and sustained plasma viremia $>10^8$ viral RNA copies/ml plasma for 3 wk (1–4 wk postchallenge). Reyes et al. (32) have recently demonstrated that i.v. $SHIV_{SF162P3}$ inoculation moderately decreases the absolute number of peripheral blood $CD4^+$ T cells at 2–3 wk postinfection, as we have observed. In contrast, all of the vaccinated macaques also developed detectable plasma viremia at 1 wk postchallenge, and the plasma viremia levels in the three vaccinated macaques peaked ~3 wk later than those in the control macaques (Fig. 7), so that the vaccinated macaques sustained plasma viremia levels between 10^4 and 3×10^6 viral RNA copies/ml plasma at 6 and 10 wk postchallenge in contrast to the control macaques (between 10^3 and 10^5 viral RNA copies/ml plasma). However, Table I shows there are differences of ~19.95- to 217.10-fold in the geometric mean viral loads of the two groups between 1 and 4 wk postchallenge, and at one time point (week 1), the difference (217.10-fold) was statistically significant ($p = 0.03$). Furthermore, Table II suggests that the higher in vitro anti-HIV and in vitro anti-SHIV activities of anti-cDDR5 sera are responsible for the low viral loads. These results suggest that the high induction of the anti-CCR5 Ab can suppress viral propagation during acute HIV-1 transmission, but only high induction of the anti-CCR5 Ab is not sufficient to clear detectable plasma-associated viruses because the anti-CCR5 Ab does not directly neutralize $SHIV_{SF162P3}$. It seems difficult to completely eliminate or inhibit HIV-1 acute infection in vivo even if only the anti-CCR5 Ab delays viral propagation during the initial HIV-1 transmission. Lopalco et al. (33) have recently reported that antivirus Abs such as IgA to gp41 are simultaneously induced with IgG to CCR5 and IgG to CD4 in some Italian ESN subjects. These humoral immune responses contribute to an extremely low level of viral replication below the detection limit of a standard assay in ESN subjects. Moreover, there is a possibility that more than one type of immunity such as anti-CCR5 and anti-HIV humoral responses must be induced by a vaccine if that vaccine is to be effective for HIV.

In conclusion, immunization with cDDR5-MAP induces CCR5-specific Abs in cynomolgus macaques and decreases viral load at peak viremia. Our results suggest that the CCR5-based cycloimmunogen strategy using cDDR5-MAP induces CCR5-specific autoantibodies capable of inhibiting R5 HIV-1 infection. With the basic knowledge of the induction of CCR5-specific Abs using cDDR5-MAP, we are currently developing cDDR5-MAP conjugated to the HIV env protein to reconstruct the immune response in ESN subjects.

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Disclosures

The authors have no financial conflict of interest.