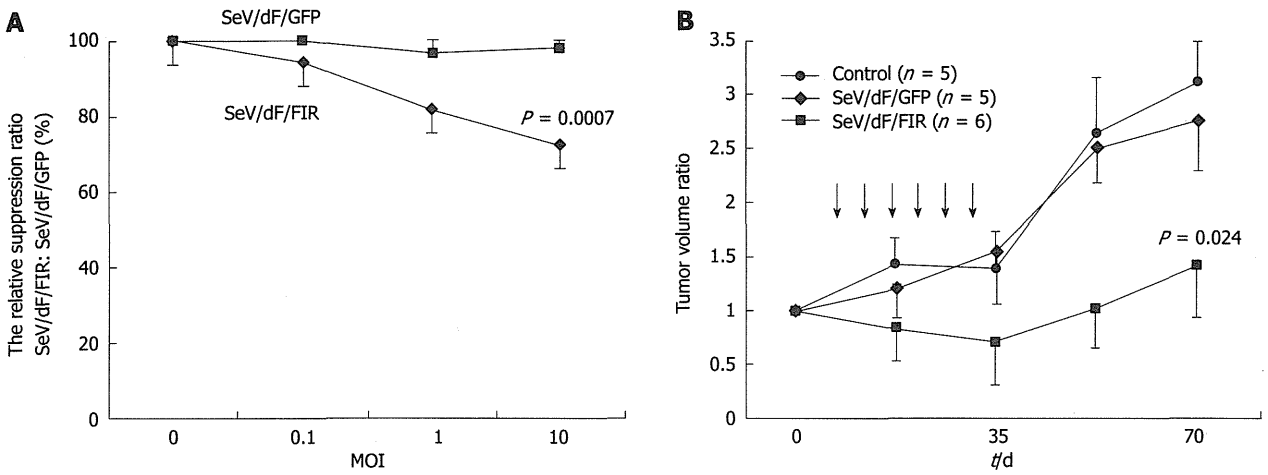

Figure 4 Sendai virus/dF/far up stream element-binding protein-interacting repressor decreased HeLa cell growth *in vitro* and in a xenograft animal model. HeLa cells (A) and SW480 cells (B) were infected with 0, 0.1, 1, and 10 MOI of SeV/dF/FIR or SeV/dF/GFP vectors and cell growth was measured by MTS assay (see Materials and Methods). Results are shown as the percentage of cell number at day 0. Points, mean of three separate experiments; bars, SD. Statistical significance was analyzed by Dunnett's test for multiple comparisons (SeV/dF/FIR *vs* SeV/dF/GFP, $P < 0.007$). Two weeks after 5×10^6 HeLa cells were xenografted into the right thigh of Balbc/nu/nu mice, the tumor size was approximately 7-8 mm. 3.0×10^7 CIU of SeV/dF/FIR or SeV/dF/GFP vector were injected directly around the tumor, and the tumor growth was observed and measured every three days as described in Materials and Methods. Results are shown as the ratio of tumor volume compared to the size at day 0. The tumor volume at day 0 of SeV/dF/FIR ($n = 6$), SeV/dF/GFP ($n = 5$), and control ($n = 5$) were 1173.1 + 259.2, 836.0 + 259.2, and 972.2 + 327.0 (average ± SD) mm^3, respectively. The average tumor volume at day 0 was estimated as 1 in each experiment. Arrows indicate the injection of SeV/dF/FIR or SeV/dF/GFP vectors. FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element; SeV: Sendai virus; GFP: Green fluorescent protein; MOI: Multiplicity of infection.

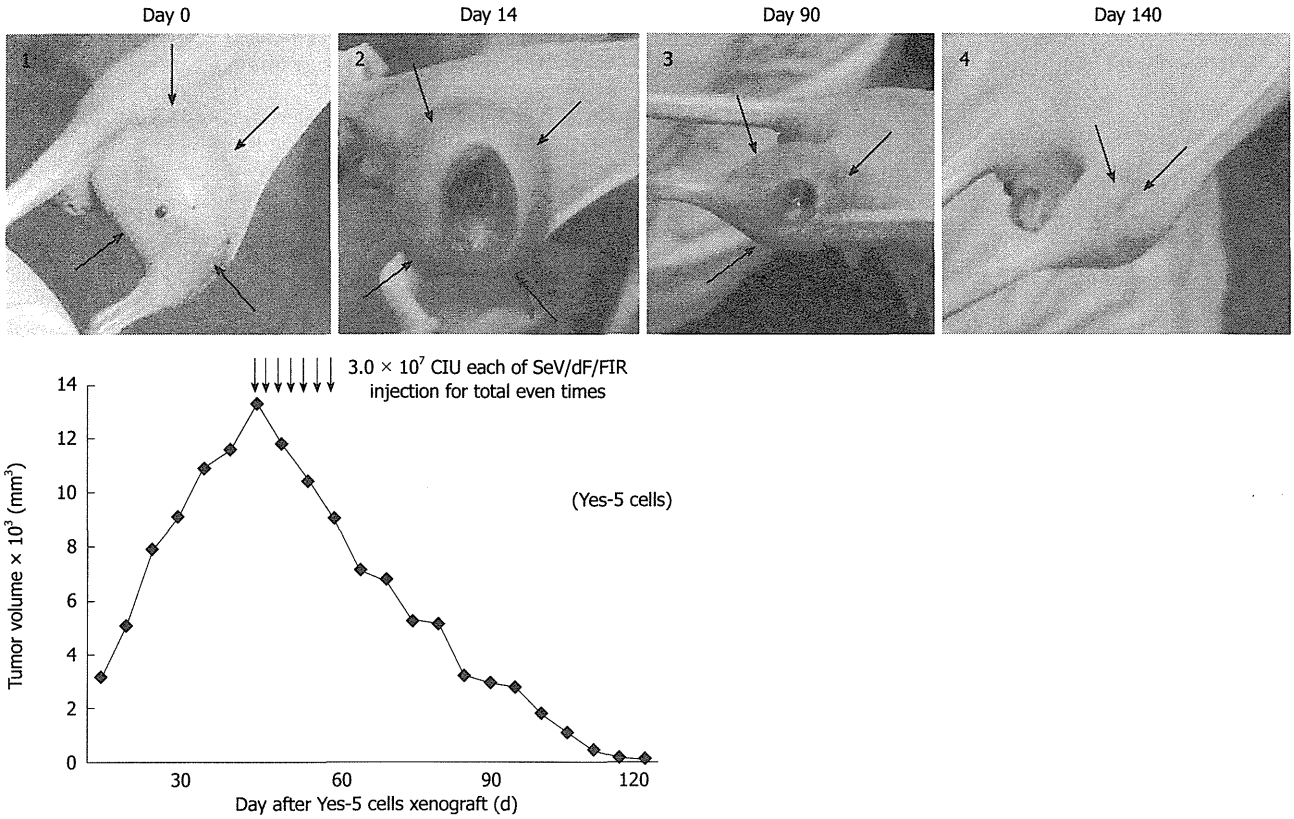

Figure 5 Sendai virus/dF/Far up stream element-binding protein-interacting repressor vector showed anti-tumor activity in a mouse xenograft model. 10^6 Yes-5 cells were xenografted into the right thigh of Balbc/nu/nu mice, and the tumor size was approximately 15 mm in diameter at Day 0. 3.0×10^7 CIU of SeV/dF/FIR vectors were injected directly around the tumor every two days, seven times in total. Tumor growth was observed and measured every two days as described in Materials and Methods. Ulcer formation was observed in the center of the tumor (day 14 after SeV/dF/FIR injection). Tumor size was significantly diminished with ulcer formation (day 90) and disappeared completely during surveillance (day 140). Thick arrows in the images indicate the tumor margin. Thin arrows indicate the injection of SeV/dF/FIR vectors into the tumor. FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element; SeV: Sendai virus; CIU: Cell-infectious units.

Table 1 Co-immunoprecipitaed proteins with far up stream element-binding protein-interacting repressor in 293T cells

Hit preys	
CDKN2AIP	CDKN2A interacting protein
CDYL	Chromodomain protein, Y chromosome-like
DAZAP1	DAZ associated protein 1
DDX17	DEAD box polypeptide 17
DDX5	DEAD (Asp-Glu-Ala-Asp) box polypeptide 5
ELAVL1	ELAV-like 1
FAM120A	Oxidative stress-associated Src activator
FUBP1 | FUBP3 | KHSRP	Far upstream element-binding protein family
FUBP1 | KHSRP	Far upstream element-binding protein family
HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1
HNRNPA1 | HNRNPA1L2	Heterogeneous nuclear ribonucleoprotein; A1 or A1-like
HNRNPA1 | HNRNPA1L2 | LOC402562	Nuclear ribonucleoprotein A1 family
HNRNPA2B1	Heterogeneous nuclear ribonucleoprotein A2/B1
HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3
HNRNPAB	Heterogeneous nuclear ribonucleoprotein A/B; isoform a
HNRNPAB | HNRNPD	Heterogeneous nuclear ribonucleoprotein; A/B or D
HNRNPD	Heterogeneous nuclear ribonucleoprotein D; isoform c
HNRNPH1	Heterogeneous nuclear ribonucleoprotein H1
HNRNPK	Heterogeneous nuclear ribonucleoprotein K
HNRNPL	Heterogeneous nuclear ribonucleoprotein L
HNRNPM	Heterogeneous nuclear ribonucleoprotein M; isoform a
HNRNPR	Heterogeneous nuclear ribonucleoprotein R
HNRNPU	Heterogeneous nuclear ribonucleoprotein U; isoform a
HNRNPUL1	Heterogeneous nuclear ribonucleoprotein U-like 1
HNRPDL	Heterogeneous nuclear ribonucleoprotein D-like
IFIT5	Interferon-induced protein with tetratricopeptide repeats 5
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
IGF2BP1 | IGF2BP3	Insulin-like growth factor 2 mRNA binding protein; 1 or 3
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2
IGF2BP3	Insulin-like growth factor 2 mRNA binding protein 3
ILF2	Interleukin enhancer binding factor 2
KHDRBS1	KH domain containing, RNA binding, signal transduction associated 1
KHDRBS1 | KHDRBS2 | KHDRBS3	KH; domain containing, RNA binding, signal transduction associated 1 Or domain-containing, RNA-binding, signal transduction-
KHSRP	KH-type splicing regulatory protein
KIAA1967	p30 DBC protein
LARP1	La related protein
LRPPRC	Leucine-rich PPR motif-containing protein
LSM12	LSM12 homolog
MAGOH | MAGOHB	Mago-nashi homolog; or B
MATR3	Matrin 3
MOV10	Mov10, Moloney leukemia virus 10, homolog
MSI2	Musashi 2; isoform a
PABP3 | PABPC1 | PABPC3	Poly (A) binding protein, cytoplasmic 1 Or poly (A) binding protein, cytoplasmic 3
PABPC1	Poly (A) binding protein, cytoplasmic 1
PABPC1 | PABPC1L | PABPC5	Poly (A) binding protein, cytoplasmic 1 or poly(A) -binding protein, Cytoplasmic 1-like or poly (A) binding protein, cytoplasmic 5
PABPC1 | PABPC4	Poly (A) binding protein, cytoplasmic 1 Or poly A binding protein, cytoplasmic 4
PABPC1L2B | PABPC4	Poly (A) binding protein, cytoplasmic 1-like 2B Or poly A binding protein, cytoplasmic 4
PABPC4	Poly A binding protein, cytoplasmic 4
PABPC4 | PABPC4L	Poly A binding protein, cytoplasmic 4
PABPN1	Poly (A) binding protein, nuclear 1
PCBP1 | PCBP2 | PCBP3 | PCBP4	Poly (rC) binding protein; 1 or 2 or 3 or 4
PCBP2 | PCBP3	Poly (rC) binding protein; 2 or 3
PTBP1	Polypyrimidine tract-binding protein 1; isoform d
SF3B1	Splicing factor 3b, subunit 1
SF3B14	Splicing factor 3B, 14 kDa subunit
SF3B3	Splicing factor 3b, subunit 3
SF3B4	Splicing factor 3b, subunit 4
SFPQ	Splicing factor proline/glutamine rich (polypyrimidine tract binding
SFRS11	Splicing factor, arginine/serine-rich 11 (SRp54)
SFRS12	Splicing factor, arginine/serine-rich 12
SNRPD1	Small nuclear ribonucleoprotein D1 polypeptide 16 kDa
SSB	Autoantigen La
SYNCRIP	Synaptotagmin binding, cytoplasmic RNA interacting protein

THADA	Thyroid adenoma associated
TRMT1	tRNA methyltransferase 1
TROVE2	TROVE domain family, member 2; isoform 2
XRN2	5'-3' exoribonuclease 2
Total hit preys:	67

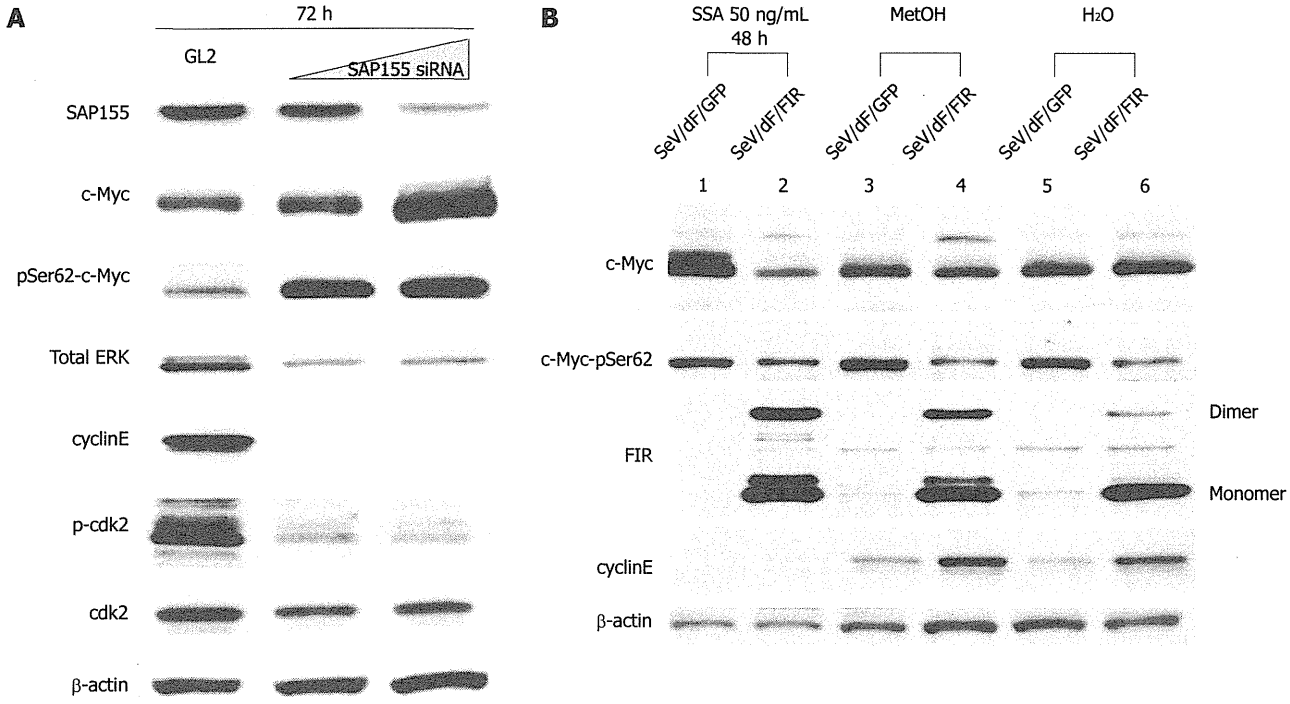

Figure 6 SAP155 siRNA induces c-Myc activation with ErK phosphorylation, but suppresses phosphorylated-cdk2/cyclinE expression. HeLa cells were treated with SAP155 siRNA for three days (72 h). A: SAP155 siRNA, as well as SSA treatment, increased not only c-Myc expression level, but also c-Myc phosphorylation at both Ser62, but suppressed phosphorylated-cdk2 and cyclinE in a dose-dependent manner. Thus, SAP155 siRNA activates c-Myc potentially *via* inhibiting endogenous FIR pre-mRNA splicing; B: FIR Sendai virus (SeV/dF/FIR) reversed the cytotoxicity of SSA by suppressing activated endogenous c-Myc. HeLa cells were treated with 50 ng/mL SSA for 48 h with control (MetOH and H_2O). 10 MOI of SeV/dF/FIR apparently suppressed activated c-Myc expression, whereas SeV/dF/FIR did not influence basal expression (MetOH or H_2O). FIR: FBP Interacting Repressor; FBP: FUSE-Binding protein; FUSE: Far Upstream Element; SeV: Sendai virus; GFP: Green fluorescent protein; MOI: Multiplicity of infection; SSA: Spliceostatin.

ment for the future development of cancer therapies based on targeting individual oncogenes such as *c-myc*. We have previously reported that FIR strongly represses endogenous *c-myc* transcription, and induces apoptosis[7] and is thus applicable for cancer treatment. In this study, first, we demonstrated that *c-myc* suppressor FBP-interacting repressor (FIR) strongly repressed endogenous *c-myc* transcription and induced apoptosis in SW480, LoVo (human colon cancer cell lines) as well as HeLa cells (human cervical squamous cancer cell line). Second, SeV/dF/FIR showed strong anti-tumor effects in both cultured cells and xenograft tumor growth in an animal model. These results provide new insight into a new therapeutic target for tumor treatment.

What type of suitable vector should be selected and how should FIR expressing vectors be conveyed to cancers? Sendai virus is an RNA virus and exists only in the cytoplasm, hence it is relatively safe as it does not affect chromosomes. In addition, SeV does not transform cells by integrating its genome into the cellular genome, thereby avoiding possible malignant transformation due to the genetic alteration of host cells; this is a safety advantage of SeV. For this reason, we chose SeV and prepared a fusion gene-deficient SeV/dF/FIR vector. The fusion gene-deficient SeV vector cannot transmit to F protein-non-expressing cells as F protein is essential for viral infection. The fusion gene-deficient SeV vector in this study does not require helper virus for reproduction, but is self-replicable in infected cells. Thus, the fusion gene-deficient SeV vector has several advantages over expressing vectors as a gene delivery system for human disease including cancer treatment. First, the fusion gene-deficient SeV vector is not pathogenic in humans. Second, the virus replicates only in the cytoplasm, therefore does not affect chromosome DNA in host cells. Third, SeV vector shows highly efficient gene transfer to a wide spectrum of cells, even to smooth muscle cells, nerve cells, or endothelial cells which are generally difficult to infect. Fourth, the SeV vector shows highly efficient gene transfer to a wide spectrum of cells, even to smooth muscle cells and does not generate wild-type virus in packaging cells. Recently, a gene-deficient SeV (SeV/dF) vector alone demonstrated tumor suppression by activating dendritic cells (DCs)[24], or if granulocyte

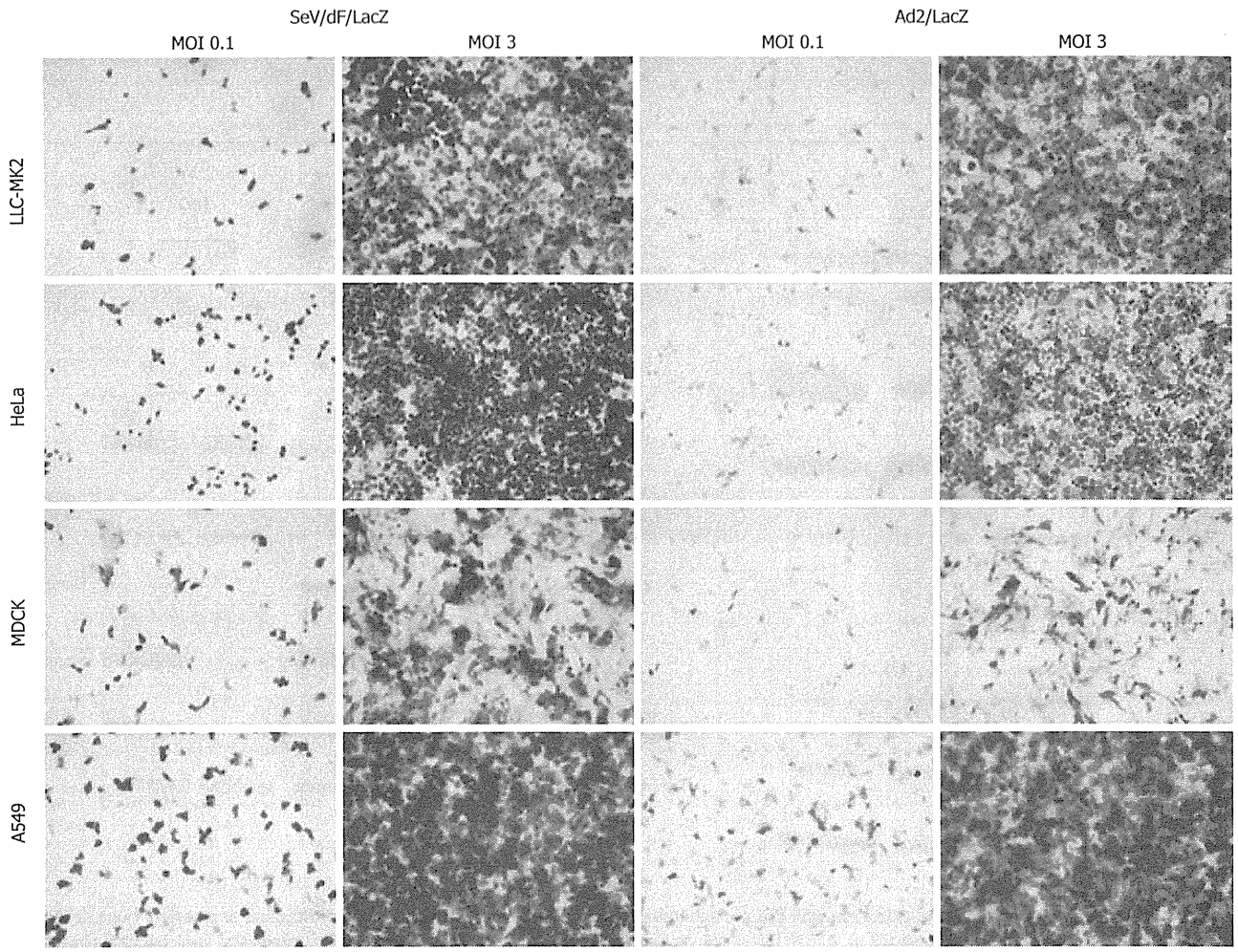

Figure 7 Sendai virus/dF/LacZ transduction efficiency was examined in some human and animal cell lines. Confluent culture of LLC-MK2 (macaque kidney fibroblasts), HeLa (human adenocarcinoma cells), MDCK (canine kidney cells), and A549 (human lung carcinoma cells) were infected with LacZ expressing SeV vector (SeV/dF/LacZ) at a MOI of 0.1 or 3.0. LacZ expressing Adenovirus vector (Ad5/LacZ) was used as a control. Two days after infection, the cells were stained with X Gal. SeV: Sendai virus.

macrophage colony-stimulating factor was encoded, it produced autologous tumor vaccines[25]. Therefore, the SeV/dF/FIR vector in this study may suppress tumor growth by a dual function through c-Myc suppression of tumor cells and DC activation. Furthermore, SeV/dF/FIR showed a synergistic effect with cisplatin in the treatment of malignant pleural mesothelioma[29]. FIR-binding proteins are basically classified into four categories (Table 1); (1) RNA binding proteins and splicing factors; (2) transcription factors and chromatin remodeling proteins; (3) actin-binding proteins; and (4) signal transduction and protein kinase families. This suggests that FIR potentially engages in some different intracellular events, such as RNA transport, DNA damage repair and pre-mRNA splicing. Accordingly, the side effects of SeV/dF/FIR need to be considered before clinical use, such as pre-mRNA splicing disturbance[8,9], DNA damage repair[37] or intracellular protein transport interference. For clinical safety, SeV/dF/FIR is preferable for local tumor growth control rather than systemic cancer therapy.

Taken together, these findings show that SeV/dF/FIR is a promising approach for cancer gene therapy, although further clinical and basic research are required to explain the precise mechanism of tumor suppression by FIR expressing vectors.

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COMMENTS

Background

Far Up Stream Element-Binding Protein-Interacting Repressor (FIR) is a *c-myc* transcriptional repressor. Thus, FIR expressing vectors are applicable for cancer therapy. In this study, the authors examined a novel therapeutic strategy to suppress *c-myc* in human cancers using a fusion gene-deficient Sendai virus (SeV/dF/FIR) which is inherently non-transmissible.

Research frontiers

As c-myc transcriptional control is largely unknown, modulation of c-myc regulation by SeV/dF/FIR for cancer therapy should be monitored, strictly and skeptically, from several aspects. This study revealed that SeV/dF/FIR is effective for cancer gene therapy without significant side effects in a xenograft model.

Innovations and breakthroughs

SeV/dF/FIR showed high gene transduction efficiency with significant antitumor effects and apoptosis induction in HeLa and SW480 cells. In the xenograft model, SeV/dF/FIR showed strong suppression of tumor growth with no significant side effects.

Applications

SeV/dF/FIR is potentially applicable for future clinical cancer treatment as SeV/dF/FIR suppresses endogenous c-Myc as well as Spliceostatin A (SSA)-activated c-Myc.

Terminology

FUSE: Far Upstream Element which is required for correct c-myc transcription. FBP: FUSE-Binding protein which has strong transcriptional activity. FIR: FBP interacting repressor which is a critical transcriptional repressor of c-myc gene. SeV: Sendai virus, a member of the Paramyxoviridae family, has envelopes and a nonsegmented negative-strand RNA genome. The SeV genome contains six major genes in tandem on a single negative-strand RNA. DC: Dendritic cell. The gene-deficient SeV (SeV/dF) vector alone demonstrates tumor suppression by activating dendritic cells (DCs).

Peer review

The authors performed the enthusiastic experiments *in vivo* and animal model to examine the SeV/dF/FIR for cancer gene therapy to minimize the side effect for the clinical use.

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Effects of different promoters on the virulence and immunogenicity of a HIV-1 Env-expressing recombinant vaccinia vaccine

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ABSTRACT

Previously, we developed a vaccination regimen that involves priming with recombinant vaccinia virus LC16m8Δ (rm8Δ) strain followed by boosting with a Sendai virus-containing vector. This protocol induced both humoral and cellular immune responses against the HIV-1 envelope protein. The current study aims to optimize this regimen by comparing the immunogenicity and safety of two rm8Δ strains that express HIV-1 Env under the control of a moderate promoter, p7.5, or a strong promoter, pSFJ1-10. m8Δ-p7.5-JRCSFenv synthesized less gp160 but showed significantly higher growth potential than m8Δ-pSFJ-JRCSFenv. The two different rm8Δ strains induced antigen-specific immunity; however, m8Δ-pSFJ-JRCSFenv elicited a stronger anti-Env antibody response whereas m8Δ-p7.5-JRCSFenv induced a stronger Env-specific cytotoxic T lymphocyte response. Both strains were less virulent than the parental m8Δ strain, suggesting that they would be safe for use in humans. These findings indicate the vaccine can be optimized to induce favorable immune responses (either cellular or humoral), and forms the basis for the rational design of an AIDS vaccine using recombinant vaccinia as the delivery vector.

1. Introduction

Despite the increasing availability and effectiveness of antiretroviral treatments, a safe and effective vaccine that prevents HIV-1 infection would be invaluable. A recent report from Thailand showed that the RV144 vaccine protocol, which involved priming with a canarypox virus vector followed by boosting with recombinant gp120 protein, reduced HIV-1 infection by approximately 30% [1]. These results are encouraging, and indicate that poxviruses may be used as vectors for HIV-1 subunit vaccines.

However, the efficacy of the RV144 vaccine was only moderate, suggesting the need to improve either the vaccination regimen or the poxvirus vector used for delivery. One improvement that may elicit a more potent protective immune response is the use of a replication-competent vaccinia virus (VV) vector rather than the non-replicating canarypox vector.

We previously reported that a heterologous prime-boost vaccination protocol using a recombinant m8Δ (rm8Δ) virus (m8Δ-pSFJ-JRCSFenv), which expresses the HIV-1JR-CSF envelope glycoprotein, and a recombinant Sendai virus (rSeV), SeV-JRCSFenv, elicited both HIV-1 Env-specific humoral and cell-mediated immune responses [2]. This may be a promising vaccination protocol to protect against HIV-1 infection. The aim of the present study is to further optimize this regimen.

The replication-competent VV strain, LC16m8, is a smallpox vaccine licensed for use in Japan. It has been used in 100,000 people without any serious adverse effects [3]. LC16m8Δ (m8Δ) is a genetically stable derivative of LC16m8, which is safer than the parental LC16m8 virus but shows the same degree of antigenicity [4]. Moreover, immunization with m8Δ protects mice against infection with virulent VV much more efficiently than the non-replicating VV strain, MVA [4]. Thus, m8Δ may be a promising VV vector for use in vaccines against infectious diseases.

Three types of promoter (early, intermediate, and late) have been identified in VV. Antigens that are highly expressed under the control of a powerful late promoter are generally considered to be potent inducers of a strong immune response [5]. However, early promoters appear to elicit stronger cytotoxic T lymphocyte (CTL) responses [6,7]. In some cases, the propagation of VV *in vitro* is suppressed in the presence of high levels of foreign antigen. Therefore, the balance between antigen expression and viral propagation *in vivo* may be crucial for optimal immunogenicity.

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The p7.5 promoter is an early–late promoter that was identified in 1984 and is widely used for the construction of live VV-vectored vaccines. Because the levels of gene expression driven by the p7.5 promoter have yet to be optimal, a more potent promoter, pSFJ1-10, was constructed, which enables the genes of interest to be expressed at higher levels during both the early and late phases of the infection cycle [8,9].

Here, we compared the immunogenicity and safety of two rm8Δs that express HIV-1 Env under the control of the p7.5 or pSFJ1-10 promoters. Both were tested in a vaccination protocol that involved priming with rm8Δ followed by boosting with rSeV. We found that one of the vectors preferably induced humoral responses against HIV-Env, whereas the other primarily induced cellular immune responses. These findings suggest that it may be possible to select vaccine vectors that induce favorable immune responses. In suckling mice, both rm8Δ-p7.5-JRCSFenv and rm8ΔpSFJ-JRCSFenv were relatively less virulent than LC16m8Δ. Our results may provide important information to develop HIV-1 vaccine for clinical trials.

2. Materials and methods

2.1. Cells and viruses

The RK13 cell line was cultured in RPMI1640 supplemented with 10% FCS at 37 °C in an atmosphere containing 5% CO_2. BHK, TZM-bl [10,11], 293T and L929 cell lines were cultured in DMEM supplemented with 10% FCS. VV LC16m8Δ, m8ΔVNC110 that harbors multiple cloning site in the HA gene of LC16m8Δ genome, m8Δ-pSFJ-JRCSFenv and SeV-JRCSFenv, which express gp160 of HIV-1 JRCSF, and canarypox virus were described previously [2].

2.2. Construction of the LC16m8Δ expressing JR-CSFenv under the control of the p7.5 promoter

To construct LC16m8Δ-p7.5-JRCSFenv, a transfer plasmid that harbors the HIV-1 JR-CSF env gene downstream of the p7.5 VV promoter was first constructed. The gp160-encoding region was amplified from pJWJRCSFenvΔEcoR1 (the template) by PCR using the following primer pair: JRCSFenv F1 (AGTGGATCCGCCACCATGAGAGTGAAGGGGATCAGGAAG; BamH1 site underlined) and JRCSFenvR1 (TTAGAGCTCTTATAGCAAAGCCCTTTCCAAGCC; Sac1 site underlined). The VV transcription termination signals (TTTTTNT) within the env gene sequence were synonymously mutated *in vitro* using a mutagenesis kit (Stratagene). The env fragment was then digested with Sac1 and ligated into the pVR1 vector [12], which had been digested with Sac1 and Sma1. BHK cells, which had been infected with canarypox virus, were then co-transfected with the resultant plasmid and LC16m8Δ genomic DNA to generate VV LC16m8Δ-p7.5-JRCSFenv. HA^- recombinants were selected using erythrocytes isolated from white leghorn chickens (Sankyo Labo Service Corporation, Inc.) [13,14]. Expression of HIV-1 Env protein was examined by Western blotting and plaque immunostaining.

2.3. Western blotting

Vaccinia-infected-RK13 cells were lyzed and the proteins separated in 10% SDS-PAGE gels. Immunoblot analysis was performed with human antiserum from a HIV-1-infected patient or monoclonal mouse anti-β actin antibody, followed by alkaline phosphatase-conjugated anti-human or mouse IgG (Promega). Proteins were visualized using NBT/BCIP (Sigma).

2.4. Plaque immunostaining

RK13 cells were cultured in 6-well plates and infected with recombinant VV (at approximately 100 plaque forming unit (pfu)/well). The cells were incubated with the virus for 72 h at 33 °C, fixed with 2% paraformaldehyde solution, and permeabilized by incubating with 0.5% Nonidet P-40 for 1 min. The fixed cells were blocked with 5% skimmed milk (in PBS) for 30 min at room temperature and incubated with the primary antibody (HIV-1 infected human serum; diluted 1000-fold) for 1 h at room temperature, followed by the secondary antibody (alkaline phosphatase-conjugated anti-human IgG (Promega); diluted 2500-fold). The plaques were then stained with NBT/BCIP.

2.5. Propagation potential of rm8Δ

To evaluate the propagation potential of LC16m8Δ and its recombinants, RK13, 293T and L929 cells (3×10^5) were infected with the viruses at a multiplicity of infection of 3 and then incubated for 24 h at 33 °C. Progeny viruses were harvested and titrated on a monolayer of RK13 cells in a plaque assay.

2.6. Immunization of mice

Seven-week-old female C57BL/6J mice (CLEA Japan) were administered with LC16m8Δ's recombinants (each at 1×10^7 pfu by skin scarification). Eight weeks later, the mice were boosted with rSeV expressing JRCSFenv (4×10^7 cell-infectious unit (CIU)) *via* intranasal administration (i.n.). Mice were sacrificed at 2 or 8 weeks after the final immunization, and serum and spleen samples were collected.

2.7. Intracellular cytokine staining (ICS) of splenocytes

Env-specific cellular immune responses were measured using an ICS assay as described previously [2]. The percentage of IFN-γ^+CD107a$^+$ T cells within the CD4- or CD8-gated lymphocyte populations were determined using a FACSCanto flow cytometer (BD biosciences) and the data were analyzed using FlowJo software (Tree Star).

2.8. Evaluation of neutralizing activity

The HIV-1 neutralizing activity of the mouse sera was measured in a TZM-bl cell-based assay as previously described [2,15,16].

2.9. Measurement of anti-Env antibody levels by ELISA

The titer and avidity of the anti-HIV-1 Env IgG antibodies in the mouse sera were determined by ELISA as described previously [2].

2.10. Safety of m8Δs

To evaluate the safety of the LC16m8Δ and m8Δ recombinants, 10 μl of a serially diluted solution that contains 10^3–10^7 pfu of rm8Δs was injected intracerebrally (i.c.) into 10 to 17 of 2–3-day-old suckling Crlj:CD1 (ICR) mice (Charles River) [17]. Survival was monitored daily for 2 weeks and the 50% lethal dose (LD50) was calculated as described in a figure legend.

2.11. Statistical analysis

Statistical analysis was performed using Student's *t*-test (Microsoft Excel version 11.6.6). *P* values of <0.05 were

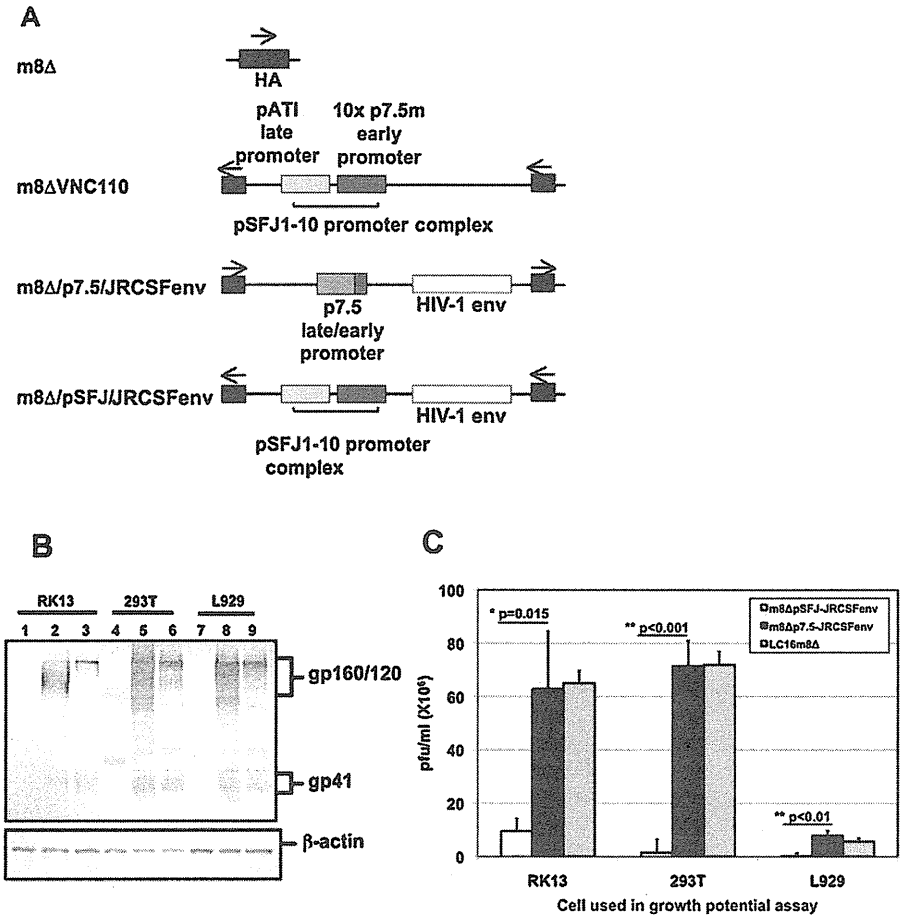


Fig. 1. Construction of the Env-expressing vaccinia vector, Env expression, and virus propagation. (A). Schematic illustration showing the structure of the hemagglutinin (HA) gene region within LC16m8Δ and its derivatives. Arrows indicate the direction of the HA coding frame. (B). Comparison of Env expression in cells infected with m8Δ-pSFJ-JRCSFenv or m8Δ-p7.5-JRCSFenv. One microgram of cell lysate derived from RK13 (rabbit kidney epithelial), 293T (human embryonal kidney cell line) and L929 (mouse fibroblastoid line) cells infected with rVV was subjected to SDS-PAGE and analyzed by Western blotting as described in Section 2. Lanes 1, 4, and 7 represent the cells infected with LC16m8Δ, lanes 2, 5, and 8, m8Δ-pSFJ-JRCSFenv; Lanes 3, 6, and 9, m8Δ-p7.5-JRCSFenv. (C) Comparison of the growth potential of the LC16m8Δ constructs. Viruses were recovered from RK13, 293T, and L929 cells 24 h after infection and titrated in a plaque assay. Data represent the mean ± SD ($n = 4$).

considered significant. The survival of virus-injected suckling mice was evaluated using the log-rank test (R version 2.15.1).

3. Results

3.1. In vitro properties of rm8Δ expressing JR-CSFenv under the control of different promoters

We previously constructed m8Δ-pSFJ-JRCSFenv, which expresses the HIV-1JR-CSF env gene under the control of the high expression pSFJ1-10 promoter, and showed that it elicited HIV-1 Env-specific cellular and humoral responses when used in combination with the Sendai vector, SeV-JRCSFenv [2]. However, because an rVV that moderately expresses a foreign gene, but propagates better, might elicit more potent immunological responses, we constructed recombinant m8Δ expressing JR-CSFenv under the control of the p7.5 promoter (which is a moderate driver of foreign gene expression) (Fig. 1A). We first compared expression of the Env protein in various cells infected with m8Δ-p7.5-JRCSFenv or m8Δ-pSFJ-JRCSFenv by Western blotting (Fig. 1B). Regardless of the cell type, m8Δ-p7.5-JRCSFenv produced several-fold less gp120/160 than m8Δ-pSFJ-JRCSFenv. In addition, the bands corresponding to gp120/160 expressed by cells infected with m8Δ-pSFJ-JRCSFenv were much broader than those expressed by cells infected with m8Δ-p7.5-JRCSFenv. Meanwhile, titration of the progeny virus after one-step growth revealed that the growth potential of rVVs in mouse L929 cells are 10 times lower than that in human 293T cells and rabbit RK13 cells (Fig. 1C). Nevertheless, m8Δ-p7.5-JRCSFenv showed growth potential similar to that of the parental m8Δ, and significantly higher (6–50-fold) than that of m8Δ-pSFJ-JRCSFenv (Fig. 1C). This indicates that overexpression of the foreign gene suppresses viral propagation.

3.2. Immunogenicity of m8Δ-p7.5-JRCSFenv and m8Δ-pSFJ-JRCSFenv

We next compared the immunogenicity of m8Δ-p7.5-JRCSFenv and m8Δ-pSFJ-JRCSFenv by using them to prime mice, which were then boosted with SeV-JRCSFenv according to the schedule outlined in Fig. 2A. Splenocytes were isolated, stimulated with a mixture of HIV-1 consensus subtype B Env (15-mer) peptides (NIH AIDS reagent program, No. 202/203; corresponding to aa 805–819 and aa 809–823 of gp160), the two most immunogenic HIV-derived peptides, and then examined by ICS [2]. The percentage of HIV-1 Env-specific IFN-γ-secreting $CD107a^+CD8^+$ T cells was then calculated. A representative gating strategy is shown in Fig. 2B. Vaccination with m8Δ-pSFJ-JRCSFenv and m8Δ-p7.5-JRCSFenv elicited HIV-1JR-CSF Env-specific CTL responses. Mice primed with m8Δ-p7.5-JRCSFenv showed higher levels of HIV-1 Env-specific IFN $\gamma^+CD107a^+CD8^+$ T cells than mice primed with m8Δ-pSFJ-JRCSFenv (Fig. 2C; 12.8 ± 1.2% *vs.* 7.8 ± 2.1%; $p = 0.002$). The proportion of IFN $\gamma^+CD107a^+CD8^+$ T cells in both groups somewhat

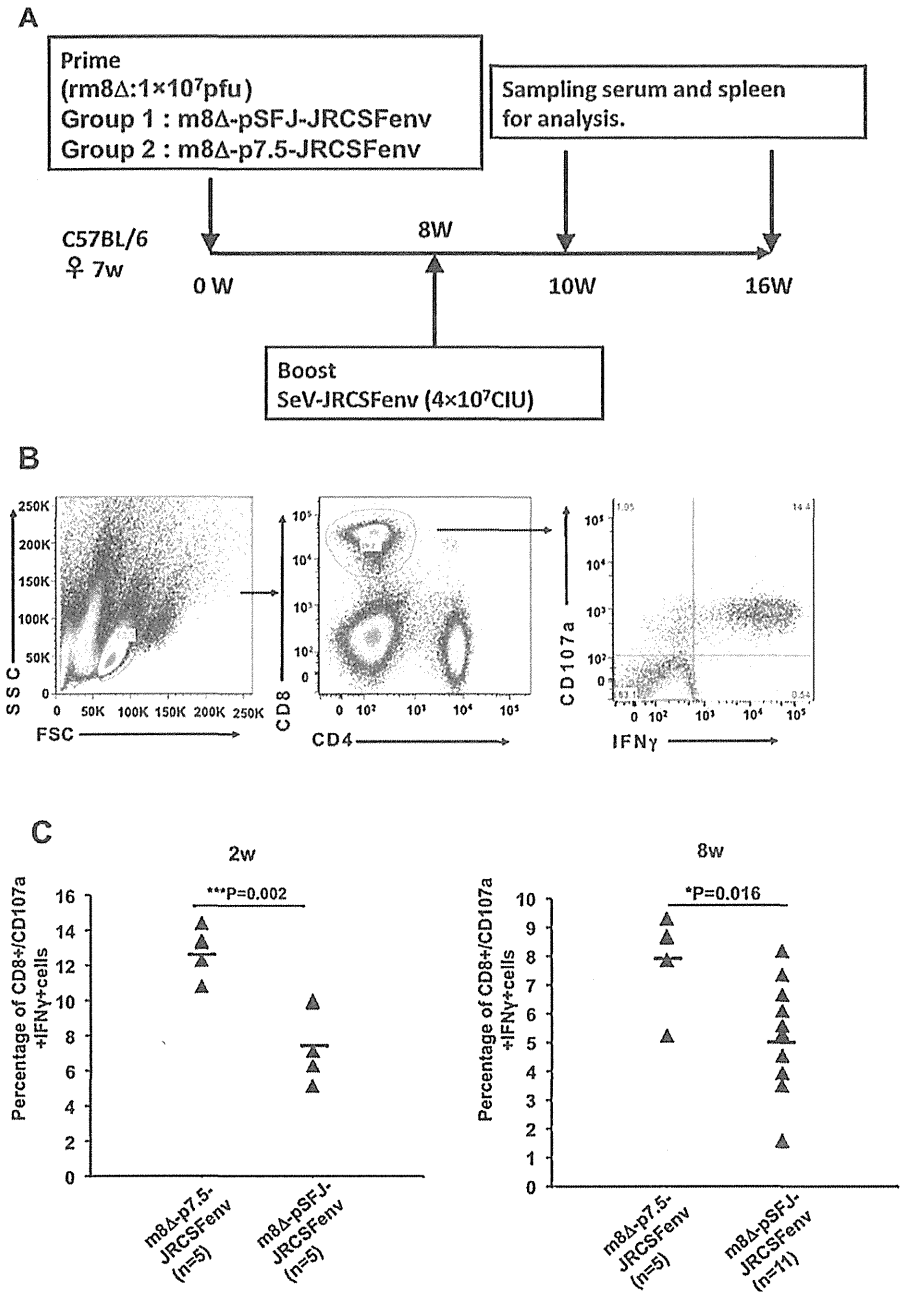


Fig. 2. The p7.5 promoter induces more efficient production of Env-specific CTL responses than the pSFJ promoter. (A) Schematic illustration showing the rm8Δ prime/rSeV boost vaccination protocol. Seven-week-old female C57BL/6J mice were vaccinated with LC16m8Δ's recombinants (16 mice for m8Δ-pSFJ-JRCSFenv (group 1) and 10 mice for m8Δ-p7.5-JRCSFenv (group 2); each at 1×10^7 pfu) followed by a boost with SeV-JRCSFenv (4×10^7 CIU). Blood samples and spleen tissues were examined at the indicated time points. (B) Representative diagram showing FACS analysis of HIV-1 Env-specific IFN-γ-secreting CD107a$^+$CD8$^+$ T cells derived from vaccinated mouse splenocytes. (C) Comparison of Env-specific cellular immune response between the two vaccinated groups at 2 and 8 weeks post-SeV boost.

declined at 8 weeks post-boost; however, the difference between the groups was maintained ($p = 0.016$). We next measured the levels of Env-specific Abs (Fig. 3A) and anti-HIV-1-neutralizing Abs (Fig. 3B) in mice sera. The levels of anti-HIV-1 Env-specific IgG were 6–7-fold higher in mice immunized with m8Δ-pSFJ-JRCSFenv than in mice immunized with m8Δ-p7.5-JRCSFenv; this was in sharp contrast to the observed cellular responses (Fig. 2). The difference of humoral immunity had already been detected 6 weeks after rm8Δ prime (supplementary data). The uneven of sample numbers between two groups did not introduce bias into the data, since we obtained the same result when two groups have the same number of animals (data not shown). Sera from both groups of mice showed neutralizing activity against a tier 1 pseudotyped HIV-1 strain, SF162, but only after rSeV boost, and no neutralization activity against tier 2 HIV-1 had been detected. At both 2 and 8 weeks post-SeV-JRCSFenv boost, the neutralizing competency of sera from mice immunized with m8Δ-pSFJ-JRCSFenv was marginally stronger than that of mice immunized with m8Δ-p7.5-JRCSFenv; however, the difference was not significant (Fig. 3B). We also measured the avidity of the anti-Env Abs in both groups: no significant difference was observed (Fig. 3C). Since the m8Δ-pSFJ-JRCSFenv prime/SeV-JRCSFenv boost elicited greater HIV-1 Env-specific antibody responses, we next asked whether this antibody titer is maintained over the long-term. We followed a subgroup of mice

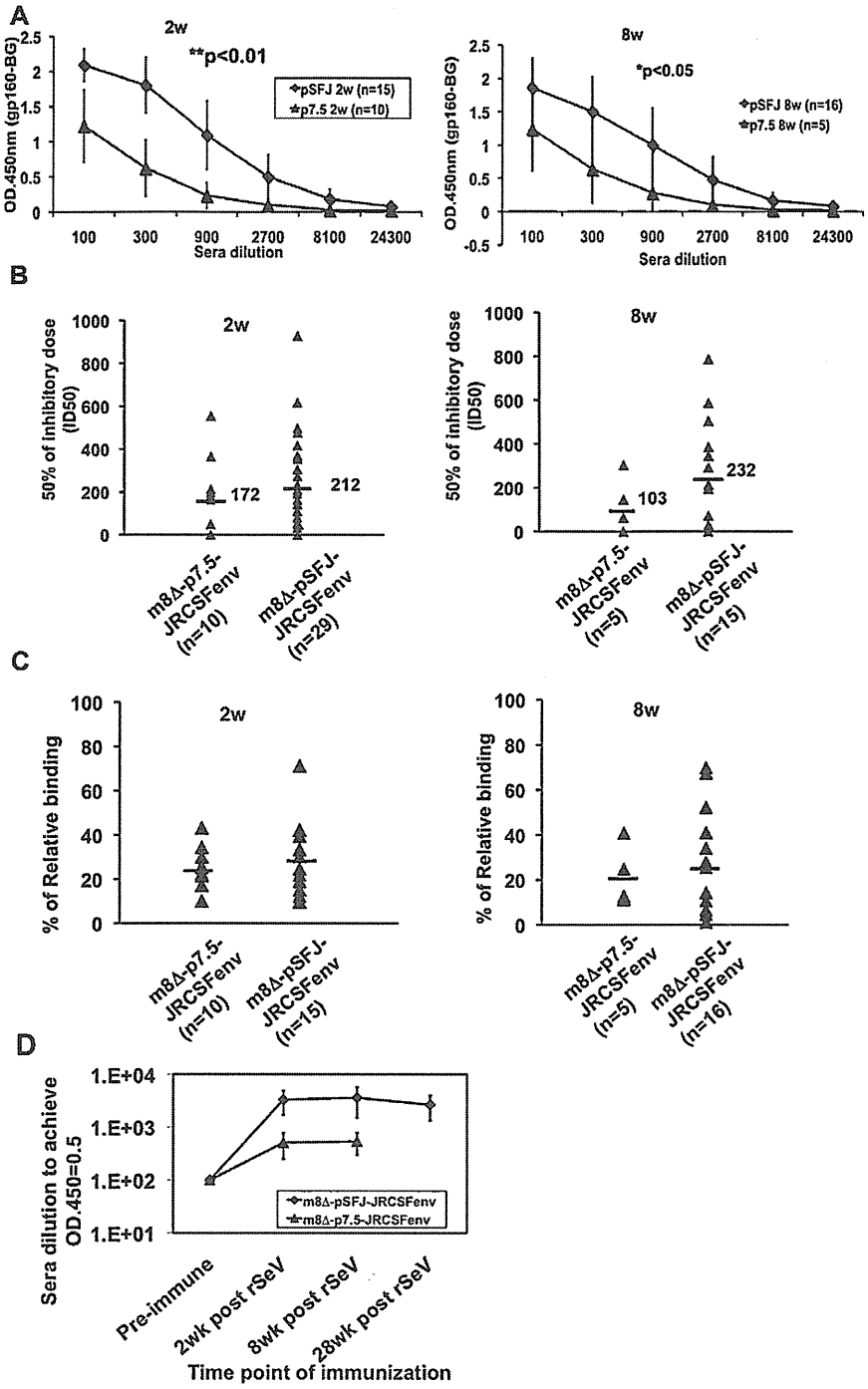
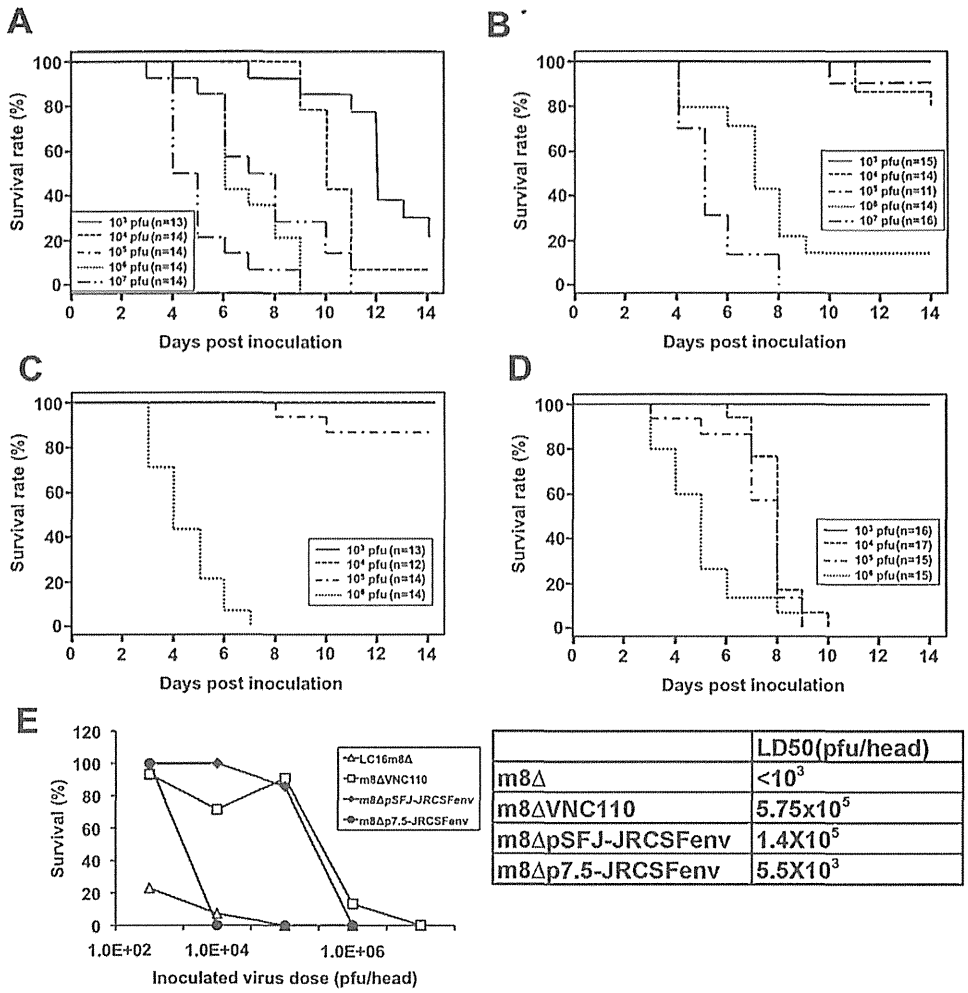


Fig. 3. m8Δ-pSFJ-JRCSFenv induces stronger humoral immune responses than m8Δ-p7.5-JRCSFenv (A). Comparison of Env-specific antibody levels. Serum from individual immunized mice was analyzed in a HIV-1JR-CSF gp160 ELISA as described previously (Ref. [2]). The plates were developed with an HRP-conjugated anti-mouse IgG antibody. The Env-specific antibody titer was determined by subtracting the background values at OD450. Data represent the mean ± SD of the Env-specific antibody titer of all animals in each group. Env binding antibody titers measured at 2 and 8 weeks post-rSeV boost are shown. (B) Comparison of anti-HIV-1 neutralizing antibody activity in sera from the two groups of immunized mice. We included more previously accumulated mice samples that subjected to the same immunization procedure as group 1 in Fig. 2. The 50% inhibitory dose (ID50) against an HIV-1 SF162 env-pseudotyped virus was measured using TZM-bl cells (a CD4- and CCR5-expressing derivative of HeLa cells). The neutralizing activity of mouse sera is shown at 2 and 8 weeks post-SeV boost. (C) Comparison of the avidity of HIV-1 Env-specific anti-sera from the two groups at 2 and 8 weeks post-SeV boost as described previously (Ref. [2]). (D) Comparison of HIV-1 Env-specific antibody induction dynamics between the two groups after the rSeV boost.

treated with this vaccination regimen for 28 weeks after the Sendai virus boost and found that the anti-HIV-1 Env antibody titer was maintained throughout the observation period (Fig. 3D).

3.3. Safety evaluation of the rm8Δ in suckling mice

To evaluate the safety of rm8Δ, we i.c.-injected suckling mice with m8Δ, m8ΔVNC110, m8Δ-pSFJ-JRCSFenv, or m8Δ-p7.5-JRCSFenv. At 2 weeks post-injection, more of the mice in the m8ΔVNC110- and m8Δ-pSFJ-JRCSFenv-injected (at 10^4 and 10^5 pfu) groups survived compared with those in the m8Δ-p7.5-JRCSFenv-injected group (Fig. 4B–D). LC16m8Δ, which should be safe for human use, showed the highest mortality (Fig. 4A). The median lethal doses (LD50) for each strain were as follows: LC16m8Δ, $<10^3$ pfu; m8Δ-p7.5-JRCSFenv, 5.5×10^3 pfu; m8Δ-pSFJ-JRCSFenv, 1.4×10^5 pfu; and m8ΔVNC110, 5.75×10^5 pfu



	LD50(pfu/head)
m8Δ	$<10^3$
m8ΔVNC110	5.75×10^5
m8ΔpSFJ-JRCSFenv	1.4×10^5
m8Δp7.5-JRCSFenv	5.5×10^3

Fig. 4. m8Δ-pSFJ-JRCSFenv is safer than m8Δ-p7.5-JRCSFenv *in vivo*. Cumulative survival curves for vaccinated suckling mice are shown. Mice were injected with LC16m8Δ (A), m8ΔVNC110 (B), m8Δ-pSFJ-JRCSFenv (C), or m8Δ-p7.5-JRCSFenv (D). Percentage of survival at 2 weeks post inoculation was plotted to make a survive curve using Microsoft Excel (version 11.6.6) and the 50% lethal dose (LD50) was calculated according to the Trendline of the curve. The LD50 for each VV is shown (E). The numbers of the mice used for each dose are indicated in the chart. Statistical analysis was performed using the log-rank test.

(Fig. 4E). These results suggest that both m8Δ-p7.5-JRCSFenv and m8Δ-pSFJ-JRCSFenv may be safer for use in humans.

4. Discussion

An effective HIV-1 vaccine should induce long-lasting humoral and cellular immunity against HIV-1. A replication-competent VV would be a good candidate for such a vaccine because recombinant VV can induce both antigen-specific CTL and antibody responses. In addition, the process of viral replication may allow the repeated presentation of viral antigens, leading to affinity maturation of both antibodies and T cell receptors. LC16m8Δ-JRCSFenv is a replication-competent vaccinia vector that induces HIV-1 Env-specific cellular and humoral immune responses when used in combination with a Sendai virus vector [2]. Here, we tried to optimize this vaccination regimen by using HIV-1 Env recombinant VV vectors expressed under the control of different promoters. We found that viruses expressed under the control of these different promoters induced different cellular and humoral immune responses. m8Δ-pSFJ-JRCSFenv induced increased production of anti-HIV-1 Env-specific Abs when compared with m8Δ-p7.5-JRCSFenv. By contrast, m8Δ-p7.5-JRCSFenv induced the production of more HIV-1 Env-specific IFN-γ-secreting $CD107a^+CD8^+$ T cells. These results suggest that the induction of Env-specific CTL and humoral responses may be dependent upon different presentation pathways and/or different structures of the Env protein. The peptides used to stimulate the splenocytes in the ICS assay correspond to the 3′ domain of gp41, since previous mapping of the consensus subtype B Env peptide pool identified peptides comprising aa 805–819 and aa 809–823 as the best immunogens [2]. It also indicated that gp41 but not gp120 is a more potent inducer of cellular immunity when liberated from gp160 than it is when buried in gp160. The amount of gp41 in m8Δ-p7.5-JRCSFenv-infected 293T and L929 cells are comparable with that infected with m8Δ-pSFJ-JRCSFenv and even more in m8Δ-p7.5-JRCSFenv-infected RK13 cells, which is different from the case of gp160/gp120 (Fig. 1B). Considering the better replication of m8Δ-p7.5-JRCSFenv (Fig. 1C), we may expect that repetitive antigenic stimulation, which favors CTL induction, strengthens the immunogenicity of gp41 that is derived from m8Δ-p7.5-JRCSFenv to induce production of Env-specific IFN-$\gamma^+CD107a^+CD8^+$ T cells. The relatively lower ratio of gp41 to gp160/gp120 in m8Δ-pSFJ-JRCSFenv-infected cells than m8Δ-p7.5-JRCSFenv cells indicates that the cleavage of gp160 to gp120 and gp41 was less efficient due to overexpression of Env.

On the other hand, the efficient production of anti-Env-specific antibodies may require higher expression of HIV-1 Env in primarily infected cells. The 6–7-fold higher level of the HIV-1 Env binding antibody titer observed in mice immunized with m8Δ-pSFJ-JRCSFenv is consistent with the higher levels of Env observed

in m8Δ-pSFJ-JRCSFenv-infected cells. The gp160/gp120 isolated from m8Δ-pSFJ-JRCSFenv-infected cells migrated more quickly and showed a broader band in PAGE gels than that from m8Δ-p7.5-JRCSFenv-infected cells. This suggests the incomplete glycosylation of gp160/gp120 due to an insufficiency of host glycosyltransferases. Nevertheless, the impact on the ability of m8Δ-pSFJ-JRCSFenv to elicit anti-Env antibody response was minimal. Although m8Δ-pSFJ-JRCSFenv induced greater production of Env binding Abs than m8Δ-p7.5-JRCSFenv, it did not induce the production of more potent anti-HIV-1 neutralizing Abs. At 8 weeks after rSeV-JRCSFenv boost, the average ID50 of serum from mice immunized with m8Δ-pSFJ-JRCSFenv was higher than that of serum from mice immunized with m8Δ-p7.5-JRCSFenv; however, the difference was not significant. This suggests that, in addition to the amount of expressed Env, the properties of the antigen (for example, the structure of the exposed epitopes) may also be important for the induction of neutralizing antibody production. There was no difference in the avidity of the anti-Env antibodies between the two groups (Fig. 3C), implicating that the process of affinity maturation was similar. This suggests that affinity maturation of antibodies is necessary, but not sufficient to induce the production of potent neutralizing antibodies. Even so, higher levels of Env binding antibodies may enable the induction of other types of antiviral immunity, such as antibody-dependent cellular cytotoxicity and antibody-dependent cell-mediated virus inhibition.

We recently showed that priming mice with an m8Δ that expresses both CD40Lm and Env induces the production of high-avidity anti-Env antibodies [2]. The above results suggest that it might be important to incorporate an adjuvant, such as CD40Lm, within the AIDS vaccine regimen to induce more potent humoral responses and produce higher levels of neutralizing antibodies.

A successful AIDS vaccine should induce the production of long-lasting antibodies. Both m8Δ-pSFJ-JRCSFenv and m8Δ-p7.5-JRCSFenv induced the production of long-lasting anti-Env antibodies when used in the rm8Δs prime/rSeV boost regimen. Immunized mice maintained high levels of anti-Env antibodies for up to 28 weeks (Fig. 3D). This supports our previous report showing that the rm8Δs prime/rSeV regimen is a good platform for the development of an HIV-1 vaccine.

Safety is critical when evaluating vaccines in clinical trials. Both m8Δ-pSFJ-JRCSFenv and m8Δ-p7.5-JRCSFenv were less virulent in newborn mice than the parental strain, LC16m8Δ. LC16m8Δ was more virulent probably because it contains an intact HA gene. The LD50 of m8Δ-p7.5-JRCSFenv was significantly lower than that of m8ΔVNC110, although their growth potential was similar. This suggests that the expression of HIV-1 Env in the mouse brain is harmful. This is supported by the fact that that virulence of m8Δ-pSFJ-JRCSFenv is similar to that of m8ΔVNC110, despite having a much lower capacity for replication. Nevertheless, our finding that recombinant VVs expressing HIV-1 env are safer than LC16m8Δ suggests that they may be promising candidates for clinical trials.

In conclusion, the results of the present study suggest that VV m8Δ vectors containing different promoters activate different arms of the immune system. That said, both strains induced long-lasting CTL and antibody responses and both appear safe enough for clinical trials. Thus, it is possible to manipulate the immune response induced by a rational AIDS vaccine by using VV m8Δs harboring different promoters.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2013.12.022.

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Control of Simian Immunodeficiency Virus Replication by Vaccine-Induced Gag- and Vif-Specific CD8$^+$ T Cells

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For development of an effective T cell-based AIDS vaccine, it is critical to define the antigens that elicit the most potent responses. Recent studies have suggested that Gag-specific and possibly Vif/Nef-specific CD8$^+$ T cells can be important in control of the AIDS virus. Here, we tested whether induction of these CD8$^+$ T cells by prophylactic vaccination can result in control of simian immunodeficiency virus (SIV) replication in Burmese rhesus macaques sharing the major histocompatibility complex class I (MHC-I) haplotype *90-010-Ie* associated with dominant Nef-specific CD8$^+$ T-cell responses. In the first group vaccinated with Gag-expressing vectors ($n = 5$ animals), three animals that showed efficient Gag-specific CD8$^+$ T-cell responses in the acute phase postchallenge controlled SIV replication. In the second group vaccinated with Vif- and Nef-expressing vectors ($n = 6$ animals), three animals that elicited Vif-specific CD8$^+$ T-cell responses in the acute phase showed SIV control, whereas the remaining three with Nef-specific but not Vif-specific CD8$^+$ T-cell responses failed to control SIV replication. Analysis of 18 animals, consisting of seven unvaccinated noncontrollers and the 11 vaccinees described above, revealed that the sum of Gag- and Vif-specific CD8$^+$ T-cell frequencies in the acute phase was inversely correlated with plasma viral loads in the chronic phase. Our results suggest that replication of the AIDS virus can be controlled by vaccine-induced subdominant Gag/Vif epitope-specific CD8$^+$ T cells, providing a rationale for the induction of Gag- and/or Vif-specific CD8$^+$ T-cell responses by prophylactic AIDS vaccines.

Human immunodeficiency virus (HIV) infection induces persistent viral replication, leading to AIDS onset in humans. Virus-specific CD8$^+$ T-cell responses play a central role in the resolution of acute peak viremia (1–4) but mostly fail to contain viral replication in HIV infection. Prophylactic vaccination resulting in more effective CD8$^+$ T-cell responses postexposure than those in natural HIV infections might contribute to HIV control. Current trials in macaque AIDS models have shown that vaccine induction of T-cell responses can result in control of postchallenge viral replication (5–10). It is now critical to define the antigens that elicit the most potent responses for development of an effective T-cell-based AIDS vaccine.

Recent studies have implicated Gag-specific CD8$^+$ T cells in the control of HIV and simian immunodeficiency virus (SIV) replication (11–16). Several HLA or major histocompatibility complex class I (MHC-I) alleles have been shown to be associated with lower viral loads (17–25). Virus control associated with some of these protective MHC-I alleles is attributed to Gag epitope-specific CD8$^+$ T-cell responses (26–29). For instance, CD8$^+$ T-cell responses specific for the HLA-B*57-restricted $Gag_{240–249}$ TW10 and HLA-B*27-restricted $Gag_{263–272}$ KK10 epitopes exert strong suppressive pressure on HIV replication and frequently select for escape mutations with viral fitness costs, leading to lower viral loads (27, 30–33). Thus, certain individuals possessing MHC-I alleles associated with dominant Gag-specific CD8$^+$ T-cell responses could have a greater chance to control HIV replication than those without these alleles. For those individuals that do not express these MHC-I alleles, the question arises as to whether prophylactic vaccination inducing Gag epitope-specific CD8$^+$ T-cell responses might contribute to HIV control. Furthermore, recent studies have shown that CD8$^+$ T-cell responses targeting SIV antigens other than Gag, such as Mamu-B*08- or Mamu-B*17-restricted Vif and Nef epitopes, exert strong suppressive pressure on SIV replication (10, 34, 35).

We previously developed a prophylactic AIDS vaccine consisting of a DNA prime and a boost with a Sendai virus (SeV) vector expressing SIVmac239 Gag (SeV-Gag) (36). Our trial showed vaccine-based control of an SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype *90-120-Ia* (5, 37). Unvaccinated animals possessing *90-120-Ia* dominantly elicited CD8$^+$ T-cell responses specific for the $Gag_{206–216}$ (IINEE AADWDL) and the $Gag_{241–249}$ (SSVDEQIQW) epitopes after SIV challenge (38, 39). DNA/SeV-Gag-vaccinated *90-120-Ia*-positive macaques showed enhanced $Gag_{206–216}$-specific and $Gag_{241–249}$-specific CD8$^+$ T-cell responses in the acute phase after SIV challenge, resulting in viremia control (37). This implies virus control by vaccine-based enhancement of Gag-specific CD8$^+$ T-cell responses in animals possessing MHC-I alleles associated with dominant Gag CD8$^+$ T-cell epitopes. However, we have not defined the efficacy of prophylactic vaccination inducing Gag-specific CD8$^+$ T-cell responses against HIV/SIV infection in the hosts pos-

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sessing MHC-I alleles not associated with dominant Gag $CD8^+$ T-cell epitopes.

In the present study, we first examined efficacy of prophylactic vaccination inducing Gag-specific $CD8^+$ T-cell responses against SIVmac239 challenge in a group of macaques that possess the *90-010-Ie* MHC-I haplotype (referred to as E) associated with dominant Nef-specific $CD8^+$ T-cell responses (39, 40). Furthermore, we examined the efficacy of prophylactic vaccination inducing Vif/Nef-specific $CD8^+$ T-cell responses in these E^+ macaques. Our results show SIV control in those vaccinees that mounted efficient Gag- or Vif-specific $CD8^+$ T-cell responses in the acute phase postchallenge.

MATERIALS AND METHODS

Animal experiments. Animal experiments were carried out in Tsukuba Primate Research Center, National Institute of Biomedical Innovation (NIBP), with the help of the Corporation for Production and Research of Laboratory Primates after approval by the Committee on the Ethics of Animal Experiments of NIBP (permission number DS21-28 and DS23-19) under the guideline for animal experiments at NIBP and National Institute of Infectious Diseases, which is in accordance with the Guidelines for Proper Conduct of Animal Experiments established by Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf). Blood collection, vaccination, and SIV challenge were performed under ketamine anesthesia.

We used Burmese rhesus macaques (*Macaca mulatta*) possessing the MHC-I haplotype *90-010-Ie* (E) (39, 40). The determination of MHC-I haplotypes was based on the family study in combination with the reference strand-mediated conformation analysis of *Mamu-A* and *Mamu-B* genes and detection of major *Mamu-A* and *Mamu-B* alleles by cloning the reverse transcription (RT)-PCR products as described previously (39–41). Confirmed MHC-I alleles consisting of the MHC-I haplotype E are *Mamu-A1*066:01*, *Mamu-B*005:02*, and *Mamu-B*015:04*. Unvaccinated R01-011, R05-007, R08-003, R08-007, R09-011, and R06-038 and Gag-vaccinated R01-010 and R01-008 used in our previous experiments (39, 42) are included in the present study. At week 1, unvaccinated macaque R06-038 was intravenously infused with 300 mg of nonspecific immunoglobulin G purified from uninfected rhesus macaques as described before (43). All animals were intravenously challenged with 1,000 50% tissue culture infective doses ($TCID_{50}$) of SIVmac239 (44).

Macaques R01-010, R05-010, R01-008, R08-002, and R08-006 received prophylactic DNA prime/SeV-Gag boost vaccination (referred to as Gag vaccination) (5). The DNA used for the vaccination, cytomegalovirus (CMV)-SHIVdEN, was constructed from *env*-deleted and *nef*-deleted simian-human immunodeficiency virus SHIVMD14YE (45) molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with 6×10^9 cell infectious units (CIU) of F-deleted replication-defective Sendai virus (SeV) expressing SIVmac239 Gag (SeV-Gag) (46).

Macaques R08-012, R10-012, R10-013, R10-010, R10-011, and R10-014 received prophylactic DNA prime/SeV-VifNef boost vaccination (referred to as Vif/Nef vaccination). The Vif-expressing DNA used for the vaccination, pcDNA-SIVvif-opt, was constructed by introducing an optimized SIVmac239 Vif cDNA (GenScript) into pcDNA3.1. The Nef-expressing DNA used for the vaccination, pcDNA-SIVnef-G2A, has an SIVmac239 Nef cDNA with a mutation resulting in glycine (G) to alanine (A) at the 2nd amino acid (aa) in Nef. Animals intramuscularly received 3 mg of Vif-expressing DNA at the first DNA vaccination and 3 mg of Vif-expressing DNA and 3 mg of Nef-expressing DNA at the second DNA vaccination. Six weeks after the first DNA prime, animals received a single boost intranasally with 1×10^9 CIU of F-deleted SeV expressing Vif-opt (SeV-Vif) and 1×10^9 CIU of F-deleted SeV expressing Nef-G2A (SeV-Nef) (47).

Analysis of antigen-specific $CD8^+$ T-cell responses. We measured virus-specific $CD8^+$ T-cell frequencies by flow cytometric analysis of gamma interferon (IFN-γ) induction after specific stimulation as described previously (48, 49). Autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) were pulsed with each peptide (at a final concentration of 1 μM) or peptide pools (at a final concentration of 1 to 2 μM for each peptide) using panels of overlapping peptides spanning the entire SIVmac239 Gag, Vif, and Nef amino acid sequences (Sigma-Aldrich Japan) for 1 h. Peripheral blood mononuclear cells (PBMCs) were cocultured with these pulsed B-LCLs in the presence of GolgiStop (monensin; BD) for 6 h. Intracellular IFN-γ staining was performed with a Cytofix/Cytoperm kit (BD) and fluorescein isothiocyanate-conjugated anti-human CD4 (BD), peridinin chlorophyll protein-conjugated anti-human CD8 (BD), allophycocyanin (APC)-Cy7-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ monoclonal antibodies (BioLegend). In the flow cytometric analysis, PBMCs were gated in forward scatter-side scatter dot plots, and B-LCLs were excluded in this step. Specific T-cell frequencies were calculated by subtracting nonspecific IFN-γ T-cell frequencies (less than 100 per million PBMCs) from those after peptide-specific stimulation. Specific T-cell frequencies lower than 100 per million PBMCs were considered negative.

Sequencing analysis of plasma viral genomes. Viral RNAs were extracted using the high pure viral RNA kit (Roche Diagnostics, Tokyo, Japan) from macaque plasma obtained around 1 year after challenge. Fragments of cDNAs encoding SIVmac239 Gag, Vif, and Nef were amplified by nested RT-PCR (25 cycles at the first RT-PCR using the PrimeScript one-step RT-PCR kit, version 2 [TaKaRa] and 30 cycles at the second PCR using KOD-Plus, version 2 [Toyobo]) from plasma RNAs and subjected to direct sequencing by using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan) as described before (39). Predominant nonsynonymous mutations were determined.

Statistical analysis. Statistical analysis was performed with Prism software version 4.03, with significance levels set at a P value of <0.050 (GraphPad Software, Inc.). Antigen-specific $CD8^+$ T-cell frequencies were compared by the nonparametric Mann-Whitney U test. Correlation was analyzed by the Pearson test.

RESULTS

Plasma viral loads after SIVmac239 challenge. We used a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-010-Ie* (E). In our previous study (39), unvaccinated E^+ macaques consistently showed persistent viremia after SIVmac239 challenge. $CD4^+$ T-cell percentage in PBMCs declined to less than 20% in a year. In the present study, we compared viral loads in vaccinated animals with those in these unvaccinated animals.

The first vaccine group of five E^+ macaques received a DNA prime and an SeV-Gag boost vaccination, followed by an SIVmac239 challenge. Two of these Gag-vaccinated animals failed to control viral replication, but the remaining three showed SIV control (Fig. 1). In the latter controllers, plasma viremia became undetectable in a few months. Macaques R01-008 and R08-006 rapidly controlled SIV replication and maintained high CD4 levels (Fig. 1).

The second group of six E^+ macaques received a DNA prime and an SeV-Vif/Nef boost vaccination, followed by an SIVmac239 challenge. The vaccine protocol first delivered Vif-expressing DNA, with the second vaccination consisting of Vif-expressing and Nef-expressing DNAs, and the third with Vif-expressing and Nef-expressing SeVs (SeV-Vif and SeV-Nef) with intervals of 3 weeks. After SIV challenge, three of these Vif/Nef-vaccinated an-

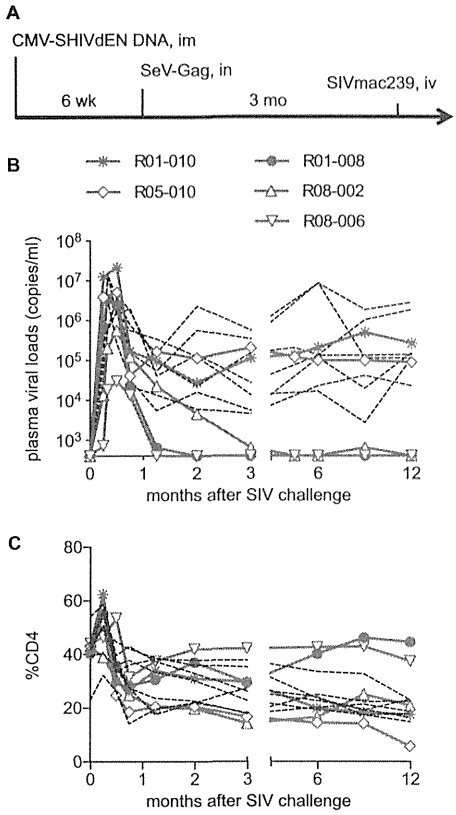

FIG 1 Viral loads and percentages of CD4 in Gag-vaccinated animals after SIVmac239 challenge. (A) Protocol of Gag vaccination and SIVmac239 challenge. (B) Plasma viral loads (SIV *gag* RNA copies/ml plasma) determined as described previously (5). The lower limit of detection is approximately 4×10^2 copies/ml. (C) Percentages of $CD4^+$ T cells in PBMCs. In panels B and C, data on unvaccinated animals ($n = 7$) are shown by dotted lines for comparison. Data on six unvaccinated (39) and two Gag-vaccinated (R01-010 and R01-008) (42) animals used in our previous studies are included.

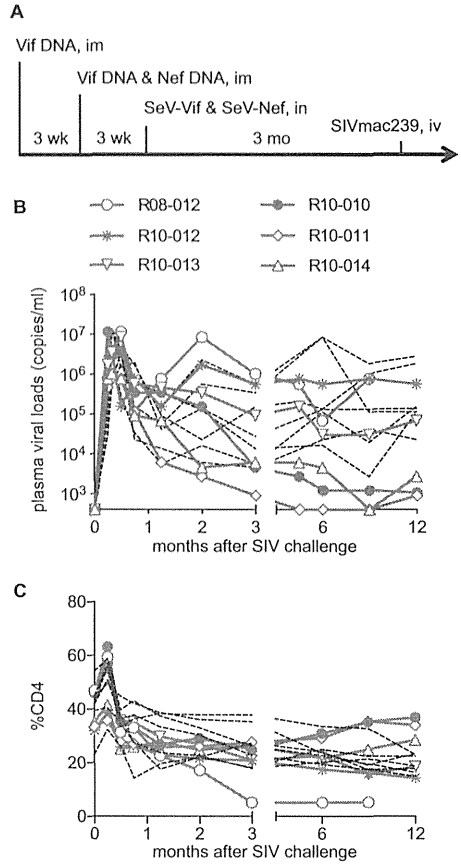

FIG 2 Viral loads and percentages of CD4 in Vif/Nef-vaccinated animals after SIVmac239 challenge. (A) Protocol of Vif/Nef vaccination and SIVmac239 challenge; (B) plasma viral loads; (C) percentages of $CD4^+$ T cells in PBMCs. In panels B and C, data on unvaccinated animals are shown by dotted lines for comparison.

imals failed to control viral replication and had high levels of set-point viral loads equivalent to those in unvaccinated macaques, but the remaining three showed SIV control with low levels of set-point viral loads (geometric mean of viral loads from 6 months to 1 year in each controller, $<2.0 \times 10^3$ copies/ml) and maintained higher CD4 levels (Fig. 2). Indeed, these six SIV controllers, consisting of three Gag-vaccinated and three Vif/Nef-vaccinated animals, showed significantly higher percentages of CD4 at 1 year than those in the remaining noncontrollers (see Fig. S1 in the supplemental material).

Gag-, Vif-, and Nef-specific $CD8^+$ T-cell responses in unvaccinated and vaccinated animals. We examined Gag-, Vif-, and Nef-specific $CD8^+$ T-cell responses in these animals. Unvaccinated macaques showed SIV-specific $CD8^+$ T-cell responses equivalent to those observed in Indian rhesus macaques (8) (Fig. 3). All of these E^+ unvaccinated macaques elicited immunodominant Nef-specific $CD8^+$ T-cell responses, consistent with our previous study analyzing other E^+ macaques (50). Gag-specific and Vif-specific $CD8^+$ T-cell responses were detected but were not immunodominant in these animals.

In contrast, all Gag-vaccinated E^+ macaques showed Gag-specific $CD8^+$ T-cell responses after the SeV-Gag boost and in the early phase after SIV challenge (Fig. 3). In these animals, Nef-specific $CD8^+$ T-cell responses mostly became immunodominant in the later phase. Importantly, all three animals that controlled SIV replication showed efficient Gag-specific $CD8^+$ T-cell responses in the acute phase postchallenge, suggesting a significant contribution of these Gag-specific $CD8^+$ T-cell responses to SIV control.

In the second group of Vif/Nef-vaccinated E^+ animals, analysis of Gag-specific, Vif-specific, and Nef-specific $CD8^+$ T-cell responses showed different patterns of responses between SIV controllers and noncontrollers (Fig. 3). In the acute phase after SIV challenge, the noncontrollers (R08-012, R10-012, and R10-013) elicited immunodominant Nef-specific $CD8^+$ T-cell responses, whereas the controllers (R10-010, R10-011, and R10-014) showed immunodominant Vif-specific $CD8^+$ T-cell responses. This suggests that the Vif-specific $CD8^+$ T-cell responses contributed to primary SIV control. In the chronic phase, Nef-specific $CD8^+$ T-cell responses were immunodominant except for one noncontroller, R10-012.

Thus, among 18 E^+ animals, consisting of seven unvaccinated, five Gag-vaccinated, and six Vif/Nef-vaccinated animals, three Gag-vaccinated and three Vif/Nef-vaccinated animals controlled SIV replication. Comparison between these six SIV controllers and the remaining 12 noncontrollers showed no significant difference in the sum of Gag-, Vif-, and Nef-specific $CD8^+$ T-cell fre-

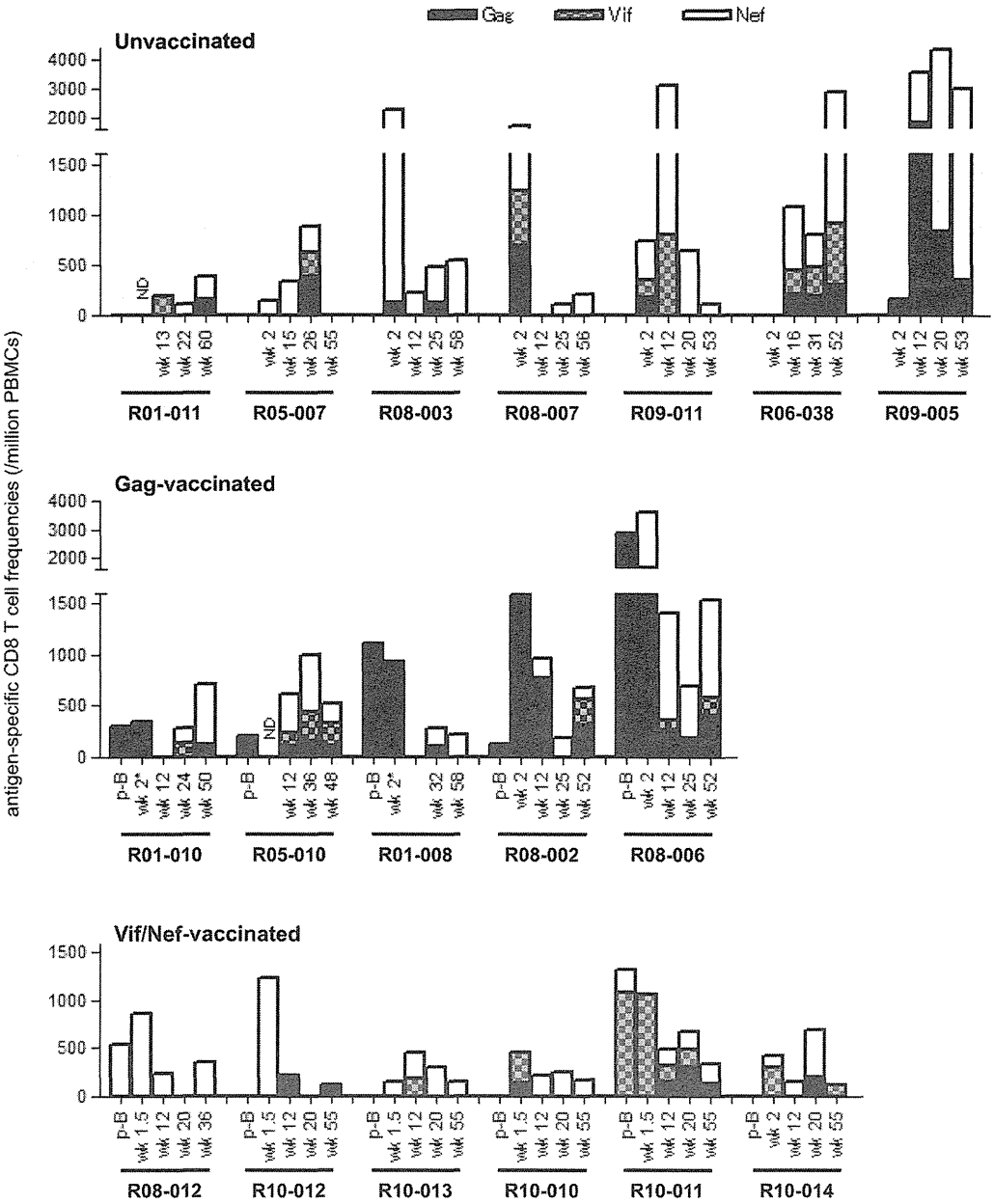

FIG 3 SIV Gag/Vif/Nef-specific $CD8^+$ T-cell responses in macaques. We examined $CD8^+$ T-cell responses specific for Gag, Vif, and Nef 1 week after SeV-Gag boost (p-B) and approximately 2 weeks, 3 months, 6 months, and 1 year after SIV challenge in unvaccinated (top), Gag-vaccinated (middle), and Vif/Nef-vaccinated (bottom) animals. We examined only Gag-specific $CD8^+$ T-cell responses but not Vif- or Nef-specific ones at week 2 in macaques R01-010 and R01-008 (indicated by asterisks). ND, not determined.

quencies in the acute phase (data not shown). The sum of Gag- and Vif-specific $CD8^+$ T-cell frequencies in the acute phase, however, was significantly higher in the controllers than in the noncontrollers ($P = 0.0031$ by Mann-Whitney U test) (Fig. 4A). Indeed, the sum of Gag- and Vif-specific $CD8^+$ T-cell frequencies in the acute phase was inversely correlated with postpeak plasma viral loads ($P = 0.0268$, $R = -0.5205$ with viral loads at 3 months [data not shown]; $P = 0.0017$, $R = -0.6849$ with viral loads at 1 year [Fig. 4B] by Pearson test). When we focused on seven unvaccinated and five Gag-vaccinated animals, three Gag-vaccinated controllers showed significantly higher Gag-specific $CD8^+$ T-cell frequencies in the acute phase than the remaining nine noncontrollers ($P = 0.0045$ by Mann-Whitney U test) (Fig. 4C). Also, in the analysis of seven unvaccinated and six Vif/Nef-vaccinated animals, Vif-specific $CD8^+$ T-cell frequencies in the acute phase were significantly higher in three Vif/Nef-vaccinated controllers than in the remaining 10 noncontrollers ($P = 0.0140$ by Mann-Whitney U test) (Fig. 4D). These results suggest that efficient Gag- or Vif-specific $CD8^+$ T-cell responses in the acute phase can result in SIV control.

Viral *gag*, *vif*, and *nef* mutations in vaccinated animals. We then tried to define the $CD8^+$ T-cell responses that might be contributing to the vaccine-based SIV control, although we were not able to map all of the $CD8^+$ T-cell epitopes because of sample

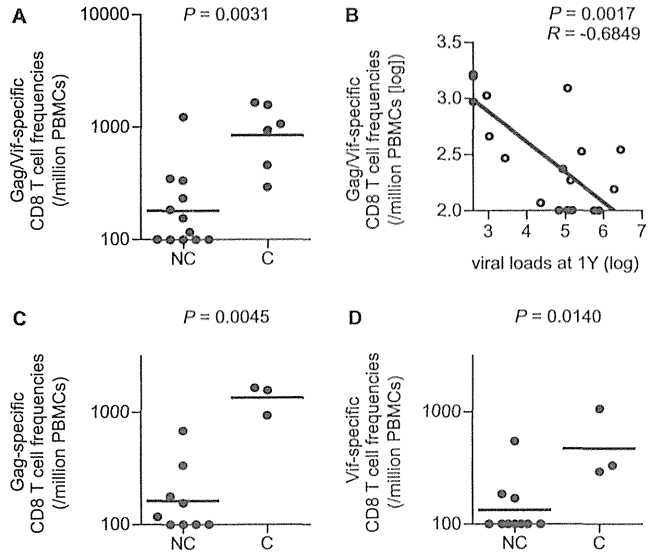

FIG 4 Comparison of Gag/Vif-specific $CD8^+$ T-cell frequencies in the acute phase between SIV controllers (C) and noncontrollers (NC). Data on Gag- and Vif-specific $CD8^+$ T-cell frequencies around week 2 postchallenge, which are shown in Fig. 3, were used. In macaques R01-011 and R05-010, samples at week 2 were unavailable, and data at week 12 were used. (A) Comparison of the sum of Gag- and Vif-specific $CD8^+$ T-cell frequencies (Gag/Vif-specific $CD8^+$ T-cell frequencies) between the controllers (three Gag-vaccinated and three Vif/Nef-vaccinated animals) and the noncontrollers in seven unvaccinated, five Gag-vaccinated, and six Vif/Nef-vaccinated animals ($n = 18$). The controllers showed significantly higher frequencies than the noncontrollers ($P = 0.0031$ by Mann-Whitney U test). (B) Correlation analysis of Gag/Vif-specific $CD8^+$ T-cell frequencies in the acute phase with plasma viral loads at 1 year. The frequencies were inversely correlated with the viral loads ($P = 0.0017$, $R = -0.6849$ by Pearson test). (C) Comparison of Gag-specific $CD8^+$ T-cell frequencies in seven unvaccinated and five Gag-vaccinated animals ($n = 12$). The three Gag-vaccinated controllers showed significantly higher frequencies than the noncontrollers ($P = 0.0045$ by Mann-Whitney U test). (D) Comparison of Vif-specific $CD8^+$ T-cell frequencies in seven unvaccinated and six Vif/Nef-vaccinated animals ($n = 13$). The three Vif/Nef-vaccinated controllers showed significantly higher frequencies than the noncontrollers ($P = 0.0140$ by Mann-Whitney U test).

limitation. Among three Gag-vaccinated controllers, R01-008, R08-002, and R08-006, our previous study found $Gag_{367-381}$-specific $CD8^+$ T-cell responses at week 5 in macaque R01-008 (5). This animal showed rapid selection of a mutation leading to an isoleucine (I)-to-threonine (T) change at the 377th aa (I377T) in SIV Gag, which results in escape from $Gag_{367-381}$-specific $CD8^+$ T-cell recognition. This suggests that these $Gag_{367-381}$-specific $CD8^+$ T-cell responses may have played an important role in SIV control. Analysis in the present study found $Gag_{385-400}$-specific $CD8^+$ T-cell responses in the acute phase with rapid selection of a mutation leading to an I-to-T change at the 391st aa (I391T) in Gag in macaque R08-006 (Fig. 5A). We confirmed that this I391T substitution results in escape from $Gag_{385-400}$-specific $CD8^+$ T-cell recognition (data not shown), suggesting a contribution of these $Gag_{385-400}$-specific $CD8^+$ T-cell responses to the control of SIV. Macaque R08-002 mounted $Gag_{273-292}$-specific $CD8^+$ T-cell responses but showed no *gag* mutation in the early phase. None of the noncontrollers selected *gag* mutations at week 5 or 6.

Among three Vif/Nef-vaccinated controllers, R10-010, R10-011, and R10-014 (Fig. 5B), macaque R10-010 mounted Vif_{65-76}-specific $CD8^+$ T-cell responses in the acute phase that resulted in the rapid selection of a mutation leading to a histidine (H)-to-tyrosine (Y) change at the 66th aa (H66Y) in Vif. Macaque R10-011 mounted $Vif_{113-132}$-specific and $Vif_{134-148}$-specific $CD8^+$ T-cell responses in the acute phase with rapid selection of a mutation leading to a Y-to-cysteine (C) change at the 143rd aa (Y143C) in Vif. We confirmed that this Y143C substitution results in escape from $Vif_{134-148}$-specific $CD8^+$ T-cell recognition (data not shown). None of the noncontrollers selected *vif* mutations at week 5 or 6. These suggest that Vif_{65-76}-specific and $Vif_{134-148}$-specific $CD8^+$ T-cell responses contributed to SIV control in macaques R10-010 and R10-011, respectively. Macaque R10-014 mounted $Vif_{113-132}$-specific $CD8^+$ T-cell responses but showed no *vif* mutation in the early phase.

In E^+ macaques, $CD8^+$ T-cell responses specific for Nef_{38-66} and $Nef_{101-138}$ regions were frequently observed (see Fig. S2 in the supplemental material). In all three Gag-vaccinated controllers, we confirmed both Nef_{38-66}-specific and $Nef_{101-138}$-specific $CD8^+$ T-cell responses in the chronic phase, although we did not have available samples for analysis of these responses in the acute phase. In five Vif/Nef-vaccinated animals, we confirmed Nef_{38-66}-specific $CD8^+$ T-cell responses in the acute phase, followed by $Nef_{101-138}$-specific $CD8^+$ T-cell induction. Nef_{38-66}-specific $CD8^+$ T-cell responses became undetectable at week 12 in all the three noncontrollers but were maintained at detectable levels in controllers R10-010 and R10-011.

Further mapping defined the Nef_{45-53} $CD8^+$ T-cell epitope. Mutations in the Nef_{45-53}-coding region were selected after 1 year in five of seven unvaccinated E^+ animals. Rapid selection of mutations at this Nef_{45-53}-coding region in a month after SIV challenge was observed in both Gag-vaccinated noncontrollers and all three Vif/Nef-vaccinated noncontrollers (Fig. 5C). In contrast, out of six Gag-vaccinated or Vif/Nef-vaccinated controllers, only one animal (R10-010) rapidly selected a mutation in this region. We confirmed that the leucine (L)-to-proline (P) substitution at the 53rd aa (L53P) in Nef results in escape from Nef_{45-53}-specific $CD8^+$ T-cell recognition (data not shown). Thus, Nef_{45-53}-specific $CD8^+$ T-cell responses may have exerted strong suppressive pressure on SIV replication in the acute phase in Gag-vaccinated or Vif/Nef-vaccinated noncontrollers.

DISCUSSION

In this study, we examined efficacy of prophylactic DNA-prime/SeV-boost vaccines against SIVmac239 challenge in a group of Burmese rhesus macaques sharing the MHC-I haplotype E. Our previous study indicated that unvaccinated E^+ animals show typical courses of SIV infection and AIDS progression (39). However, three of five Gag-vaccinated and three of six Vif/Nef-vaccinated E^+ animals controlled SIV replication, indicating a possibility of virus control by prophylactic vaccination.

Unvaccinated E^+ animals showed high-frequency Nef-specific $CD8^+$ T-cell responses, particularly specific for the Nef_{38-66} and $Nef_{101-138}$ regions, after SIVmac239 challenge. The Nef_{45-53} region is a candidate for a $CD8^+$ T-cell target associated with MHC-I haplotype E, and the NefL53P mutation resulting in escape from Nef_{45-53}-specific $CD8^+$ T-cell recognition was often selected in E^+ animals. These results imply suppressive pressure on SIV replication by Nef-specific $CD8^+$ T-cell responses in macaques sharing this MHC-I haplotype.

Gag-vaccinated animals elicited detectable Gag-specific $CD8^+$ T-cell responses after SeV-Gag boost. All three Gag-vaccinated

A

Gag-vaccinated controllers	Gag CD8 T cell targets	gag mutations at wk 5
R08-002	Gag273-292	none
R08-006	Gag385-400	I391T

B

Vif/Nef-vaccinated controllers	Vif CD8 T cell targets	vif mutations at wk 6
R10-010	Vif65-76	H66Y
R10-011	Vif113-132 & 134-148	Y143C
R10-014	Vif113-132	none

C

		Nef45-53 GLDKGLSSL			Nef45-53 GLDKGLSSL
Unvaccinated					
R01-011	1 mo	--------F	R09-011	1 mo	---------
	6 mo	-S-------		6 mo	--------P
	1 yr	----C----		1 yr	--G-----P
R05-007	1 mo	---------	R06-038	1 mo	---------
	6 mo	---------		6 mo	---------
	1 yr	---------		1 yr	---------
R08-003	1 mo	---------	R09-005	1 mo	---------
	6 mo	--G------		6 mo	--G------
	1 yr	--------R		1 yr	--G-----H
R08-007	1 mo	---------			
	6 mo	-S-------			
	1 yr	-S-------			
Gag-vaccinated non-controllers			controllers		
R01-010	1 mo	--------P	R01-008	1 mo	---------
	6 mo	--G-----P	R08-002	1 mo	---------
	1 yr	--G------		6 mo	E--------
R05-010	1 mo	E--------		1 yr	E--------
	6 mo	E-G------	R08-006	1 mo	---------
	1 yr	--G------		6 mo	---------
				1 yr	---------
Vif/Nef-vaccinated non-controllers			controllers		
R08-012	1 mo	--------P	R10-010	1 mo	A--------
	6 mo	--------P		6 mo	E-------P
R10-012	1 mo	E----S---		1 yr	E--------
	6 mo	----D---P	R10-011	1 mo	---------
	1 yr	-----L--P		6 mo	---------
R10-013	1 mo	--------R		1 yr	---------
	6 mo	--------R	R10-014	1 mo	---------
	1 yr	--G------		6 mo	---------
				1 yr	---------

FIG 5 Predominant nonsynonymous mutations in CD8$^+$ T-cell target-coding regions. (A) Gag target regions for CD8$^+$ T-cell responses in the acute phase in Gag-vaccinated controllers. Macaque R01-008 induced $Gag_{367-381}$-specific CD8$^+$ T-cell responses and selected I377T mutation in 5 weeks as described before (5). (B) Vif target regions for CD8$^+$ T-cell responses in the acute phase in Vif/Nef-vaccinated controllers. (C) Nonsynonymous mutations in Nef_{45-53} CD8$^+$ T-cell epitope-coding regions of viral cDNAs at 1 month (1 mo), 6 months (6 mo), and 1 year (1 yr). Amino acid substitutions are shown.

controllers showed efficient Gag-specific CD8$^+$ T-cell responses in the acute phase after SIV challenge. In particular, macaques R01-008 and R08-006 showed rapid SIV control without detectable plasma viremia after week 5. $Gag_{367-381}$-specific CD8$^+$ T-cell responses with rapid selection of a $Gag_{367-381}$-specific CD8$^+$ T-cell escape mutation, I377T, were observed in R01-008, whereas $Gag_{385-400}$-specific responses were associated with an escape mutation, I391T, in R08-006. Our results suggest that the prophylactic Gag vaccination results in the efficient induction of these Gag-specific CD8$^+$ T-cell responses in the acute phase, which then played an important role in the control of primary SIV replication. The MHC-I haplotypes other than E (see Table S1 in the supplemental material) may be associated with these effective Gag epitope-specific CD8$^+$ T-cell responses. Nef-specific CD8$^+$ T-cell responses became predominant after 3 or 6 months.

Vif/Nef-vaccinated animals induced Vif- or Nef-specific CD8$^+$ T-cell responses in the acute phase after SIVmac239 challenge. Before challenge, detectable Vif-specific CD8$^+$ T-cell responses were elicited after SeV-Vif/Nef boost only in macaque R10-011. It should be noted, however, that all three Vif/Nef-vaccinated controllers showed high-frequency Vif-specific CD8$^+$ T-cell responses in the acute phase, while the three noncontrollers exhibited Nef-specific CD8$^+$ T-cell responses. In particular, our results implicate Vif_{65-76}-specific and $Vif_{134-148}$-specific CD8$^+$ T-cell responses in the control of primary viral replication in macaques R10-010 and R10-011, respectively. These CD8$^+$ T-cell responses may be associated with the second MHC-I haplotypes (see Table S1 in the supplemental material). Even Vif/Nef-vaccinated controllers inducing Vif-specific CD8$^+$ T-cell responses in the acute phase showed predominant Nef-specific CD8$^+$ T-cell responses in the chronic phase.

Vif/Nef-vaccinated noncontrollers showed no Vif-specific CD8$^+$ T-cell responses but mounted Nef-specific CD8$^+$ T-cell responses in the acute phase. All three noncontrollers rapidly se-

lected *nef* mutations in the Nef_{45-53}-coding regions, and Nef_{45-53}-specific $CD8^+$ T-cell responses were undetectable after 3 months postchallenge. Interestingly, both Gag-vaccinated noncontrollers also showed rapid selection of *nef* mutations in the Nef_{45-53}-coding regions. We speculate that, in these Gag-vaccinated or Vif/Nef-vaccinated noncontrollers, dominant Nef_{45-53}-specific $CD8^+$ T-cell responses may have exerted strong suppressive pressure on primary SIV replication without the help of other vaccine antigen-specific, effective $CD8^+$ T-cell responses, leading to failure in virus control with rapid selection of escape mutations. Unvaccinated macaque R08-007 elicited Gag- and Vif-specific as well as Nef-specific $CD8^+$ T-cell responses in the acute phase but failed to control SIV replication. The high magnitude of responses may reflect the highest peak viral loads (1.4×10^7 copies/ml) at day 10 in this animal among the unvaccinated. These naive-derived Gag- and Vif-specific $CD8^+$ T-cell responses may have been less functional and insufficient for SIV control. In contrast, in vaccinated controllers, prophylactic vaccination resulted in effective Gag- or Vif-specific $CD8^+$ T-cell responses postexposure, leading to primary SIV control, followed by Nef-specific $CD8^+$ T-cell responses possibly contributing to maintenance of virus control. Induction of $CD8^+$ T-cell responses specific for dominant Nef epitopes by prophylactic vaccination may not be good for SIV control in E^+ animals. Several studies have indicated contribution of subdominant $CD8^+$ T-cell responses to HIV or SIV suppression (51–53). Thus, induction of $CD8^+$ T-cell responses specific for subdominant but not dominant epitopes by prophylactic vaccination may be a promising AIDS vaccine strategy resulting in effective, broader $CD8^+$ T-cell responses postexposure.

In summary, this study demonstrates SIV control by prophylactic vaccination in hosts possessing MHC-I alleles associated with dominant non-Gag antigen-specific $CD8^+$ T-cell responses. Our results suggest that prophylactic vaccination resulting in effective subdominant Gag/Vif epitope-specific $CD8^+$ T-cell responses in the acute phase postexposure can lead to primary HIV control. This may imply a rationale of altering the hierarchy of postexposure $CD8^+$ T-cell immunodominance toward HIV control.

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