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<tr>
<td>Citation</td>
<td>PLoS One, 7(12): e51633</td>
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<tr>
<td>Issue Date</td>
<td>2012-12-07</td>
</tr>
<tr>
<td>Doc URL</td>
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Elicitation of Both Anti HIV-1 Env Humoral and Cellular Immunities by Replicating Vaccinia Prime Sendai Virus Boost Regimen and Boosting by CD40Lm

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Abstract

For protection from HIV-1 infection, a vaccine should elicit both humoral and cell-mediated immune responses. A novel vaccine regimen and adjuvant that induce high levels of HIV-1 Env-specific T cell and antibody (Ab) responses was developed in this study. The prime-boost regimen that used combinations of replication-competent vaccinia LC16m8Δ (m8Δ) and Sendai virus (SeV) vectors expressing HIV-1 Env efficiently produced both Env-specific CD8⁺ T cells and anti-Env antibodies, including neutralizing antibodies (nAbs). These results sharply contrast with vaccine regimens that prime with an Env expressing plasmid and boost with the m8Δ or SeV vector that mainly elicited cellular immunities. Moreover, co-priming with combinations of m8Δ expressing Env or a membrane-bound human CD40 ligand mutant (CD40Lm) enhanced Env-specific CD8⁺ T cell production, but not anti-Env antibody production. In contrast, priming with an m8Δ that coexpresses CD40Δm and Env elicited more anti-Env Abs with higher avidity, but did not promote T cell responses. These results suggest that the m8Δ prime/SeV boost regimen in conjunction with CD40Lm expression could be used as an immunization platform for driving both potent cellular and humoral immunities against pathogens such as HIV-1.

Introduction

An effective HIV vaccine should elicit both antibodies [1] and cell-mediated immune responses in order to control HIV infection. Since the majority of clinical isolates of human immunodeficiency virus type 1 (HIV-1) are highly resistant to neutralizing antibodies and antigenically variable, major efforts have been aimed at eliciting cellular immunity against less variable antigens. Typical prime/boost strategies using DNA and replication-defective viral vectors have been extensively examined. These regimens efficiently elicit cellular responses including cytotoxic T cells (CTL), but are less effective at eliciting humoral responses. For example, adenovirus and vaccinia virus-based vectors expressing Gag, Nef, and other components of HIV-1 have been shown, in nonhuman primates [2–5] and in human trials [6,7], to elicit considerable multifunctional T cell responses and control early viral replication to some extent. These preparations, however, did not induce a sufficient level of immunity to protect vaccinees from HIV/simian immunodeficiency virus (SIV) infection in the absence of neutralizing antibodies [8]. Therefore, more potent immunogens and better vaccination regimens are required.

The RV144 trial that included priming with a recombinant canarypox vector, ALVAC-HIV vCP1521, followed by booster with the HIV-1 envelope gp120 protein, AIDSVAX gp120 clades B and E, plus an alum adjuvant showed a modest level of efficacy in reducing HIV-1 infection rates in Thailand [9]. Extended analysis of this HIV vaccine trial showed that it is the vaccine trial to succeed in eliciting IgG antibodies to the V1V2 region of Env, and the presence of these antibodies were inversely correlated to the rate of infection [10], suggesting an importance to elicit anti HIV-1 specific antibodies. Accordingly, both antibodies and cell-mediated immune responses should be considered for the vaccine development in order to control HIV infection.

Replication-competent vaccinia virus (VV) that has been proven to be safe in human vaccination against smallpox may be a good vehicle candidate. Among several vaccinia strains, LC16m8 has an extremely low neurovirulence profile, comparable to the replication incompetent vaccinia viruses MVA and NYVAC, and is safe in immune compromised animals [11–13]. LC16m8 is able to induce immunity at levels similar to the original Lister (LO) strain and the US licensed vaccine dryvax strain [11–13], and no serious adverse effects were detected in the administration of LC16m8 to...
100,000 infants and 3,000 adults [14]. However, LC16m8 is genetically unstable and can spontaneously generate more virulent revertants. To improve the safety of LC16m8, we identified the B5R gene responsible for the reversions and constructed the genetically stable LC16m8Δ (m8Δ), which is essentially the same as LC16m8 in antigenicity, safe in mice and rabbits, and much more immunogenic than the MVA strain [15]. Thus, m8Δ may be a better vehicle for vaccines. Indeed, immunization in a prime-boost strategy using DNA and m8Δ expressing SIV Gag elicited 7–30 fold more IFN-γ producing T cells in mice than were produced using the non-replicating vaccinia DIs strain [15].

The Sendai virus (SeV) is a non-segmented negative-strand RNA virus belonging to the paramyxovirus family and is considered nonpathogenic in humans [16–19]. A SeV vector expressing the SIV gag gene elicits SIV-specific CTL very efficiently and controlled SIV replication in a subset of immunized macaques [20,21]. Thus, the SeV vector may be another candidate for a better immunogen.

In addition to adopting better vaccination vehicles, combining these with an immune stimulating factor could produce a better efficacy. The CD40 ligand (CD40L, CD154), which belongs to the tumor necrosis factor (TNF) family, is a 39 kDa type II membrane glycoprotein that is predominantly expressed on activated CD4+ T cells [22]. CD40, the TNF receptor superfamily member that is the CD40L receptor, is expressed on all antigen-presenting cells (APCs), including macrophages, dendritic cells (DCs) and B lymphocytes [23]. Interactions between these receptors and ligand play a central role in adaptive immune responses including maturation of DCs and class switching of immunoglobulin genes [24]. Coexpression of CD40L with immunogens has the potential to enhance both humoral and cellular immune responses in various regimens [25–29]. However, one concern is that high levels of CD40L, mainly resulting from cleavage to produce a soluble form, may have deleterious side effects and could lead to systemic inflammatory responses and cardiovascular disease. A non-cleavable CD40L, CD40Lm, which was constructed with point mutations in the membrane proximal region, was reported to be less toxic in vivo [30]. Therefore, coexpression of CD40Lm may further enhance the induction of immune responses to HIV-1 without adverse effect.

To identify an improved vaccination regimen that elicits higher levels of anti-HIV-1 humoral and cellular responses, various combinations of vaccine preparations were tested in this study using the vaccinia virus m8Δ and SeV vectors expressing HIV-1 Env in conjunction with the coexpression of human CD40Lm (hCD40Lm).

Materials and Methods

Ethics Statement

All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals, Institute for Genetic Medicine, Hokkaido University. Study approval was issued by the Animal Care Committees of Hokkaido University.

Construction of HIV-1 envelope expression plasmids

The region encoding the Rev and Env genes of the HIV-1 JR- CSF genome (5981–8782 nt) was inserted into the EcoR1 restriction site of the mammalian expression vector pCAGGS [31,32] to generate pCAGGS-JRCSFrev/env. To confirm the expression of gp160, a sequence-verified pCAGGS-JRCSFrev/env was transfected into 293T cells using polyethylenimine (Polysciences, Warrington, US) [33]. Forty-eight hours after transfection, 293T cell lysates were collected and proteins were fractioned on 10% SDS polyacrylamide gels and transferred to a nitrocellulose filter (Schleicher & Schuell). Immunoblot analysis was performed with HIV-1-infected human antiserum and alkaline phosphatase-conjugated anti-human IgG (Promega, Sunnyvale, US), and then visualized using NBT/BCIP.
Construction of recombinant LC16m8Δ

The four strains of recombinant m8Δ used in this study are constructed as follows. To construct a m8Δ that expresses the full HIV-1 JR-CSF Env gene under the control of the vaccinia virus promoter in pSFJ1-10 [21], the AvrII-XhoI fragment of the JR-CSF genome was subcloned into the pJW322 plasmid [34] that had been digested with AvrII and SalI, and the vaccinia virus transcription termination signals (TTTTTNT) in the envelope gene were synonymously mutated using an in vitro mutagenesis kit (Stratagene). The coding region for gp160 was then amplified by PCR using the forward primer TTT
CGGACCGCCACCATGAGTGAAGGGGATCAGG (underline shows the RsrII site) and the reverse primer ATA
GGCCGGCCTTATAGCAAAAGCCCTTTCCAAGC (underline shows the FseI site). The PCR product was ligated into the LC16m8Δvnc110 [15] genome that had been digested with FseI and RsrII. The ligated DNA was transfected into BHK cells that were infected with

Figure 2. Cellular immunity elicited by a regimen consisting of DNA priming followed by vaccinia m8Δ boosts. (A) Schematic schedules of DNA prime/m8Δ-Env boost vaccination protocol. Mice were immunized twice with pCAGGS-JRCSFrev/env and boosted with various vaccinia viruses as follows. Group A: m8Δ; group B: m8Δ-Env; group C: m8Δ-Env/hCD40Lm; group D: m8Δ-Env plus m8Δ; group E: m8Δ-Env plus m8Δ-p7.5hCD40Lm; and group F: m8Δ-Env plus m8Δ-pSFJ1-10hCD40Lm. (B) Comparison of Env peptide-specific CD8+ T cell responses. The frequencies of IFN-γ+ CD8+ T cells in gated CD8+ T cell compartment was determined by intracellular cytokine staining (ICS) and FACScalibur/FACScanto analysis.
doi:10.1371/journal.pone.0051633.g002

Table 1. Mice immunization protocol used in this study.

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</tr>
<tr>
<td>G</td>
<td>m8Δ-Env + m8Δ-p7.5hCD40Lm</td>
<td>SeV-JRCSF env</td>
</tr>
<tr>
<td>H</td>
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</tr>
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Figure 2. Cellular immunity elicited by a regimen consisting of DNA priming followed by vaccinia m8Δ boosts. (A) Schematic schedules of DNA prime/m8Δ-Env boost vaccination protocol. Mice were immunized twice with pCAGGS-JRCSFrev/env and boosted with various vaccinia viruses as follows. Group A: m8Δ; group B: m8Δ-Env; group C: m8Δ-Env/hCD40Lm; group D: m8Δ-Env plus m8Δ; group E: m8Δ-Env plus m8Δ-p7.5hCD40Lm; and group F: m8Δ-Env plus m8Δ-pSFJ1-10hCD40Lm. (B) Comparison of Env peptide-specific CD8+ T cell responses. The frequencies of IFN-γ+ CD8+ T cells in gated CD8+ T cell compartment was determined by intracellular cytokine staining (ICS) and FACScalibur/FACScanto analysis.
doi:10.1371/journal.pone.0051633.g002
canarypox virus, as described previously [14]. The vaccinia virus constructed was designated LC16m8. For the m8D-p7.5hCD40Lm construct, the human CD40Lm gene [35] was inserted into pVR1 containing the p7.5 vaccinia virus promoter [36] at the BamHI and AvaI sites, which interrupts the HA gene sequence [37,38]. The construct was transfected into m8D-infected BHK cells, followed by selection based on an HA2 phenotype, as described previously [38,39]. For the m8D-pSFJ1-10hCD40Lm construct, the hCD40Lm gene was inserted into the XmaI and Not I sites of pBHAR that contains the pSFJ1-10 sequence inserted within the vaccinia virus HA gene [40]. For the m8D-Env/hCD40Lm construct that coexpresses pSFJ1-10-driven Env and p7.5-driven hCD40Lm, the Env gene fragment with EcoRI and SacI sites, and the p7.5 promoter-hCD40Lm fragment with SacI and XmaI sites were generated by PCR and the construct was inserted into the EcoRI and XmaI sites of pBR322. Then, the entire env-p7.5 region was amplified by PCR and ligated into the m8Dvnc110 genome, as above. A schematic diagram of each m8D construct is shown in Fig. 1A. To verify protein expression, RK13 cells were infected with these recombinant m8D viruses at a moi of 5 and cultured for 24 h at 33°C. Cell lysates were prepared, and analyzed by immunoblot analysis using the HIV-1-infected human antiserum or mouse anti-hCD40Lm mAb as the primary antibodies.

Construction of SeV expressing the JR-CSF env gene

Potential EIS sequences that may affect transcription of SeV were identified in the JR-CSF Env gene, and nucleotide substitutions that did not alter the amino acid sequence were made using in vitro mutagenesis with the following primers: mutation 1 forward primer, CCATCGTCTTCAGTCTTCCAGGGAGTACGAAAATTG; mutation 1 reverse primer, GAATTAACATTCAAAAAGAGAGGAGGCCTGATCGGTCTCCGCATCGTGTTCTCTGTACTTTCTATAG. The mutated env fragment was subcloned into pSeV/D and replication incompetent SeVJRCSFenv recombinant virus was constructed as previously described [41].

Immunization of mice

Seven-week-old female C57BL/6J mice (CLEA Japan, Tokyo) were primed twice at 2 week intervals by intramuscular (i.m.) injection with 100 μl PBS containing 50 μg pCAGGS-JRCSFrev/env. Eight weeks later, animals were boosted with 1×107 PFU of the recombinant m8Δ by skin scarification (s.s.) [42]. Alternatively, mice were primed with 1×106 PFU of the recombinant m8Δ by s.s. and eight weeks later boosted with 4×107 CIU of recombinant SeV intranasally (i.n.). Two weeks after the last immunization, vaginal fluids were collected, and mice were then sacrificed to collect sera and spleens.

Intracellular cytokine staining (ICS)

Splenocytes were stimulated with 10 μg/ml HIV-1 consensus subtype B Env (15-mer) peptides (AIDS Research and Reference Reagent Program) in the presence of Alexa Fluor-488 labeled anti-mouse CD107a (2.5 μg/ml) and brefeldin A (BD Biosciences) for 6 h. The cells were then washed and stained with PE-labeled anti-CD8 (eBioscience, San Diego, US) and Pacific Blue-labeled anti-CD4 (eBioscience, San Diego, US) mAbs for 30 min at 4°C.
After washing, the cells were permeabilized with Cytofix/Cytoperm solution (BD Bioscience, Franklin lakes, US) and stained with APC-labeled anti-mouse IFN-γ (eBiosience, San Diego, US) mAb. Then, the samples were subjected to analysis using a FACScalibur or FACScantoII instrument (BD Bioscience, Franklin lakes, US). Data were analyzed with the FlowJo software (Tree Star). The frequencies of IFN-γ+ or IFN-γ, CD107a double positive T cells among CD4 or CD8 gated lymphocytes were determined.

Purification of IgG from mouse serum

Twenty micro liter of mice sera randomly selected from group G, I, J and control were mixed with 20 μl of protein A Sepharose 4 Fast Flow resins (GE healthcare life science, Tokyo, Japan) that was pre-washed with TBS buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl), and incubate at 4°C for 1 hr with rotation. The Protein A sepharose resins were then rinsed 3 times with TBS buffer. The antibodies were eluted with 50 μl of elution buffer (50 mM glycine-HCl, pH 2.5) at 4°C for 5 min followed by addition of 2.5 μl of 1 M Tris-HCl (pH 9.0) to adjust pH to 7. The isotype of the purified antibodies were analyzed with BD cytometric bead array system according to the manufacturer’s instruction.

Serum antibody measurements by enzyme-linked immunosorbent assay (ELISA)

To prepare ELISA plates coated with HIV-1JR-CSF gp160, 293T cells (2.5x10⁶ cells) that had been transfected with 10 μg of pCAGGS;JRCSFrev/env 2 days before were lysed in 1 ml of TMN buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM MgCl₂, 140 mM NaCl, 0.5% NP40 and 1X complete protease inhibitor cocktail (Roche Applied Science, Sandhofer Strasse, Germany)) and desalted by ultrafiltration using a Centricron YM-100 (Millipore). After dilution to a final concentration of 3.0 μg/ml protein with coating buffer (eBioscience, San Diego, US), the cell lysates were added to MaxiSorp 96 well ELISA plates (NUNC) at 100 μl aliquots/well and incubated overnight at 4°C. The wells were washed twice with PBS plus 0.05% Tween 20 (PBS-T) and blocked with PBS-T containing 5% skim milk for 1 h at room temperature. Subsequently, 100 μl samples of sera, serially diluted with the blocking solution, were added to the wells and incubated for 2 h at room temperature. Plates were washed five times with HCl, pH 2.5) at 4°C for 5 min followed by addition of 2.5 μl of 1 M Tris-HCl (pH 9.0) to adjust pH to 7. The isotype of the purified antibodies were analyzed with BD cytometric bead array system according to the manufacturer’s instruction.
PBS-T. Aliquots (100 μl) of horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Promega, Sunnyvale, US), diluted 1:2500 from the stock solution, were added and incubated for 1 h at room temperature. The plates were washed five times with PBS-T and TMB ELISA substrate solution (eBioscience, San Diego, US) was added. After a 15 min incubation at room temperature, the reaction was stopped by addition of 1 M H₃PO₄. Optical density at 450 nm (OD₄₅₀) was measured using a plate reader (PerkinElmer). To calculate the amounts of Env-specific antibodies, OD values were background subtracted using the OD₄₅₀ for wells coated with cell lysates prepared from pCAGGS-transfected 293T cells.

To assess the avidity of anti-Env antibodies, 300 fold-diluted sera were added to the wells of ELISA plates and incubated as above. The plates were then washed with 3–9 M urea and subjected to the above procedure for quantification of anti-Env antibodies. Relative avidity was estimated as the ratio of absorbance after and before the urea wash. The concentration of urea required to release 50% of the bound antibody (half-maximal effective dose (ED₅₀)) was calculated by linear regression analysis of plots of the probit values vs. urea concentration.

Neutralizing antibody measurements

The titer of neutralizing Abs (nAbs) was assessed based on the reduction of luciferase reporter gene expression in TZM-bl cells after a single round of Env-pseudotyped virus infection, as described previously [35,43]. HIV-1 Env-pseudotyped virus (1000 TCID₅₀/ml) was incubated with five-fold serially-diluted test sera in triplicate. One set of control wells received the cells plus
The amount of HIV-1 gp160-specific IgA or IgG was determined
Montgomery, US) according to the manufacturer's instructions.
a mouse IgA/IgG ELISA quantitation kit (Bethy Laboratories,
IgA or IgG concentration in the vaginal fluid was quantified using
mucosal washings were centrifuged at 1000 g for 10 min. The
conjugated anti-mouse IgA (Bethyl Laboratories; 1:10,000) or
of two-fold serially-diluted vaginal samples were used. HRP-
assay. The ELISA was performed as indicated in Fig. 4. The data shown
are representative results of three independent experiments.
doi:10.1371/journal.pone.0051633.g008

virus (virus control), another set of wells received cells only
(background control). To examine the non-specific effect of sera,
MuLV Env or VSV glycoprotein-coated pseudotype virus was
expressed in the recombinant Env expression plasmid pCAGGS-
expression of Env and hCD40Lm are expressed by the separate virus.
Although we tried to detect HIV-1JR-CSF Env-specific antibodies
in the antigen, and Env of Env/hCD40Lm carrying the envelope
CD40L delivered by m8Δ Env/hCD40Lm (group D), the m8Δ
expression of an optimal amount of hCD40Lm was important for
immunogenicity of m8Δ Env/hCD40Lm (Fig. 1B). These data are consistent with the
relative strong potency of the pSFJ1-10 promoter vs. the p7.5
promoter [40].

Effect of hCD40Lm in the Env expressing DNA prime/
LC16m8Δ boost regime
The effect of CD40Lm in improving the immunogenicity of the
vaccination regime was first examined using the most frequently
tested regime of priming with an Env-expressing plasmid followed
by boosting with Env-expressing vaccinia m8Δ (Table 1 group A-
F). Female C57BL/6j mice were primed with 50 µg of pCAGGS-
JRCSev/env followed by boosting with 1 x 10⁶ PFU of various
m8Δ recombinants: m8Δ (group A), m8Δ-Env (group B), m8Δ-
Env/hCD40Lm (group C), m8Δ-Env and m8Δ (group D), m8Δ-
Env and m8Δ-p7.5-hCD40Lm (group E), and m8Δ-Env and m8Δ-
pSFJ1-10-hCD40Lm [group F (Fig. 2A)]. Mice were sacrificed two
weeks after the final immunization, and splenocytes and sera were
collected for immunological assays. An ICS FACS analysis was
conducted to identify HIV-1 Env-specific IFN-γ secreting CD8+ T
cells after stimulation with a consensus subtype B Env (15-mer)
peptide pool. Initially, all overlapping Env peptides representing
entire JR-CSF Env were tested for their ability to induce most
effective response (frequency of IFN-γ secreting CD8+ T cells),
then the two most immunogenic peptides, 805–819 aa and 809–
823 aa, were selected and the mixture of these two peptides was
used thereafter. Co-immunization of m8Δ-Env with the lower
expression hCD40Lm vector, m8Δ-p7.5-hCD40Lm (group E),
significantly enhanced the number of IFN-γ secreting CD8+ T
cells by approximately 2–4 fold compared to the groups boosted
with m8Δ-JRCSFenv alone (group B) or m8Δ-JRCSFenv together
with empty m8Δ (group D). In contrast, the higher expression
hCD40Lm vector, m8Δ-pSFJ1-10-hCD40Lm (group F), decreased the
number of IFN-γ expressing CD8+ T cells. There was no
significant difference in the number of IFN-γ secreting CD8+ T
cells between mice boosted with the coexpression vector, m8Δ-
Env/hCD40Lm (group C), and mice boosted with m8Δ-Env
(group B) [Fig. 2B]. IFN-γ and CD107a double positive CD8+ T
cell fractions were also quantified to determine the number of
functional CD8+ T cells. As in the case of IFN-γ secreting CD8+ T
cells, co-immunization with m8Δ-p7.5-hCD40Lm enhanced the
immunogenicity of m8Δ-env (data not shown). Interestingly, the
expression of an optimal amount of hCD40Lm was important for
the enhancement since high levels of expression were not effective
(Compare group E and F in Fig. 2B). The m8Δ-pSFJ1-10-hCD40Lm vector (group F) was therefore omitted from
subsequent experiments. The data above suggest that uncleaved
CD40L delivered by m8Δ vector help the Env expression pox
vector to generate more effective T cell responses, but only when
CD40Lm are expressed by the separate virus.

Although we tried to detect HIV-1 JR-CSF Env-specific antibodies
by ELISA and TZM-bl cell-based assays, the DNA prime/m8Δ

Figure 8. Env-specific mucosal IgG elicited by the regimen of
Env expressing LC16m8Δ prime/SeV boost. Vaginal fluid pooled
from 5–6 immunized mice was analyzed by HIV-1JR-CSF gp160 ELISA
assay. The ELISA was performed as indicated in Fig. 4. The data shown
are representative results of three independent experiments.

Measurement of IgA and IgG in vaginal fluid
To collect vaginal samples, a total of 400 µl of PBS was flushed
into the vagina and collected into a microcentrifuge tube. The
mucosal washings were centrifuged at 1000 g for 10 min. The
supernatants were collected and stored at −20°C until assay. Total
IgA or IgG concentration in the vaginal fluid was quantified using
a mouse IgA/IgG ELISA quantitation kit (Bethyl Laboratories,
Montgomery, US) according to the manufacturer’s instructions.
The amount of HIV-1 gp160-specific IgA or IgG was determined
using ELISA plates prepared as described above. Aliquots (100 µl)
of two-fold serially-diluted vaginal samples were used. HRP-
conjugated anti-mouse IgA (Bethyl Laboratories; 1:10,000) or
HRP-conjugated anti-mouse IgG (Promega, Sunnyvale, US) was
used as the secondary antibody at a dilution of 1:2500.

Statistical analysis
Data were expressed as arithmetic mean ± standard deviation
(mean ± SD). Data analysis was conducted using Student’s t-test
(EXCEL version 11.5, Microsoft). A P-value of <0.05 was
considered significant.

Results
In vitro Expression of Env and hCD40Lm

We first confirmed the expression of the Env and hCD40Lm
genes by the vectors constructed in this study by immunoblot
assay. As shown in Fig. 1B, 293T cells transfected or infected with
the recombinant Env expression plasmid pCAGGS-JRCSFrev/
Env, or the Sendai virus (SeV) vector expressing HIV-1 JR-CSF
envelope gene, produced gp120 and gp160. Four recombinant
m8Δs were examined: m8Δ-Env carrying only the HIV-1 JR-CSF
envelope protein; m8Δ-Env/hCD40Lm carrying the envelope
protein and hCD40Lm; and m8Δ-pSFJ1-10-hCD40Lm and m8Δ-
p7.5-hCD40Lm, carrying hCD40Lm under different promoters,
Cellular immunity elicited in the Env expressing LC16mΔA prime/SeV boost regimen

To identify vaccination methods that may elicit both cellular and humoral immunity, a novel immunization regimen using SeV and m8A expressing HIV-1JR-CSF Env was tested. At first we performed a preliminary experiment to optimize the order of prime-boost regime, in which, an m8A-Env prime/SeV-env boost regime produced better responses than a SeV-env prime/m8A-Env boost regime (data not shown). The priming effects of various combinations of m8A recombinants such as m8A-Env plus m8A-p7.5hCD40Lm (group G), m8A-Env plus m8Δ (group H), m8A-Env/hCD40Lm (group I), m8Δ-Env (group J), and m8A-Env/hCD40Lm (group K) were examined; untreated mice were the control (group L).

The inclusion of hCD40Lm delivered by a separate vector enhances the anti-Env antibody production compared to priming with m8A-Env alone or m8A-Env plus m8Δ (group J and H). Comparison of the endpoint dilution titer of the antibodies, suggests that m8Δ-Env/hCD40Lm priming (group I) elicited approximately 5-6 times more anti-Env antibody than the other immunization groups (Fig. 4B).

Next, the avidity of the anti-Env antibodies was assessed based on the relative amount of antibody that remained on the ELISA plate after a urea wash (Fig. 5). The ED50 of the urea wash for the anti-Env antibodies prepared from mice primed with m8A-Env/hCD40Lm, m8AEnv + m8ΔhCD40Lm, m8ΔEnv and m8AEnv+m8Δ (group I, G, J and H) is 5.16 M, 4.22 M, 3.84 M and 2.57 M, respectively (Fig. 5A). The relative amount of residual anti-Env antibody from group I was significantly higher than any other group after washing with 7 M urea (Fig. 5B). These results indicate that priming with the coexpression vector m8A-Env/hCD40Lm markedly enhanced the avidity of the Env antibodies produced, compared to the Env expression vector alone; co-immunization with m8A-Env and m8A-p7.5hCD40Lm (group G) did not enhance avidity.

Finally, the titer of neutralizing antibodies was determined using pseudotyped viruses coated with gp160 of JR-CSF, tier 1 SF162, and several tier 2 viruses belonging to clade B and C using TZM-bl reporter cells [35,43]. All m8A prime/SeV boost regimens except the control groups K and L elicited neutralizing antibodies against SF162: the mean titers (ID50) of the sera prepared from mice primed with m8A-Env/hCD40Lm, m8A-Env+m8Δ-hCD40Lm, and m8A-Env (group I, G and J) were 954, 287, and 205, respectively. The m8A-Env/hCD40Lm-primed group I exhibited a significant enhancement of neutralizing antibody titer against SF162 (Fig. 6), Co-immunization with m8A-Env and m8A-p7.5hCD40Lm (group G) did not show significantly enhanced nAbs to SF162. Sera from untreated mice contained low levels of a non-specific inhibitor to SF162 (titer approximately 100), but the level is insignificant. However, these regimens, irrespective of the inclusion of hCD40Lm, were not effective in eliciting nAbs against tier 2 viruses, including JR-CSF. Immunization twice with the plasmid expressing gp160 followed by boosting with SeV-env did not elicit detectable anti-Env antibody (see Fig. 4), indicating the importance of priming with m8A-Env. Priming and boosting with the same m8Δ-Env did not elicit significant titers of nAb (data not shown). To further confirm that the antibodies but not other factors were responsible for the neutralizing activity of the sera, we purified antibodies using protein A Sepharose. All the purified antibodies showed IgG isotypes. We performed neutralizing antibody measurement using TZM-bl reporter cells as described above. The neutralization capacities of purified immunoglobulins showed high identicalness with the neutralizing titer of original sera (R² = 0.777) (Fig. 6B) confirming that neutralizing antibodies were elicited using our m8A prime/SeV boost regimens.

An immunological correlate analysis was conducted using Spearman rank correlates to evaluate the relationship between the SF162 neutralization capacity and the avidity of JR-CSF Env binding antibodies; a significant positive correlation was present (R = 0.686, P<0.0001; Fig. 7A). A weaker, but still significant, positive correlation between the nAb titers and the amount of the Env-binding antibodies was identified (R = 0.501, P = 0.0013; Fig. 7B). Moreover, the amounts of Env-binding antibodies showed a positive correlation with the antibody avidity (R = 0.555, P = 0.000377; Fig. 7C). These results verify that better immunized mice produced not only greater amounts of antibody,
but also higher affinity antibodies with enhanced neutralizing capacity.

Secretion of anti-Env IgG and IgA into vaginal fluid was examined. Because of the limited amounts of mouse vaginal fluid, fluid from 5–6 mice was mixed for these assays. The amounts of total IgG in the fluid were relatively constant. Fluid prepared from the DNA prime/SeV-env boost group contained 277 ng/ml IgG, the m8Δ-Env prime/SeV-env boost group contained 270 ng/ml, the m8Δ-Env/hCD40Lm prime/SeV-env boost group contained 252 ng/ml, and the m8Δ-Env + m8Δ/hCD40Lm prime/SeV-env group contained 242 ng/ml. Similar levels of anti-gp160 IgG were detected in all the m8Δ primed groups, but no gp160-specific IgG was detected in the DNA primed group (Fig. 8). Anti-Env IgA was not detected in significant amounts in any immunization group (data not shown).

**Discussion**

Although researchers have proposed either antibodies or T cells as the most effective means to elicit protective immunity, a central theme of HIV-1 vaccine design now is to elicit coordinated antiviral CD8+ cytotoxic T lymphocytes (CTL) to control HIV-1 infection and CD4+ T cells that help induce and maintain CD8+ and B cell responses [44–46]. In this study, we found that a novel immunization schedule including a HIV-1 Env expressing m8Δ prime/SeV boost regimen is able to elicit both Env-specific CD8+ T cells and antibodies. These results sharply contrast the results of conventional DNA virus/vector boost regimens that have elicited CD8+ T cell responses with poor antibody response, which is consistent with the previous report [47]. Moreover, this regimen elicited anti-Env IgG secreted into the vaginal fluid. Thus, the m8Δ prime/SeV boost regimen should provide a basis for an improved immunization protocol. Although nAbs against tier 2 viruses were absent, recent reports have suggested that non-neutralizing Env binding antibodies play a role in preventing infection by HIV-1/SIV [9,48]. Thus, the humoral immunity elicited by the m8Δ prime/SeV boost regimen may also have an improved efficacy against HIV-1 infection. It is an important future theme to elicit broad nAbs against tier 2 viruses on the basis of this regimen.

In m8Δ prime/SeV boost regimen only priming with m8Δ-Env did not elicit any anti-Env antibody before rSeV boost (data not shown), indicating the necessity of rSeV booster. Intranasal Sendai virus was well tolerated and had good immunogenicity [19], making it a good candidate for HIV vaccine. Indeed, involvement of rSeV in prime-boost-boost HIV vaccine strategies had been reported to elicit persistent humoral response in BALB/c mice and rhesus macaques [49] as well as in pre-clinical trials [50]. However, no elicitation of neutralizing antibody has been reported. Our results that m8Δ prime/SeV boost in combination with hCD40Lm adjuvant regimen efficiently elicited neutralizing antibody suggests that this regimen may be a better vaccine strategy.

Recent advances in immunology have shown that several types of molecules may be used as novel adjuvants to enhance the immunogenicity of vaccines. In addition to alum and MF59, other adjuvants including cytokines, chemokines, toll-like receptor ligands and some co-stimulatory molecules may have potential for clinical use [51–54]. Among these molecules, CD40L is one of the most potent stimuli for DCs, which activate CTL and B cells [15,25–28]. We showed that hCD40Lm expressing m8Δ in conjunction with Env expressing vaccinia virus enhanced production of HIV-1 specific CTL, but not antibodies. In contrast, the coexpression m8Δ-Env/hCD40Lm vector did not increase the induction of Env-specific CD8+ T cells compared to m8Δ-Env alone, but did elicit more anti-Env antibodies with higher avidity and neutralizing capacity against tier 1 SF162. The high avidity antibodies elicited by m8Δ-Env/hCD40Lm may constitute nAbs. Indeed, we found a positive correlation between the avidity of Env-binding antibodies and neutralizing activity against HIV-1 SF162 (Fig. 7). The enhancement of antiviral immunity by hCD40Lm in this mouse model also suggests that human CD40Lm is functional in mouse, encouraging us to use it as an AIDS vaccine adjuvant. We have to admit that our novel vaccine regimen still has room to be improved. The species variation between human and mouse CD40Lms should be considered when noting that the enhancement of humoral immunity to be less impressive. Using homogenous CD40Lm or further inclusion of other adjuvant is encouraged to magnitude antibody response of our novel vaccine regimen. Although the reason for the divergent effects of hCD40Lm expressed from different constructs on the production of viral specific immunity is unclear, Given that co-immunization with the two vectors may result in gp160 and CD40Lm being expressed on the same cell or on different cells, whereas immunization with the coexpression vector m8Δ-RC5Fenv/hCD40Lm results in gp160 and CD40Lm being expressed on the same cells. Simultaneous expression of gp160 and CD40Lm may preferentially stimulate B cells through a direct interaction with the vector-infected cells because only B cells express both CD40 and a B cell receptor to gp160. The expression of CD40Lm alone may promote the maturation of DCs that were sensitized by gp160 and lead to the activation of cellular immunity. Alternatively, the expression levels of Env and hCD40Lm in cells that have been infected with both m8Δ-Env and m8Δ-hCD40Lm may be different from levels in cells infected with m8Δ-Env/hCD40Lm, leading to different immune responses. Other hypothesis to explain these phenomena cannot be excluded at this point.

The detection of anti-Env IgG in vaginal fluid indicates that our vaccine regimen can also elicit mucosal immunity. However, in contrast to the enhancing effect of hCD40Lm on production of anti-Env antibodies in sera, we didn’t find that inclusion of hCD40Lm promote levels of anti-gp160 IgG in vaginal fluid as seen in Fig. 8. The reason for this difference is currently under elucidation. Further investigation of mechanism responsible for this divergent effect may be profitable to improve our vaccine regimen to achieve more potent mucosal immunity against HIV.

Recently, monoclonal antibodies (mAbs) that broadly neutralize most HIV-1 strains have been isolated from chronically infected subjects, and analyses of the epitopes recognized by these antibodies may direct the way to devise antigens that elicit broad nAbs [55–57]. These studies suggest the possibility of a vaccine that elicits the production of broadly neutralizing antibodies that could prevent HIV-1 infection. However, detailed analyses of broadly neutralizing mAbs have indicated a requirement for extensive affinity maturation of the cognate immunoglobulin genes [57]. The observation that broad neutralizing Abs are generated after a long incubation period in HIV-1-infected individuals also supports this idea and, furthermore, suggests the importance of repetitive antigenic stimulation [38,39]. Therefore, in addition to devising specific antigens, the development of methods to promote the affinity maturation of antibodies and maturation of B cells should be equally important. This study has demonstrated that CD40Lm, which can activate class switching of immunoglobulin genes and maturation of dendritic cells [22,23], is suitable for eliciting more potent nAbs. In addition, replication-competent m8Δ that repeatedly presents native antigens in vivo may be important for effective immunization.
In conclusion, this study showed that a novel vaccine regimen which includes the expression of HCD104m in the context of LC16m8 priming and Sendai virus vector boosting was able to elicit both HIV-1 Env-specific cellular and humoral immunoresponses. Thus, such a regimen may provide a platform for HIV-1 vaccine development, as well as other infectious pathogens.

Acknowledgments

We are grateful for the assistance of Ms. Y. Ishida and R. Narita, and for the HIV-1 Env peptide panel supplied by the NIH AIDS Research and Reagent Program.

References

13. Kawada M, Igarashi H, Takeda A, Tsukamoto T, Yamamoto H, et al. (2006) Initiation of LC16m8 that includes the expression of hCD40Lm in the context of Sendai virus vector boosting was able to elicit both HIV-1 Env-specific cellular and humoral immunoresponses. Thus, such a regimen may provide a platform for HIV-1 vaccine development, as well as other infectious pathogens.

Author Contributions


