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 LC16 m8Δ (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 r m8Δ strains that express HIV-1 Env under the control of a moderate promoter, p7.5, or a strong promoter, pSFJ1-10. m8 Δ -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.

Keywords: LC16m8Δ, promoter pSFJ1-10, promoter p7.5, HIV-1 Env, immunogenicity. Safety

Highlights

1. HIV-1 Env expressing replication competent vaccinia m8Δ prime/Sendai virus vector boost elicited both long-lasting cellular and humoral immunities.

2. VV m8Δ vectors containing different promoters activate different arms of the immune system.

3. The vaccine regimen can be optimized to selectively induce either cellular or humoral immune response.

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 by 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]. The balance between antigen expression and viral propagation *in vivo* may be crucial for optimal immunogenicity. 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 m8Δ-p7.5-JRCSFenv and m8ΔpSFJ-JRCSFenv were relatively less virulent than LC16m8Δ. Our results may provide important information to develop HIV-1 vaccine for clinical trials.

2. Materials & Methods

2.1 Cells and viruses

The RK13 (rabbit kidney epithelial) cell line was cultured in RPMI1640 supplemented with 10% FCS at 37°C in an atmosphere containing 5% CO_2. BHK (baby hamster kidney), TZM-bl (a CD4- and CCR5-expressing derivative of HeLa cells) cell lines [10, 11], 293T (human embryonal kidney cell line) and L929 (mouse fibroblastoid line) 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 m8 Δ recombinants, 10 μl of a serially diluted solution that contains 10^3–10^7 pfu of rm8 Δ s was injected intracerebrally (i.c.) into 10 to 17 of 2–3-day-old suckling Crlj:CD1 (ICR) mice (Charles River). Survival was then monitored daily for 2 weeks and the 50% lethal dose (LD50) was calculated [17].

2.11 Statistical analysis

Statistical analysis was performed using Student's *t*-test (Microsoft Excel version 11.6.6). P values of <0.05, <0.01, and <0.001 were 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 m8 Δ 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 to 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 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 results 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-1s 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

252 difference was not significant (Fig. 3B). We also measured the avidity of the anti-Env
253 Abs in both groups: no significant difference was observed (Fig. 3C). Since the m8Δ-
254 pSFJ-JRCSFenv prime/SeV-JRCSFenv boost elicited greater HIV-1 Env-specific
255 antibody responses, we next asked whether this antibody titer is maintained over the
256 long-term. We followed a subgroup of mice treated with this vaccination regimen for
257 28 weeks after the Sendai virus boost and found that the anti-HIV-1 Env antibody titer
258 was maintained throughout the observation period (Fig. 3D).
259 *3.3 Safety evaluation of the rm8 Δ in suckling mice*
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261 To evaluate the safety of rm8 Δ, we i.c.-injected suckling mice with m8 Δ,
262 m8ΔVNC110, m8 Δ-pSFJ-JRCSFenv, or m8 Δ-p7.5-JRCSFenv. At 2 weeks post-
263 injection, more of the mice in the m8 Δ VNC110- and m8 Δ-pSFJ-JRCSFenv-injected
264 (at 10^4 and 10^5 pfu) groups survived compared with those in the m8 Δ-p7.5-
265 JRCSFenv-injected group (Fig. 4B, C, and D). LC16m8 Δ, which should be safe for
266 human use, showed the highest mortality (Fig 4A). The median lethal doses (LD50)
267 for each strain were as follows: LC16m8 Δ, $<10^3$ PFU; m8 Δ-p7.5-JRCSFenv, $5.5 \times$
268 10^3 PFU; m8 Δ-pSFJ-JRCSFenv, 1.4×10^5 PFU; and m8 Δ VNC110, 5.75×10^5 PFU
269 (Fig. 4E). These results suggest that both m8 Δ-p7.5-JRCSFenv and m8 Δ-pSFJ-
270 JRCSFenv may be safer for use in humans.
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272
273 **4. Discussion**
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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 m8 Δ -p7.5-JRCSFenv to induce production of Env-specific IFN- γ $^{+}$CD107a^{+}CD8^{+} T cells. The relative lower ratio of gp41to 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 titre observed in mice immunized with m8Δ-pSFJ-JRCSFenv is consistent with the higher levels of Env observed in m8Δ-pSFJ-JRCSFenv-infected cells. The Gp120/160 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 gp120/160 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 m8 Δ -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 new-born mice than the parental strain, LC16m8Δ. LC16m8Δ was more virulent probably because it contains an intact HA gene. The LD50 of m8 Δ -p7.5-JRCSFenv was significantly lower than that of m8 Δ VNC110, 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 m8 Δ -pSFJ-JRCSFenv is similar to that of m8 Δ VNC110, despite having a much lower capacity for replication. Nevertheless, our finding that

recombinant VVs expressing HIV-1 env are safer than LC16m8 Δ 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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450

451 **Figure legends**

452

453 Fig 1. **Construction of the Env-expressing vaccinia vector, Env expression, and**
454 **virus propagation**. (A). Schematic illustration showing the structure of the
455 hemagglutinin (HA) gene region within LC16m8Δ and its derivatives. Arrows
456 indicate the direction of the HA coding frame. (B). Comparison of Env expression in
457 cells infected with m8Δ-pSFJ-JRCSFenv or m8Δ-p7.5-JRCSFenv. One microgram of
458 cell lysate derived from RK13, 293T, and L929 cells infected with rVV was subjected
459 to SDS-PAGE and analysed by Western blotting as described in "Materials and
460 Methods". Lanes 1, 4, and 7 represent the cells infected with LC16m8Δ, lanes 2, 5,
461 and 8, m8Δ-pSFJ-JRCSFenv; Lanes 3, 6, and 9, m8Δ-p7.5-JRCSFenv.
462 (C). Comparison of the growth potential of the LC16m8Δ constructs. Viruses were
463 recovered from RK13, 293T, and L929 cells 24 h after infection and titrated in a
464 plaque assay. Data represent the mean ± SD (n=4).

465

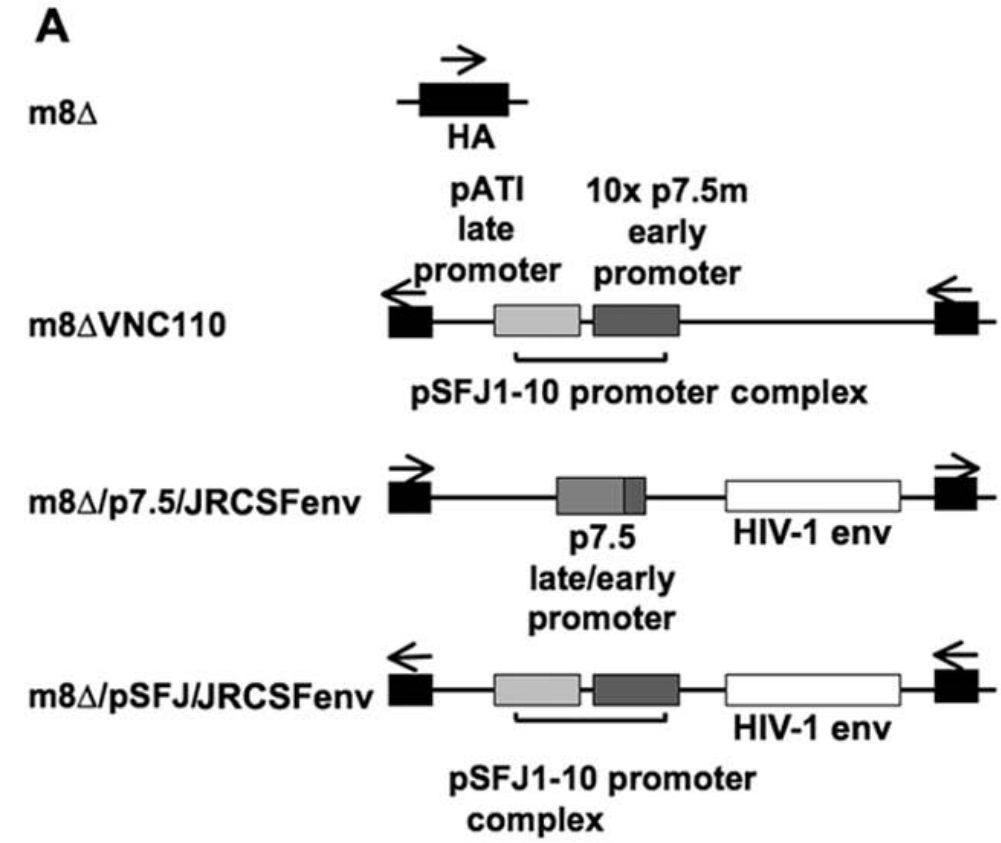
466 **Fig 2. The p7.5 promoter induces more efficient production of Env-specific CTL**
467 **responses than the pSFJ promoter.** (A). Schematic illustration showing the rm8Δ
468 prime/rSeV boost vaccination protocol. Seven-week-old female C57BL/6J mice were
469 vaccinated with LC16m8Δ's recombinants (16 mice for m8Δ-pSFJ-JRCSFenv (group
470 1) and 10 mice for m8Δ-p7.5-JRCSFenv (group2); each at 1×10^7 PFU) followed by
471 a boost with SeV-JRCSFenv (4×10^7 CIU). Blood samples and spleen tissues were
472 examined at the indicated time points. (B). Representative diagram showing FACS
473 analysis of HIV-1 Env-specific IFN- γ -secreting $CD107a^+CD8^+$ T cells derived

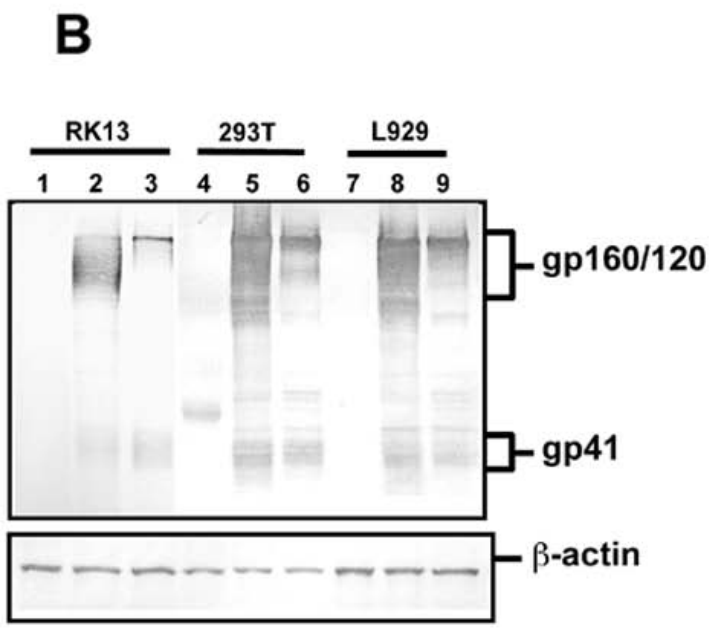
from vaccinated mouse splenocytes. (C). Comparison of Env-specific cellular immune response between the two vaccinated groups at 2 and 8 weeks post-SeV boost.

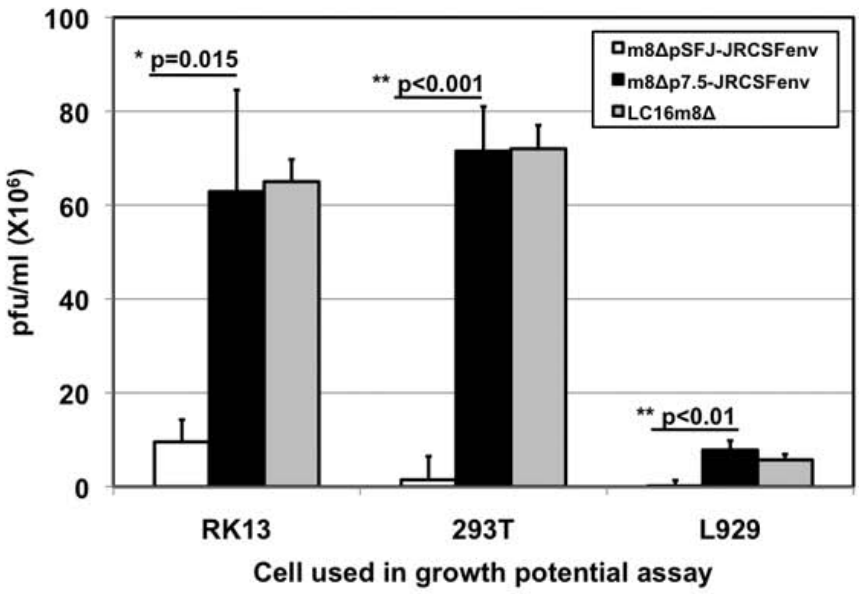
Fig 3. **m8Δ-pSFJ-JRCSFenv induces stronger humoral immune responses than m8Δ-p7.5-JRCSFenv** (A). Comparison of Env-specific antibody levels. Serum from individual immunized mice was analyzed in a HIV-1JR-CSF gp160 ELISA as described previously (Ref.2). The plates were developed with an HRP-conjugated 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. 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.

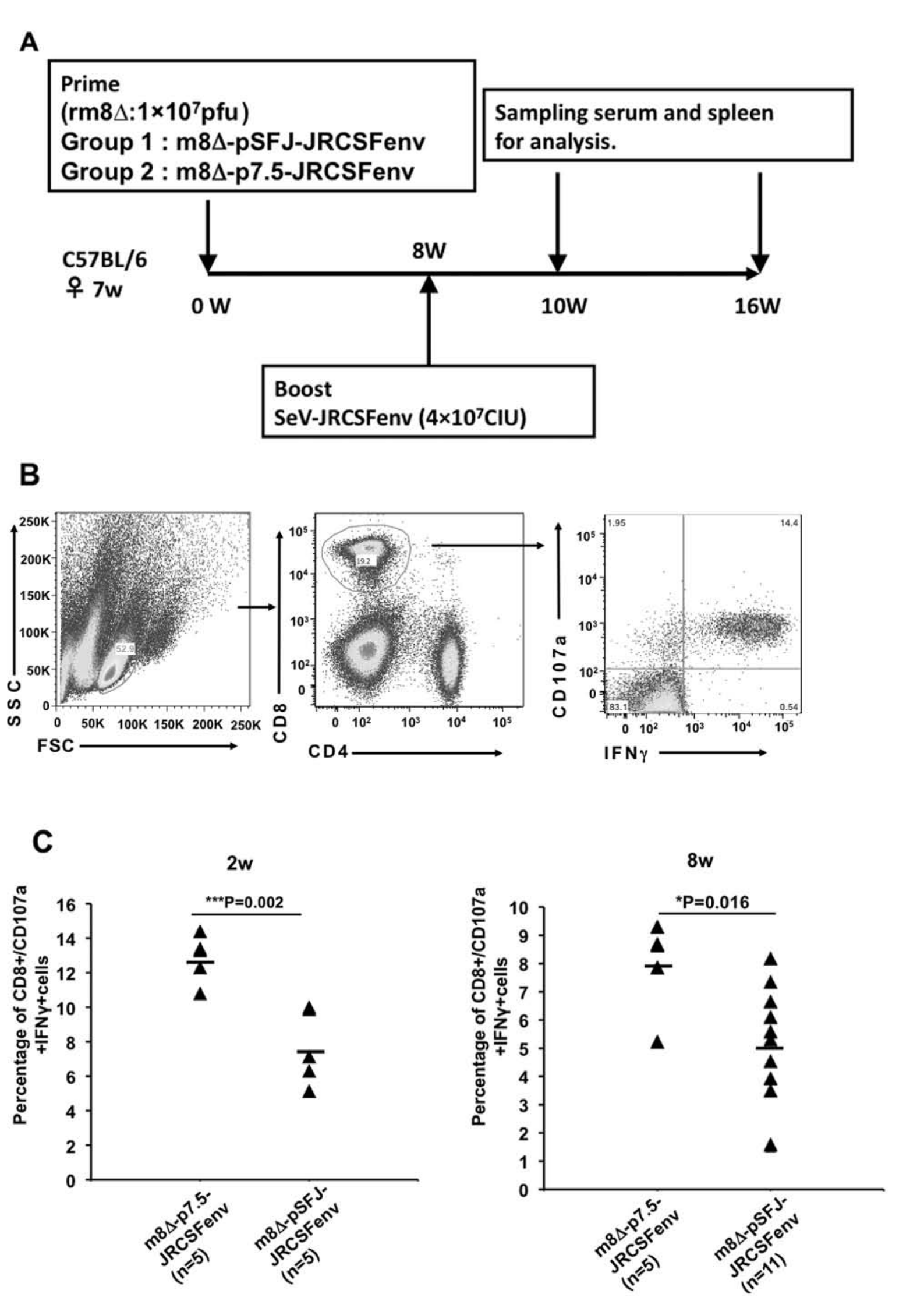
Fig 4. m8Δ-pSFJ-JRCSFenv is safer than m8Δ-p7.5-JRCSFenv *in vivo*. Cumulative survival curves for vaccinated suckling mice are shown. Mice were injected with LC16m8Δ (A), m8ΔVNC110 (B), m8Δ-pSFJ-JRCSFenv (C), or m8Δ-

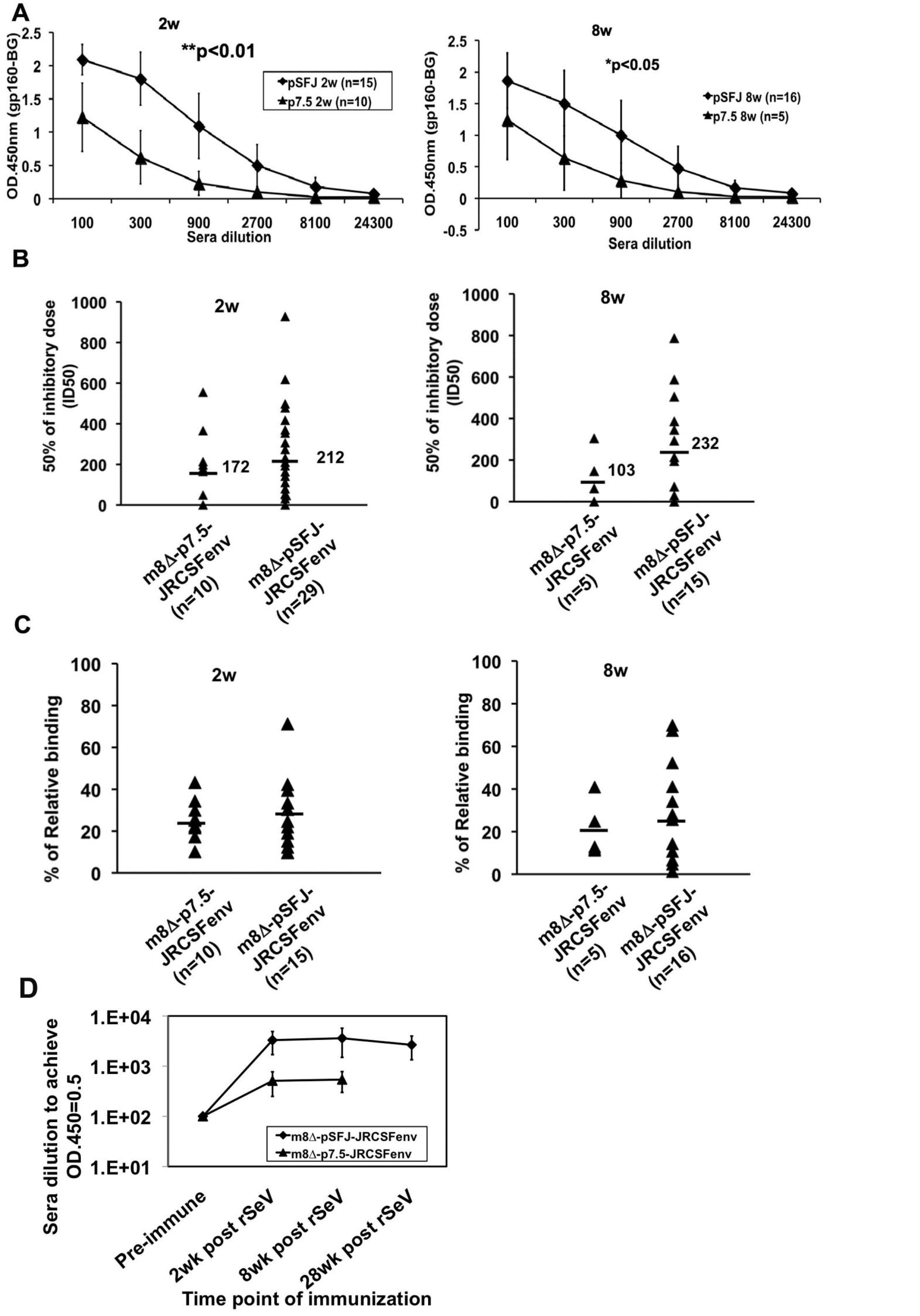
p7.5-JRCSFenv (D). Percentage of survival at 2 weeks post inoculation was plotted to make a survive curve and the 50% lethal dose (LD50) was calculated according to the Trendline of the curve. The LD50 for each VV is shown (E). The numbers of the mice used for each dose are indicated in the chart. Statistical analysis was performed using the log-rank test.

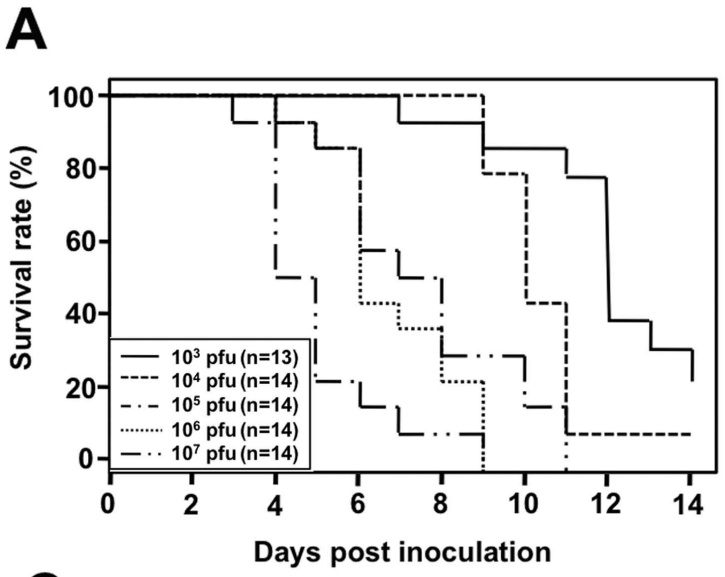

A
m8Δ
HA
pATI late promoter
10x p7.5m early promoter
m8ΔVNC110
pSFJ1-10 promoter complex
m8Δ/p7.5/JRCSFenv
p7.5 late/early promoter
HIV-1 env
m8Δ/pSFJ/JRCSFenv
HIV-1 env
pSFJ1-10 promoter complex

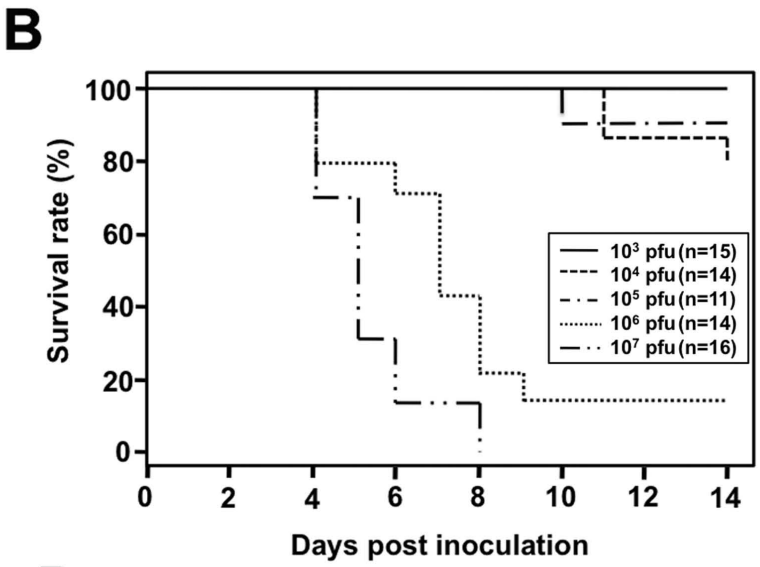

B
RK13
293T
L929
1 2 3 4 5 6 7 8 9
gp160/120
gp41
β-actin

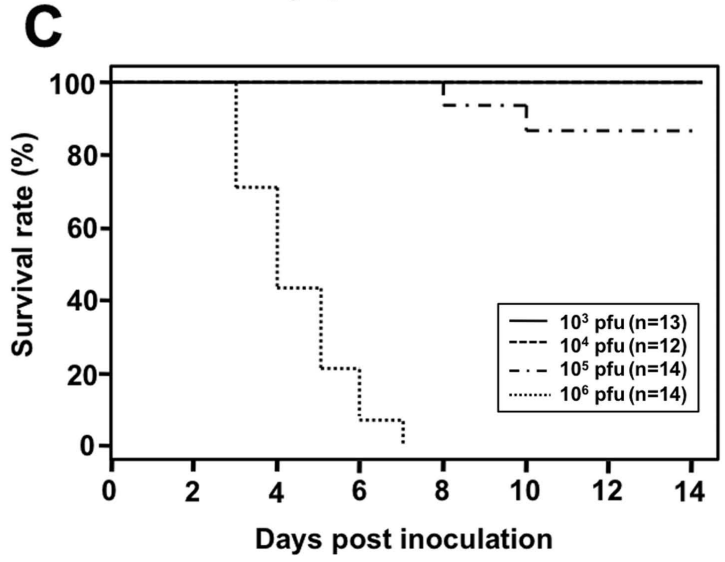

C
* p=0.015
** p<0.001
** p<0.01
m8ΔpSFJ-JRCSFenv
m8Δp7.5-JRCSFenv
LC16m8Δ
pfu/ml (X10⁶)
100
80
60
40
20
0
RK13
293T
L929
Cell used in growth potential assay

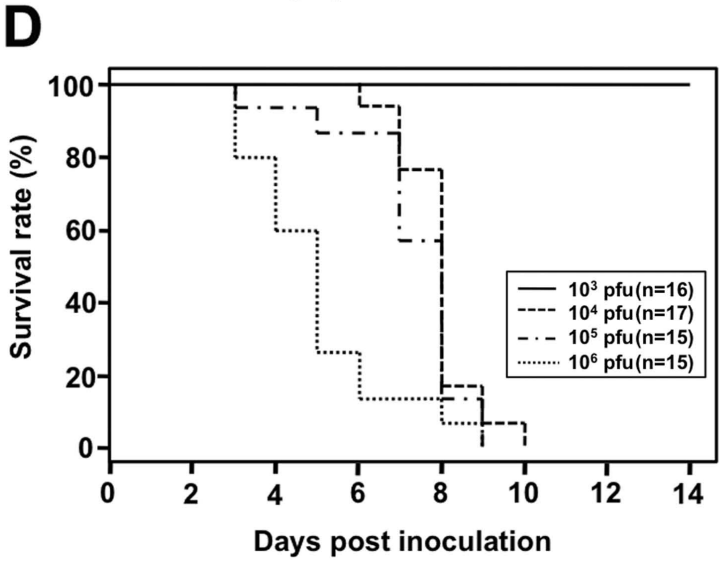

A
Prime
(rm8Δ:1×10⁷pfu)
Group 1 : m8Δ-pSFJ-JRCSFenv
Group 2 : m8Δ-p7.5-JRCSFenv
Sampling serum and spleen
for analysis.
C57BL/6
♀ 7w
8W
0 W
10W
16W
Boost
SeV-JRCSFenv (4×10⁷CIU)
B
SSC
FSC
CD8
CD4
CD107a
IFNγ
52.9
19.2
1.95
14.4
83.1
0.54
C
2w
***P=0.002
Percentage of CD8+/CD107a +IFNγ+cells
m8Δ-p7.5-JRCSFenv (n=5)
m8Δ-pSFJ-JRCSFenv (n=5)
8w
*P=0.016
m8Δ-p7.5-JRCSFenv (n=5)
m8Δ-pSFJ-JRCSFenv (n=11)

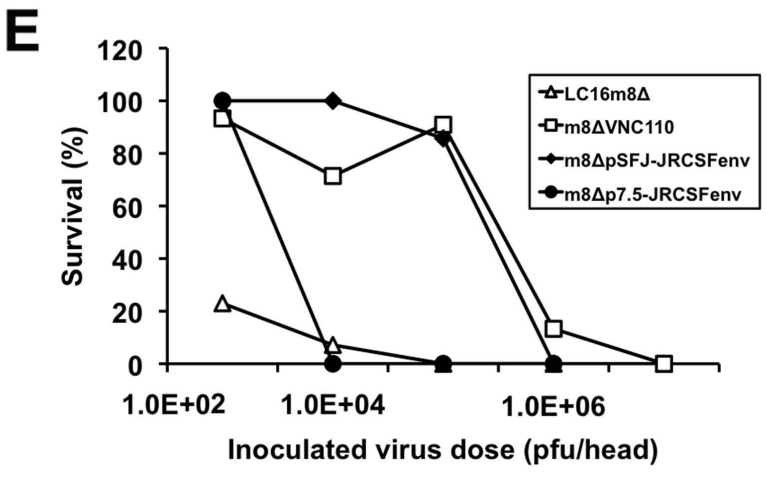

A
2w
**p<0.01
pSFJ 2w (n=15)
p7.5 2w (n=10)
OD.450nm (gp160-BG)
Sera dilution
8w
*p<0.05
pSFJ 8w (n=16)
p7.5 8w (n=5)
B
50% of inhibitory dose (ID50)
172
212
103
232
m8Δ-p7.5-JRCSFenv (n=10)
m8Δ-pSFJ-JRCSFenv (n=29)
m8Δ-p7.5-JRCSFenv (n=5)
m8Δ-pSFJ-JRCSFenv (n=15)
C
% of Relative binding
m8Δ-p7.5-JRCSFenv (n=10)
m8Δ-pSFJ-JRCSFenv (n=15)
m8Δ-p7.5-JRCSFenv (n=5)
m8Δ-pSFJ-JRCSFenv (n=16)
D
Sera dilution to achieve OD.450=0.5
m8Δ-pSFJ-JRCSFenv
m8Δ-p7.5-JRCSFenv
Pre-immune
2wk post rSeV
8wk post rSeV
28wk post rSeV
Time point of immunization











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