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Author(s)	Isshiki, Mao; Zhang, Xianfeng; Sato, Hirotaka; Ohashi, Takashi; Inoue, Makoto; Shida, Hisatoshi
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1 Effects of different promoters on the virulence and immunogenicity of a HIV-1 Env-
2 expressing recombinant vaccinia vaccine

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4 Mao Isshiki¹, Xianfeng Zhang^{1*}, Hiroataka Sato^{1,2}, Takashi Ohashi¹, Makoto Inoue³,
5 Hisatoshi Shida¹

6

7 ¹Institute for Genetic Medicine, Hokkaido University, Kita-ku, Sapporo 060-0815,
8 Japan

9 ²Current address: Viral Infectious Diseases Unit, RIKEN, Hirosawa, Wako, Saitama,
10 351-0198, Japan

11 ³DNAVEC Corporation, Techno Park Oho, 6 Ohkubo, Tsukuba, Ibaraki 300-2611,
12 Japan

13

14

15

16 * Corresponding author: Institute for Genetic Medicine, Hokkaido University, Kita-
17 ku, Sapporo 060-0815, Japan

18 Tel/Fax: +81-11-706-7543

19 Mail: zhangxf@igm.hokudai.ac.jp

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26 **Abstract**

27

28 Previously, we developed a vaccination regimen that involves priming with
29 recombinant vaccinia virus LC16 m8Δ (rm8Δ) strain followed by boosting with a
30 Sendai virus-containing vector. This protocol induced both humoral and cellular
31 immune responses against the HIV-1 envelope protein. The current study aims to
32 optimize this regimen by comparing the immunogenicity and safety of two r
33 m8Δ strains that express HIV-1 Env under the control of a moderate promoter, p7.5,
34 or a strong promoter, pSFJ1-10. m8 Δ -p7.5-JRCSFenv synthesized less gp160 but
35 showed significantly higher growth potential than m8Δ-pSFJ-JRCSFenv. The two
36 different rm8Δ strains induced antigen-specific immunity; however, m8Δ-pSFJ-
37 JRCSFenv elicited a stronger anti-Env antibody response whereas m8Δ-p7.5-
38 JRCSFenv induced a stronger Env-specific cytotoxic T lymphocyte response. Both
39 strains were less virulent than the parental m8Δ strain, suggesting that they would be
40 safe for use in humans. These findings indicate the vaccine can be optimized to
41 induce favorable immune responses (either cellular or humoral), and forms the basis
42 for the rational design of an AIDS vaccine using recombinant vaccinia as the delivery
43 vector.

44

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

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52 **Highlights**

53 1. HIV-1 Env expressing replication competent vaccinia m8Δ prime/Sendai virus

54 vector boost elicited both long-lasting cellular and humoral immunities.

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

56 immune system.

57 3. The vaccine regimen can be optimized to selectively induce either cellular or

58 humoral immune response.

59

60 **1. Introduction**

61

62 Despite the increasing availability and effectiveness of antiretroviral treatments, a safe

63 and effective vaccine that prevents HIV-1 infection would be invaluable. A recent

64 report from Thailand showed that the RV144 vaccine protocol, which involved

65 priming with a canarypox virus vector followed by boosting with recombinant gp120

66 protein, reduced HIV-1 infection by approximately 30% [1]. These results are

67 encouraging, and indicate that poxviruses may be used as vectors for HIV-1 subunit

68 vaccines.

69 . However, the efficacy of the RV144 vaccine was only moderate, suggesting the need

70 to improve either the vaccination regimen or the poxvirus vector used for delivery.

71 One improvement that may elicit a more potent protective immune response is the use

72 of a replication-competent vaccinia virus (VV) vector rather than the non-replicating

73 canarypox vector.

74 We previously reported that a heterologous prime-boost vaccination protocol using a

75 recombinant m8Δ (rm8Δ) virus (m8Δ-pSFJ-JRCSFenv), which expresses the HIV-

76 1JR-CSF envelope glycoprotein, and a recombinant Sendai virus (rSeV), SeV-

77 JRCSFenv, elicited both HIV-1 Env-specific humoral and cell-mediated immune
78 responses [2]. This may be a promising vaccination protocol to protect against HIV-1
79 infection. The aim of the present study is to further optimize this regimen.

80 The replication-competent VV strain, LC16m8, is a smallpox vaccine licensed for use
81 in Japan. It has been used in 100,000 people without any serious adverse effects [3].
82 LC16m8 Δ (m8 Δ) is a genetically stable derivative of LC16m8, which is safer than the
83 parental LC16m8 virus but shows the same degree of antigenicity [4]. Moreover,
84 immunization with m8 Δ protects mice against infection by virulent VV much more
85 efficiently than the non-replicating VV strain, MVA [4]. Thus, m8 Δ may be a
86 promising VV vector for use in vaccines against infectious diseases.

87 Three types of promoter (early, intermediate, and late) have been identified in VV.
88 Antigens that are highly expressed under the control of a powerful late promoter are
89 generally considered to be potent inducers of a strong immune response [5]. However,
90 early promoters appear to elicit stronger cytotoxic T lymphocyte (CTL) responses
91 [6,7]. The balance between antigen expression and viral propagation *in vivo* may be
92 crucial for optimal immunogenicity. The p7.5 promoter is an early-late promoter that
93 was identified in 1984 and is widely used for the construction of live VV-vectored
94 vaccines. Because the levels of gene expression driven by the p7.5 promoter have yet
95 to be optimal, a more potent promoter, pSFJ1-10, was constructed, which enables the
96 genes of interest to be expressed at higher levels during both the early and late phases
97 of the infection cycle [8,9].

98 Here, we compared the immunogenicity and safety of two rm8 Δ s that express HIV-1
99 Env under the control of the p7.5 or pSFJ1-10 promoters. Both were tested in a
100 vaccination protocol that involved priming with rm8 Δ followed by boosting with
101 rSeV. We found that one of the vectors preferably induced humoral responses against

102 HIV-Env, whereas the other primarily induced cellular immune responses. These
103 findings suggest that it may be possible to select vaccine vectors that induce favorable
104 immune responses. In suckling mice, both m8Δ-p7.5-JRCSFenv and m8ΔpSFJ-
105 JRCSFenv were relatively less virulent than LC16m8Δ. Our results may provide
106 important information to develop HIV-1 vaccine for clinical trials.

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108

109 **2. Materials & Methods**

110

111 *2.1 Cells and viruses*

112 The RK13 (rabbit kidney epithelial) cell line was cultured in RPMI1640
113 supplemented with 10% FCS at 37°C in an atmosphere containing 5% CO₂. BHK
114 (baby hamster kidney), TZM-bl (a CD4- and CCR5-expressing derivative of HeLa
115 cells) cell lines [10, 11], 293T (human embryonal kidney cell line) and L929 (mouse
116 fibroblastoid line) were cultured in DMEM supplemented with 10% FCS. VV
117 LC16m8Δ, m8ΔVNC110 that harbors multiple cloning site in the HA gene of
118 LC16m8Δ genome, m8Δ-pSFJ-JRCSFenv and SeV-JRCSFenv, which express gp160
119 of HIV-1 JRCSF, and canarypox virus were described previously [2].

120

121 *2.2 Construction of the LC16m8Δ expressing JR-CSFenv under the control of the p7.5* 122 *promoter*

123 To construct LC16m8Δ-p7.5-JRCSFenv, a transfer plasmid that harbors the HIV-1
124 JR-CSF env gene downstream of the p7.5 VV promoter was first constructed. The
125 gp160-encoding region was amplified from pJWJRCSFenvΔEcoR1 (the template) by
126 PCR using the following primer pair: JRCSFenv F1

127 (AGTGGATCCGCCACCATGAGAGTGAAGGGGATCAGGAAG; BamH1 site
128 underlined) and JRCSFenvR1
129 (TTAGAGCTCTTATAGCAAAGCCCTTTCCAAGCC; Sac1 site underlined). The
130 VV transcription termination signals (TTTTTNT) within the env gene sequence were
131 synonymously mutated *in vitro* using a mutagenesis kit (Stratagene). The env
132 fragment was then digested with Sac1 and ligated into the pVR1 vector [12], which
133 had been digested with Sac1 and Sma1. BHK cells, which had been infected with
134 canarypox virus, were then co-transfected with the resultant plasmid and LC16m8Δ
135 genomic DNA to generate VV LC16m8Δ-p7.5-JRCSFenv. HA⁻ recombinants were
136 selected using erythrocytes isolated from white leghorn chickens (Sankyo Labo
137 Service Corporation, Inc.) [13,14]. Expression of HIV-1 Env protein was examined
138 by Western blotting and plaque immunostaining.

139

140 2.3 Western Blotting

141 Vaccinia-infected-RK13 cells were lysed and the proteins separated in 10% SDS-
142 PAGE gels. Immunoblot analysis was performed with human antiserum from a HIV-
143 1-infected patient or monoclonal mouse anti-β actin antibody, followed by alkaline
144 phosphatase-conjugated anti-human or mouse IgG (Promega). Proteins were
145 visualized using NBT/BCIP (Sigma).

146

147 2.4 Plaque immunostaining

148 RK13 cells were cultured in 6-well plates and infected with recombinant VV (at
149 approximately 100 plaque forming unit (pfu)/well). The cells were incubated with the
150 virus for 72 h at 33°C, fixed with 2% paraformaldehyde solution, and permeabilized
151 by incubating with 0.5% Nonidet P-40 for 1 min. The fixed cells were blocked with

152 5% skimmed milk (in PBS) for 30 min at room temperature and incubated with the
153 primary antibody (HIV-1 infected human serum; diluted 1000-fold) for 1 h at room
154 temperature, followed by the secondary antibody (alkaline phosphatase-conjugated
155 anti-human IgG (Promega); diluted 2500-fold). The plaques were then stained with
156 NBT/BCIP.

157

158 *2.5 Propagation potential of rm8Δ*

159 To evaluate the propagation potential of LC16m8Δ and its recombinants, RK13, 293T
160 and L929 cells (3×10^5) were infected with the viruses at a multiplicity of infection of
161 3 and then incubated for 24 h at 33°C. Progeny viruses were harvested and titrated on
162 a monolayer of RK13 cells in a plaque assay.

163

164 *2.6 Immunization of mice*

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

170

171 *2.7 Intracellular cytokine staining (ICS) of splenocytes*

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

177

178 *2.8 Evaluation of neutralizing activity*

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

181

182 *2.9 Measurement of anti-Env antibody levels by ELISA*

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

185 *2.10 Safety of m8Δs*

186 To evaluate the safety of the LC16m8Δ and m8 Δ recombinants, 10 μl of a serially
187 diluted solution that contains 10³–10⁷ pfu of rm8 Δ s was injected intracerebrally (i.c.)
188 into 10 to 17 of 2–3-day-old suckling Crlj:CD1 (ICR) mice (Charles River). Survival
189 was then monitored daily for 2 weeks and the 50% lethal dose (LD50) was calculated
190 [17].

191 *2.11 Statistical analysis*

192 Statistical analysis was performed using Student's *t*-test (Microsoft Excel version
193 11.6.6). P values of <0.05, <0.01, and <0.001 were considered significant. The
194 survival of virus-injected suckling mice was evaluated using the log-rank test (R
195 version 2.15.1).

196

197

198 **3. Results**

199

200 *3.1 In vitro properties of rm8Δ expressing JR-CSFenv under the control of different*
201 *promoters.*

202

203 We previously constructed m8Δ-pSFJ-JRCSFenv, which expresses the HIV-1JR-CSF
204 env gene under the control of the high expression pSFJ1-10 promoter, and showed
205 that it elicited HIV-1 Env-specific cellular and humoral responses when used in
206 combination with the Sendai vector, SeV-JRCSFenv [2]. However, because an rVV
207 that moderately expresses a foreign gene, but propagates better, might elicit more
208 potent immunological responses, we constructed recombinant m8 Δ expressing JR-
209 CSFenv under the control of the p7.5 promoter (which is a moderate driver of foreign
210 gene expression) (Fig.1A). We first compared expression of the Env protein in
211 various cells infected with m8Δ-p7.5-JRCSFenv or m8Δ-pSFJ-JRCSFenv by Western
212 blotting (Fig. 1B). Regardless of the cell type, m8Δ-p7.5-JRCSFenv produced several-
213 fold less gp120/160 than m8Δ-pSFJ-JRCSFenv. In addition, the bands corresponding
214 to gp120/160 expressed by cells infected with m8Δ-pSFJ-JRCSFenv were much
215 broader than those expressed by cells infected with m8Δ-p7.5-JRCSFenv. Meanwhile,
216 titration of the progeny virus after one-step growth revealed that the growth potential
217 of rVVs in mouse L929 cells are 10 times lower than that in human 293T cells and
218 rabbit RK13 cells (Fig 1C), Nevertheless, m8Δ-p7.5-JRCSFenv showed growth
219 potential similar to that of the parental m8Δ, and significantly higher (6 to 50 fold)
220 than that of m8Δ-pSFJ-JRCSFenv (Fig. 1C). This indicates that overexpression of the
221 foreign gene suppresses viral propagation.

222

223 *3.2 Immunogenicity of m8Δ-p7.5-JRCSFenv and m8Δ-pSFJ-JRCSFenv.*

224

225 We next compared the immunogenicity of m8Δ-p7.5-JRCSFenv and m8Δ-pSFJ-
226 JRCSFenv by using them to prime mice, which were then boosted with SeV-

227 JRCSFenv according to the schedule outlined in Fig.2A. Splenocytes were isolated,
228 stimulated with a mixture of HIV-1 consensus subtype B Env (15-mer) peptides (NIH
229 AIDS reagent program, No. 202/203; corresponding to aa 805–819 and aa 809–823 of
230 gp160), the two most immunogenic HIV-derived peptides, and then examined by ICS
231 [2]. The percentage of HIV-1 Env-specific IFN- γ -secreting CD107a⁺CD8⁺ T cells
232 was then calculated. A representative gating strategy is shown in Fig. 2B. Vaccination
233 with m8Δ-pSFJ-JRCSFenv and m8Δ-p7.5-JRCSFenv elicited HIV-1JR-CSF Env-
234 specific CTL responses. Mice primed with m8Δ-p7.5-JRCSFenv showed higher levels
235 of HIV-1 Env-specific IFN γ ⁺CD107a⁺CD8⁺ T cells than mice primed with m8Δ-
236 pSFJ-JRCSFenv (Fig. 2C; 12.8 ± 1.2% vs. 7.8 ±2.1%; p=0.002). The proportion of
237 IFN γ ⁺CD107a⁺CD8⁺ T cells in both groups somewhat declined at 8 weeks post-
238 boost; however, the difference between the groups was maintained (p=0.016). We
239 next measured the levels of Env-specific Abs (Fig. 3A) and anti-HIV-1-neutralizing
240 Abs (Fig. 3B) in mice sera. The levels of anti-HIV-1 Env-specific IgG were 6–7-fold
241 higher in mice immunized with m8Δ-pSFJ-JRCSFenv than in mice immunized with
242 m8Δ-p7.5-JRCSFenv; this was in sharp contrast to the observed cellular responses
243 (Fig. 2). The difference of humoral immunity had already been detected 6 weeks after
244 m8Δ prime (supplementary data). [The uneven of sample numbers between two](#)
245 [groups did not introduce bias into the data, since we obtained the same results when](#)
246 [two groups have the same number of animals \(data not shown\)](#). Sera from both groups
247 of mice showed neutralizing activity against a tier 1 pseudotyped HIV-1 strain, SF162,
248 but only after rSeV boost, and no neutralization activity against tier 2 HIV-1s had
249 been detected. At both 2 and 8 weeks post-SeV-JRCSFenv boost, the neutralizing
250 competency of sera from mice immunized with m8Δ-pSFJ-JRCSFenv was marginally
251 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*

260

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 10⁴ and 10⁵ 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 , <10³ PFU; m8 Δ -p7.5-JRCSFenv, 5.5 ×
268 10³ PFU; m8 Δ -pSFJ-JRCSFenv, 1.4 × 10⁵ PFU; and m8 Δ VNC110, 5.75 × 10⁵ 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.

271

272

273 **4. Discussion**

274

275 An effective HIV-1 vaccine should induce long-lasting humoral and cellular
276 immunity against HIV-1. A replication-competent VV would be a good candidate for
277 such a vaccine because recombinant VV can induce both antigen-specific CTL and
278 antibody responses. In addition, the process of viral replication may allow the
279 repeated presentation of viral antigens, leading to affinity maturation of both
280 antibodies and T cell receptors. LC16m8Δ-JRCSFenv is a replication-competent
281 vaccinia vector that induces HIV-1 Env-specific cellular and humoral immune
282 responses when used in combination with a Sendai virus vector [2]. Here, we tried to
283 optimize this vaccination regimen by using HIV-1 Env recombinant VV vectors
284 expressed under the control of different promoters. We found that viruses expressed
285 under the control of these different promoters induced different cellular and humoral
286 immune responses. m8Δ-pSFJ-JRCSFenv induced increased production of anti-HIV-1
287 Env-specific Abs when compared with m8Δ-p7.5-JRCSFenv. By contrast, m8Δ-p7.5-
288 JRCSFenv induced the production of more HIV-1 Env-specific IFN- γ -secreting
289 CD107a⁺CD8⁺ T cells. These results suggest that the induction of Env-specific CTL
290 and humoral responses may be dependent upon different presentation pathways and/or
291 different structures of the Env protein. The peptides used to stimulate the splenocytes
292 in the ICS assay correspond to the 3' domain of gp41, since previous mapping of the
293 consensus subtype B Env peptide pool identified peptides comprising aa 805–819 and
294 aa 809–823 as the best immunogens [2]. It also indicated that gp41 but not gp120 is a
295 more potent inducer of cellular immunity when liberated from gp160 than it is when
296 buried in gp160. The amount of gp41 in m8Δ-p7.5-JRCSFenv-infected 293T and
297 L929 cells are comparable with that infected with m8Δ-pSFJ-JRCSFenv and even
298 more in m8Δ-p7.5-JRCSFenv-infected RK13 cells, which is different from the case of
299 gp160/gp120 (Fig 1B). Considering the better replication of m8Δ-p7.5-JRCSFenv

300 (Fig 1C), we may expect that repetitive antigenic stimulation, which favors CTL
301 induction, strengthens the immunogenicity of gp41 that is derived from m8 Δ -p7.5-
302 JRCSFenv to induce production of Env-specific IFN- γ ⁺CD107a⁺CD8⁺ T cells. The
303 relative lower ratio of gp41 to gp160/gp120 in m8 Δ -pSFJ-JRCSFenv-infected cells
304 than m8 Δ -p7.5-JRCSFenv cells indicates that the cleavage of gp160 to gp120 and
305 gp41 was less efficient due to overexpression of Env.

306 On the other hand, the efficient production of anti-Env-specific antibodies may
307 require higher expression of HIV-1 Env in primarily-infected cells. The 6–7 fold
308 higher level of the HIV-1 Env binding antibody titre observed in mice immunized
309 with m8 Δ -pSFJ-JRCSFenv is consistent with the higher levels of Env observed in
310 m8 Δ -pSFJ-JRCSFenv-infected cells. The Gp120/160 isolated from m8 Δ -pSFJ-
311 JRCSFenv-infected cells migrated more quickly and showed a broader band in PAGE
312 gels than that from m8 Δ -p7.5-JRCSFenv-infected cells. This suggests the incomplete
313 glycosylation of gp120/160 due to an insufficiency of host glycosyltransferases.

314 Nevertheless, the impact on the ability of m8 Δ -pSFJ-JRCSFenv to elicit anti-Env
315 antibody response was minimal. Although m8 Δ -pSFJ-JRCSFenv induced greater
316 production of Env binding Abs than m8 Δ -p7.5-JRCSFenv, it did not induce the
317 production of more potent anti-HIV-1 neutralizing Abs. At 8 weeks after rSeV-
318 JRCSFenv boost, the average ID50 of serum from mice immunized with m8 Δ -pSFJ-
319 JRCSFenv was higher than that of serum from mice immunized with m8 Δ -p7.5-
320 JRCSFenv; however, the difference was not significant. This suggests that, in addition
321 to the amount of expressed Env, the properties of the antigen (for example, the
322 structure of the exposed epitopes) may also be important for the induction of
323 neutralizing antibody production. There was no difference in the avidity of the anti-
324 Env antibodies between the two groups (Fig 3C), implicating that the process of

325 affinity maturation was similar. This suggests that affinity maturation of antibodies is
326 necessary, but not sufficient to induce the production of potent neutralizing antibodies.
327 Even so, higher levels of Env binding antibodies may enable the induction of other
328 types of antiviral immunity, such as antibody-dependent cellular cytotoxicity and
329 antibody-dependent cell-mediated virus inhibition.

330 We recently showed that priming mice with an m8 Δ that expresses both CD40Lm and
331 Env induces the production of high-avidity anti-Env antibodies [2]. The above results
332 suggest that it might be important to incorporate an adjuvant, such as CD40Lm,
333 within the AIDS vaccine regimen to induce more potent humoral responses and
334 produce higher levels of neutralizing antibodies.

335 A successful AIDS vaccine should induce the production of long-lasting antibodies.
336 Both m8 Δ -pSFJ-JRCSFenv and m8 Δ -p7.5-JRCSFenv induced the production of long-
337 lasting anti-Env antibodies when used in the rm8 Δ s prime/rSeV boost regimen.
338 Immunized mice maintained high levels of anti-Env antibodies for up to 28 weeks
339 (Fig 3D). This supports our previous report showing that the rm8 Δ s prime/rSeV
340 regimen is a good platform for the development of an HIV-1 vaccine.

341 Safety is critical when evaluating vaccines in clinical trials. Both m8 Δ -pSFJ-
342 JRCSFenv and m8 Δ -p7.5-JRCSFenv were less virulent in new-born mice than the
343 parental strain, LC16m8 Δ . LC16m8 Δ was more virulent probably because it contains
344 an intact HA gene. The LD50 of m8 Δ -p7.5-JRCSFenv was significantly lower than
345 that of m8 Δ VNC110, although their growth potential was similar. This suggests that
346 the expression of HIV-1 Env in the mouse brain is harmful. This is supported by the
347 fact that that virulence of m8 Δ -pSFJ-JRCSFenv is similar to that of m8 Δ VNC110,
348 despite having a much lower capacity for replication. Nevertheless, our finding that

349 recombinant VVs expressing HIV-1 env are safer than LC16m8 Δ suggests that they
350 may be promising candidates for clinical trials.

351 In conclusion, the results of the present study suggest that VV m8 Δ vectors containing
352 different promoters activate different arms of the immune system. That said, both
353 strains induced long-lasting CTL and antibody responses and both appear safe enough
354 for clinical trials. Thus, it is possible to manipulate the immune response induced by a
355 rational AIDS vaccine by using VV m8 Δ s harboring different promoters.

356

357

358

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360

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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

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

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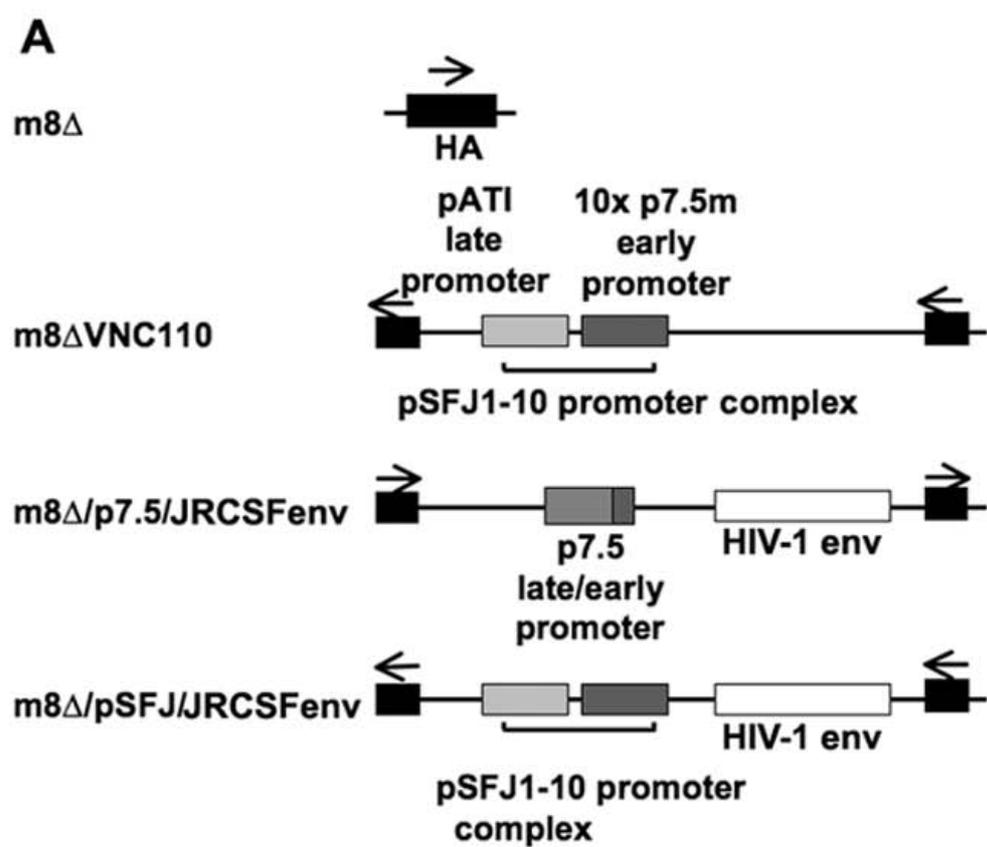
478 **Fig 3. m8Δ-pSFJ-JRCSFenv induces stronger humoral immune responses than**
479 **m8Δ-p7.5-JRCSFenv (A).** Comparison of Env-specific antibody levels. Serum from
480 individual immunized mice was analyzed in a HIV-1JR-CSF gp160 ELISA as
481 described previously (Ref.2). The plates were developed with an HRP-conjugated
482 anti-mouse IgG antibody. The Env-specific antibody titer was determined by
483 subtracting the background values at OD450. Data represent the mean ± SD of the
484 Env-specific antibody titer of all animals in each group. Env binding antibody titers
485 measured at 2 and 8 weeks post-rSeV boost are shown. (B). Comparison of anti-HIV-
486 1 neutralizing antibody activity in sera from the two groups of immunized mice. We
487 included more previously accumulated mice samples that subjected to the same
488 immunization procedure as group 1 in Fig 2. The 50% inhibitory dose (ID50) against
489 an HIV-1 SF162 env-pseudotyped virus was measured using TZM-bl cells. The
490 neutralizing activity of mouse sera is shown at 2 and 8 weeks post-SeV boost. (C).
491 Comparison of the avidity of HIV-1 Env-specific anti-sera from the two groups at 2
492 and 8 weeks post-SeV boost as described previously (Ref. 2). (D). Comparison of
493 HIV-1 Env-specific antibody induction dynamics between the two groups after the
494 rSeV boost.

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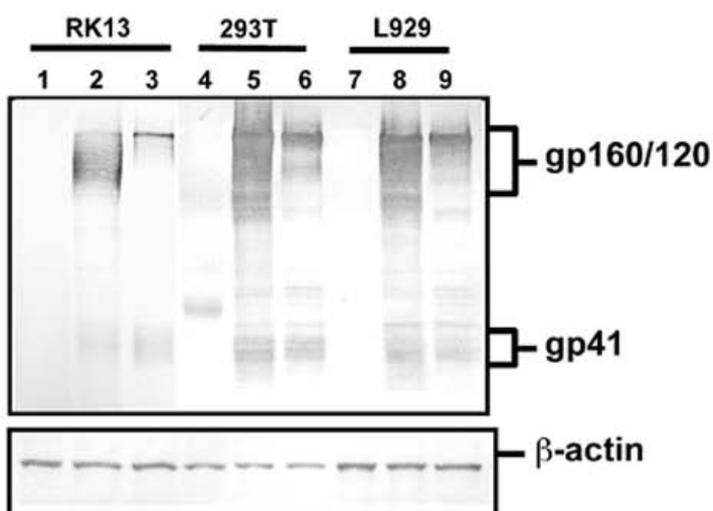
496 **Fig 4. m8Δ-pSFJ-JRCSFenv is safer than m8Δ-p7.5-JRCSFenv *in vivo*.**

497 Cumulative survival curves for vaccinated suckling mice are shown. Mice were
498 injected with LC16m8Δ (A), m8ΔVNC110 (B), m8Δ-pSFJ-JRCSFenv (C), or m8Δ-

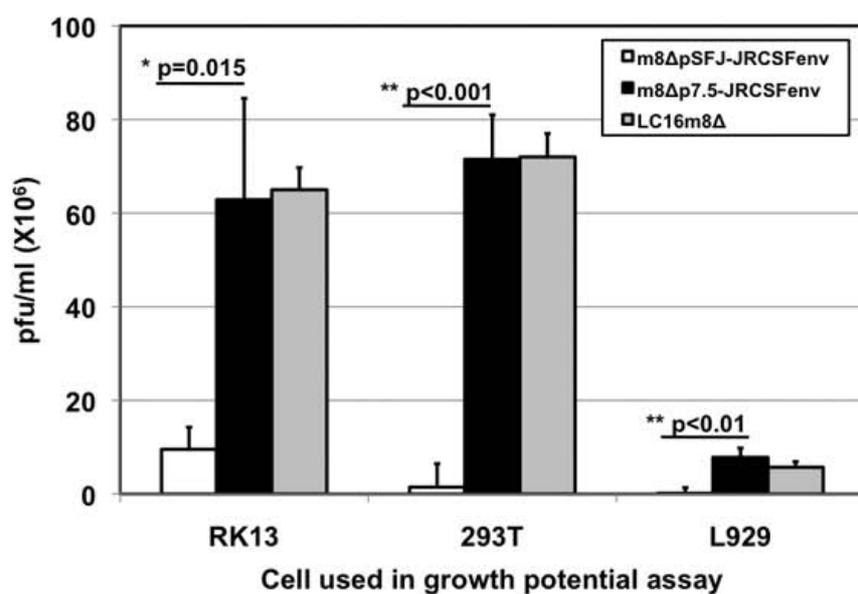
499 p7.5-JRCSFenv (D). Percentage of survival at 2 weeks post inoculation was plotted to
500 make a survive curve and the 50% lethal dose (LD50) was calculated according to the
501 Trendline of the curve. The LD50 for each VV is shown (E). The numbers of the mice
502 used for each dose are indicated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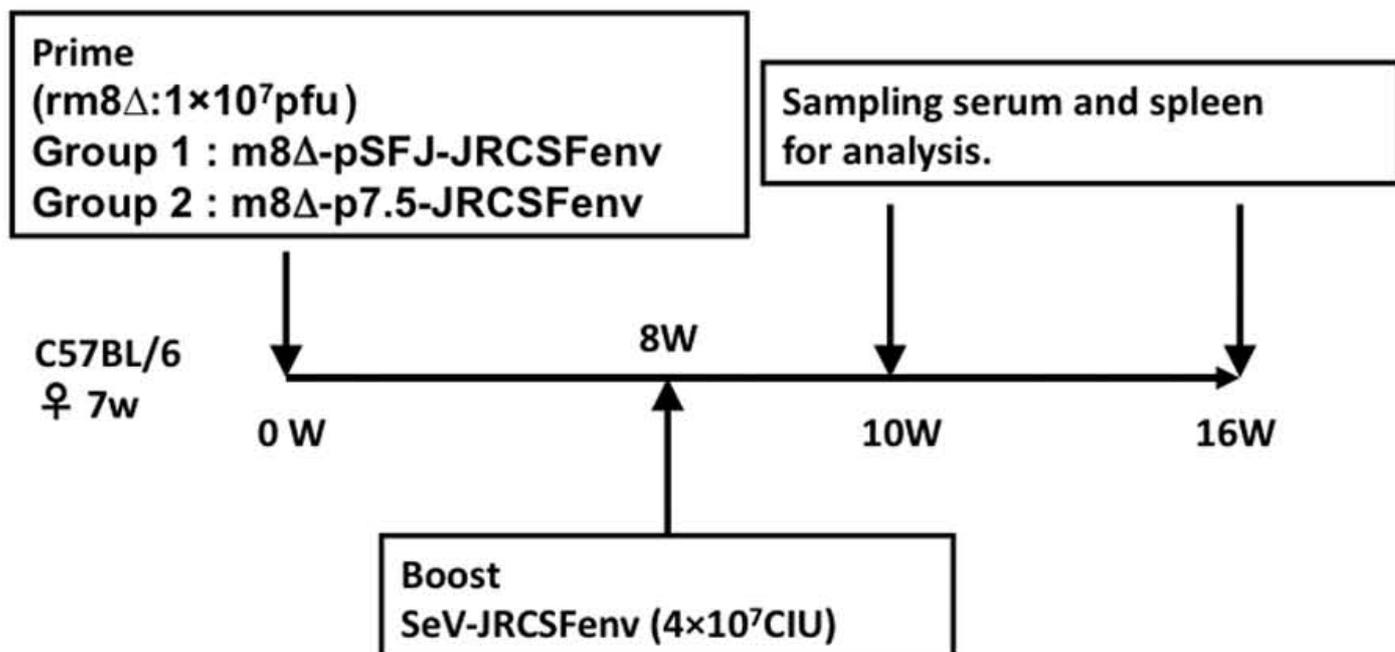
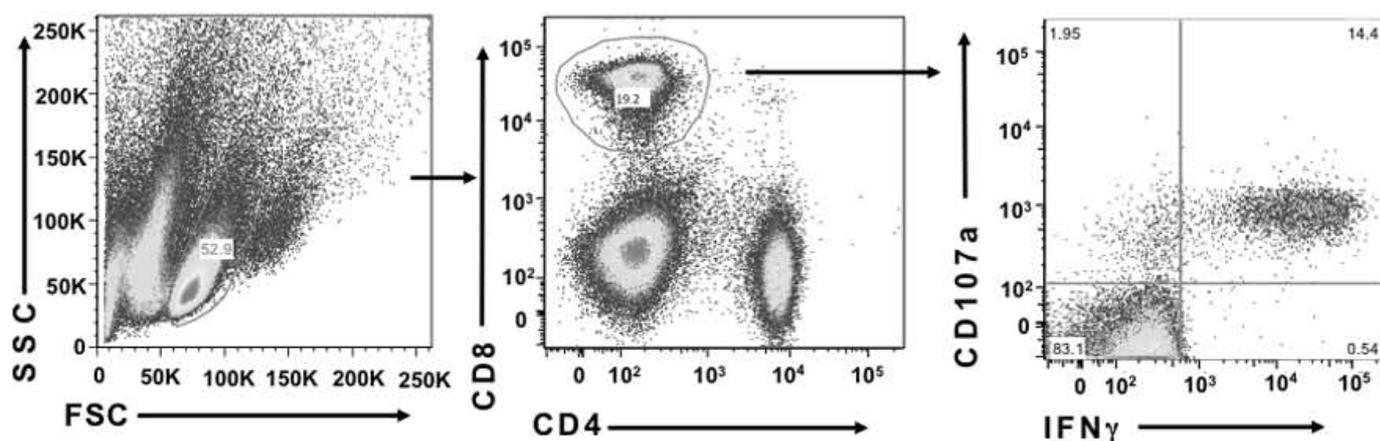
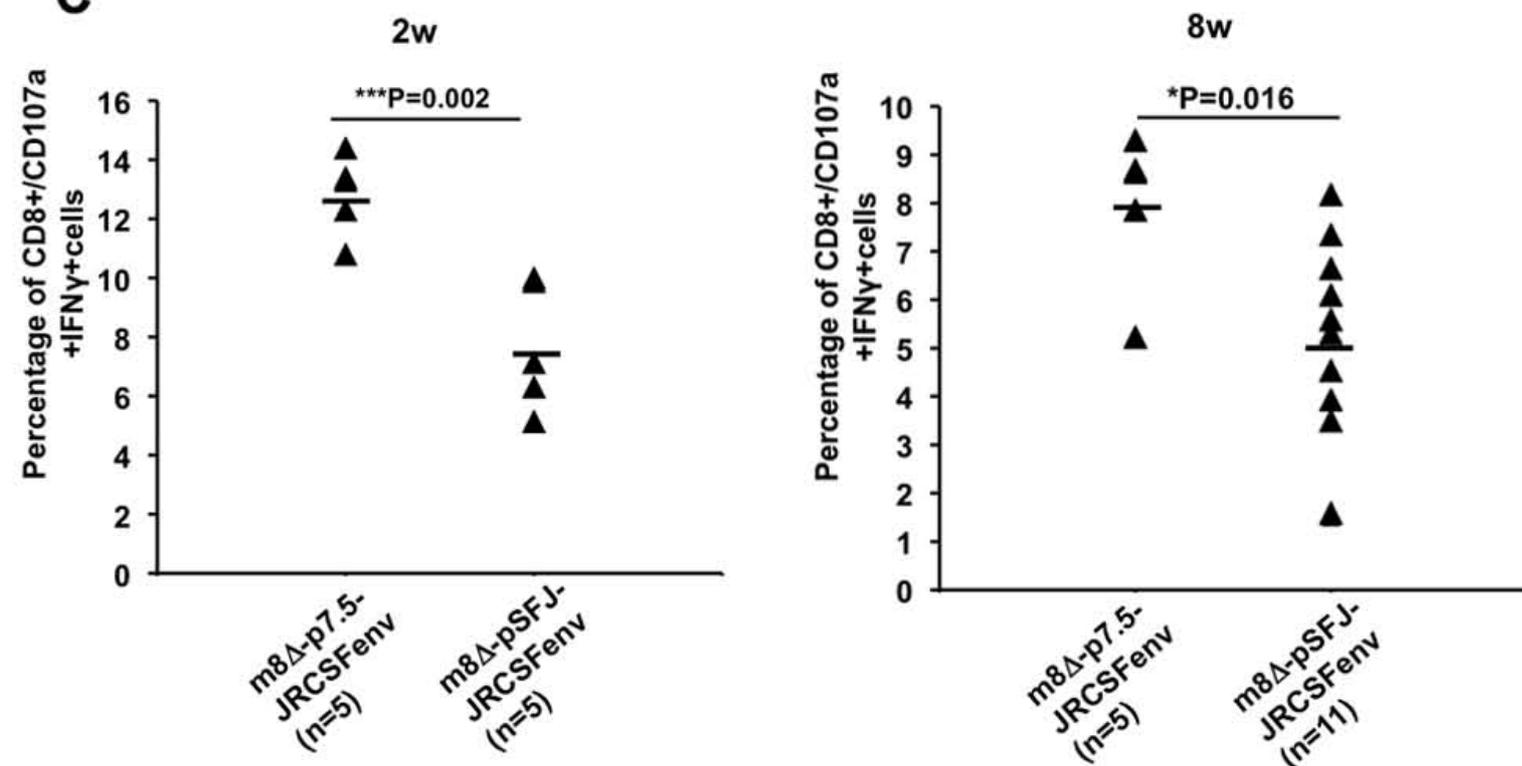


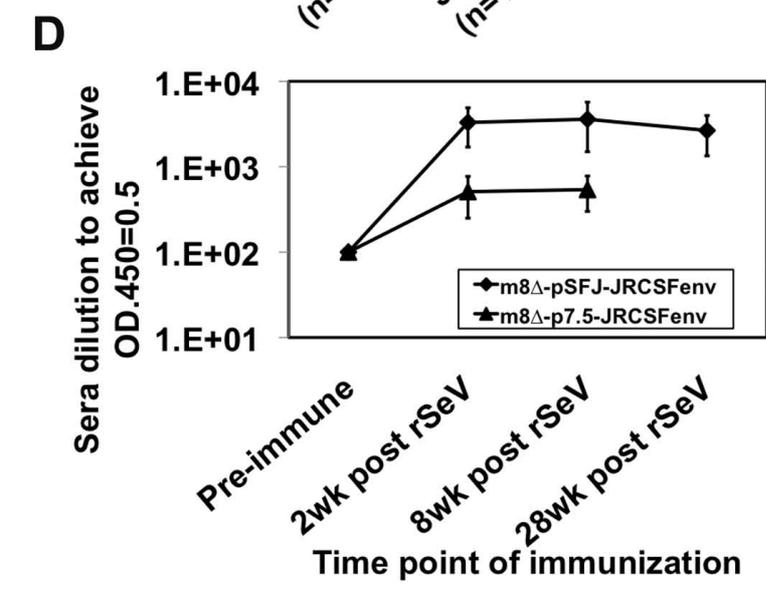
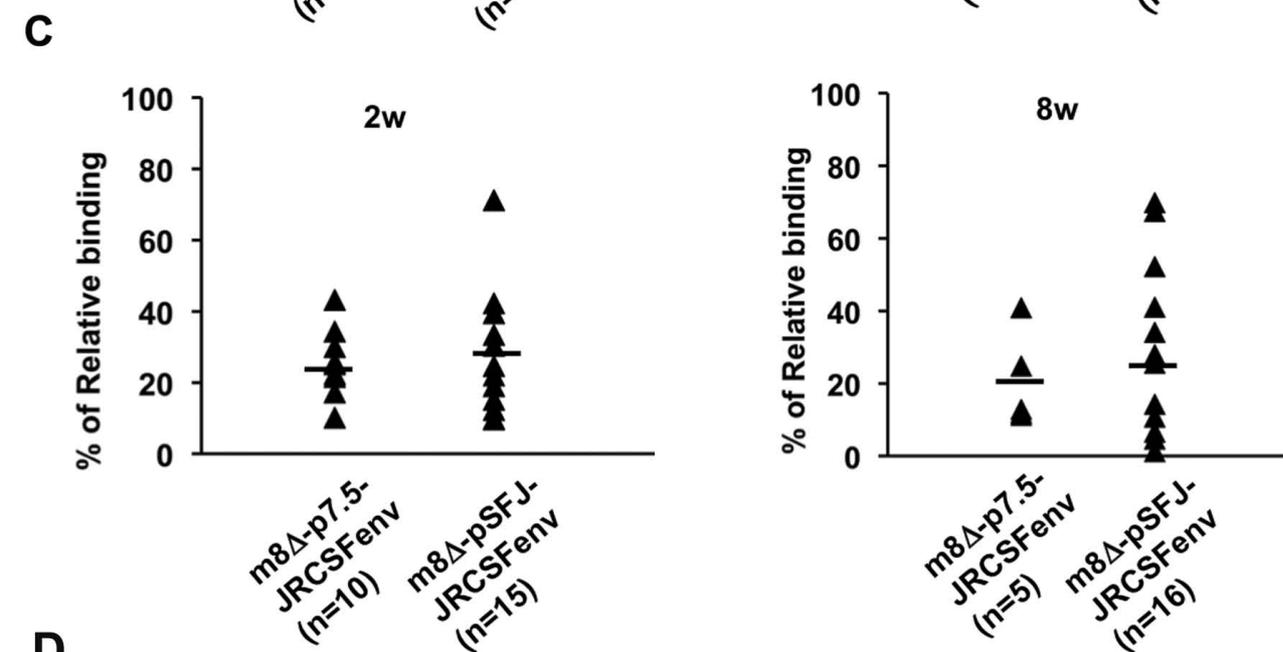
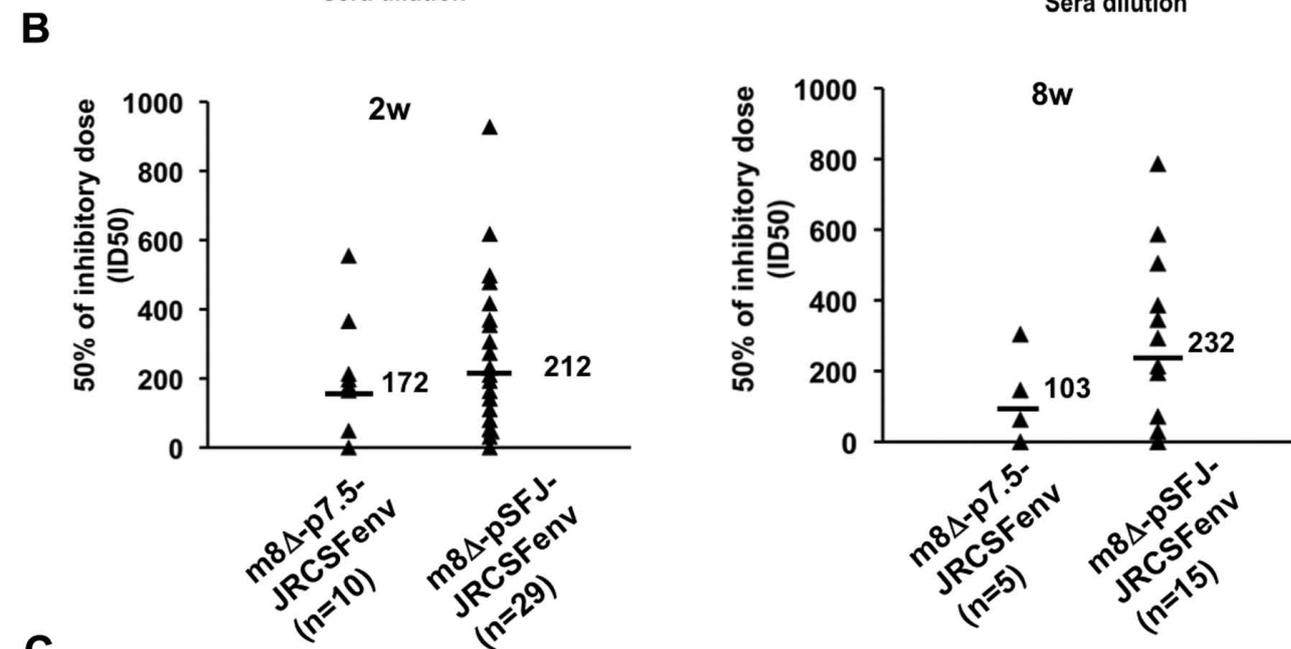
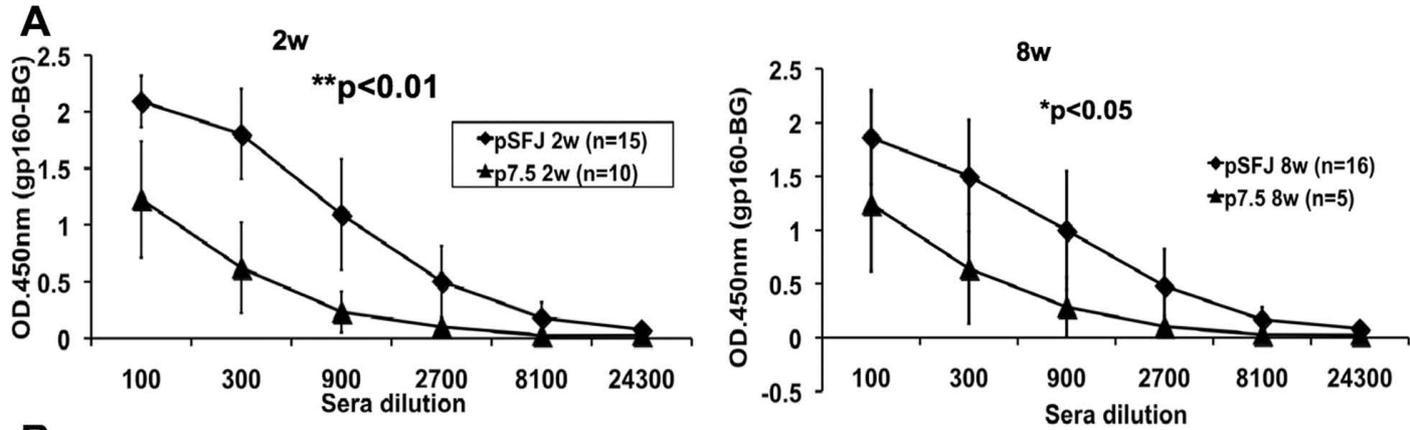
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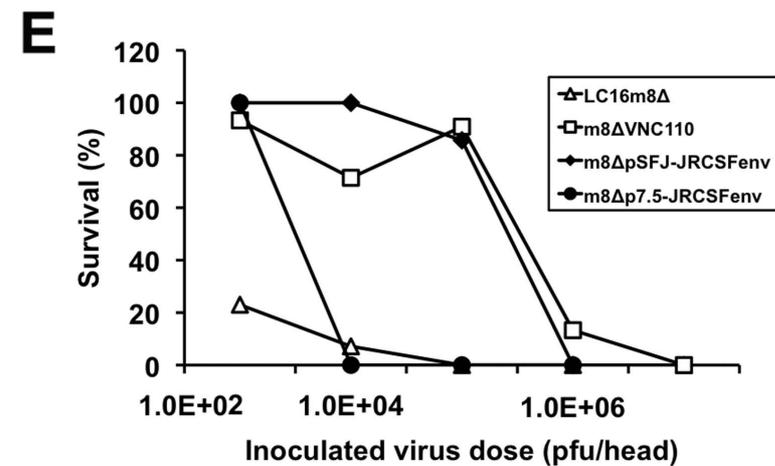
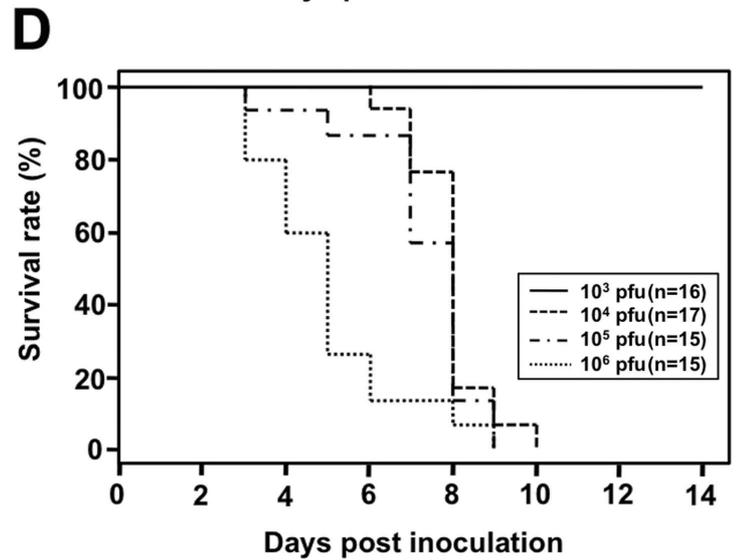
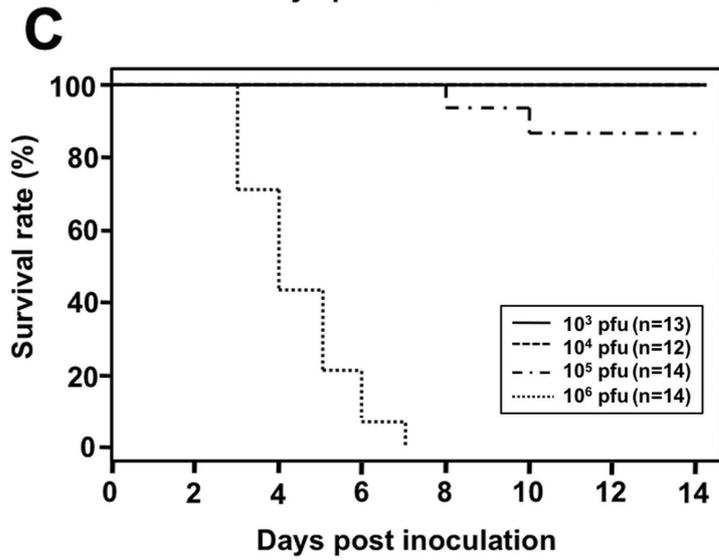
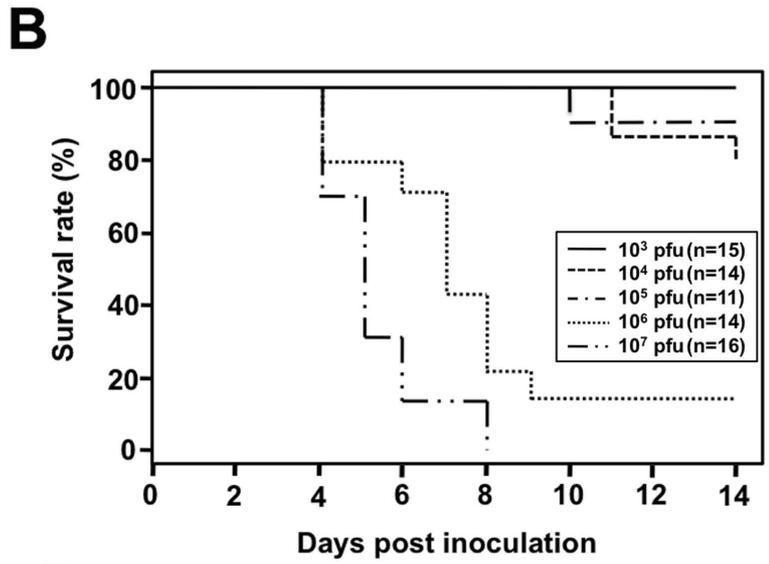
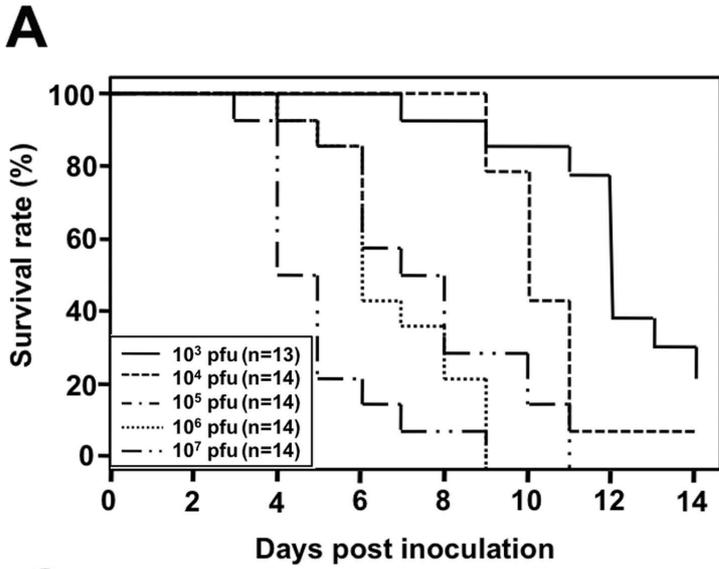


C



A**B****C**





	LD50(pfu/head)
m8Δ	<10 ³
m8ΔVNC110	5.75x10 ⁵
m8ΔpSFJ-JRCSFenv	1.4X10 ⁵
m8Δp7.5-JRCSFenv	5.5X10 ³