

32 **Abstract**

33 Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne disease
34 caused by SFTS virus (SFTSV) infection. Despite a gradual increase of SFTS cases and high
35 mortality in endemic regions, no specific viral therapy nor vaccine is available. Here, we
36 developed a single recombinant plasmid DNA encoding SFTSV genes, Gn and Gc together
37 with NP-NS fusion antigen, as a vaccine candidate. The viral antigens were fused with Fms-
38 like tyrosine kinase-3 ligand (Flt3L) and IL-12 gene was incorporated into the plasmid to
39 enhance cell-mediated immunity. Vaccination with the DNA provides complete protection of
40 IFNAR KO mice upon lethal SFTSV challenge, whereas immunization with a plasmid
41 without IL-12 gene resulted in partial protection. Since we failed to detect antibodies against
42 surface glycoproteins, Gn and Gc, in the immunized mice, antigen-specific cellular immunity,
43 as confirmed by enhanced antigen-specific T cell responses, might play major role in
44 protection. Finally, we evaluated the degree of protective immunity provided by protein
45 immunization of the individual glycoprotein, Gn or Gc. Although both protein antigens
46 induced a significant level of neutralizing activity against SFTSV, Gn vaccination resulted in
47 relatively higher neutralizing activity and better protection than Gc vaccination. However,
48 both antigens failed to provide complete protection. Given that DNA vaccines have failed to
49 induce sufficient immunogenicity in human trials when compared to protein vaccines,
50 optimal combinations of DNA and protein elements, proper selection of target antigens, and
51 incorporation of efficient adjuvant, need to be further investigated for SFTSV vaccine
52 development.

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54 **Keywords:** Severe fever with thrombocytopenia syndrome, DNA vaccine, T cells, protective
55 immunity

56 **Author summary**

57 Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne infection
58 endemic to East Asia including China, Korea, and Japan. Gradual rise of disease incidence
59 and relatively high mortality have become a serious public health problem in the endemic
60 countries. In this study, we developed a recombinant plasmid DNA encoding four antigens,
61 Gn, Gc, NP, and NS, of SFTS virus (SFTSV) as a vaccine candidate. In order to enhance cell-
62 mediated immunity, the viral antigens were fused with Flt3L and IL-2 gene was incorporated
63 into the plasmid. Immunization with the DNA vaccine provides complete protection against
64 lethal SFTSV infection in IFNAR KO mice. Antigen-specific T cell responses might play a
65 major role in the protection since we observed enhanced T cell responses specific to the viral
66 antigens but failed to detect neutralizing antibody in the immunized mice. When we
67 immunized with either viral glycoprotein, Gn protein induced relatively higher neutralizing
68 activity and better protection against SFTSV infection than Gc antigen, but neither generated
69 complete protection. Therefore, an optimal combination of DNA and protein elements, as
70 well as proper selection of target antigens, might be required to produce an effective SFTSV
71 vaccine.

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80 Introduction

81 Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne infectious
82 disease caused by SFTS virus (SFTSV), belonging to the *Phenuiviridae* family of
83 *Bunyavirales* [1, 2]. The genome of SFTSV is composed of three segmented RNAs: large (L)
84 segment encoding RNA-dependent RNA polymerase (RdRp), medium (M) encoding the
85 envelop glycoproteins, Gn/Gc, and small (S) encoding the nucleocapsid and nonstructural
86 proteins (NP and NS) [1]. Clinical manifestations include fever, gastrointestinal symptoms,
87 leukocytopenia, and thrombocytopenia [3, 4]. Disease mortality of SFTS patients has been
88 estimated to be 5 ~ 20% [3]. Even though the majority of SFTS cases has been reported from
89 China [3], Korea [4], and Japan [5], SFTSV infections in southern Asia, including Vietnam,
90 have been recently reported in a retrospective survey [6]. Currently, no specific viral therapy
91 nor vaccine is available. An effective vaccine is needed to combat its relatively high mortality,
92 especially in elderly patients, and spread of SFTSV between humans [7, 8].

93 Vaccine development for SFTS is at an early discovery phase and there have only been a few
94 studies on vaccine candidates using animal infection models [8-11]. Immunization of NS
95 antigen with Freund's adjuvant in C57BL/6 mice, which are naturally resistant to SFTSV but
96 partially mimic human infections [12], failed to enhance viral clearance, although it induced
97 high titer of anti-NS antibodies and significantly elevated IFN- γ levels in sera upon viral
98 challenge [9]. Vaccination of plasmid DNAs encoding NP and NS peptides also enhanced
99 antigen-specific cellular immunity of T cells, such as IFN- γ and TNF- α secreting CD4⁺ and
100 CD8⁺ T cells, in BALB/c mice when applied by nano-patterned microneedles [10]. However,
101 the protective effect of cellular immunity induced by DNA vaccination was not confirmed by
102 *in vivo* infection with SFTSV. Recently, Dong F. *et al.* reported that a single dose of live

103 attenuated recombinant vesicular stomatitis virus (rVSV) vaccine expressing the SFTSV
104 Gn/Gc glycoproteins elicited high titers of protective neutralizing antibodies in both wild type
105 and interferon α/β receptor knockout (IFNAR KO) mice [11]. They clearly showed that a
106 single dose rVSV carrying SFTSV Gn/Gc could provide complete protection against lethal
107 challenge with SFTSV in young and old IFNAR KO mice, a promising infection model for
108 severe human infection of SFTSV [11, 13, 14]. Nevertheless, the potential role of cellular
109 immunity against the viral antigens in complete protection was not examined in this study,
110 although adoptive transfer of immune sera from mice immunized with rVSV-Gn/Gc to naïve
111 IFNAR KO mice provide partial (~ 60%) protection against lethal SFTSV challenge [11].
112 In this study, we developed a recombinant plasmid DNA (pSFTSV) encoding extracellular
113 domains of Gn and Gc, and NP-NS fusion antigen as a DNA vaccine candidate. We
114 examined whether it could provide protective immunity against lethal SFTSV infection in
115 IFNAR KO mice. In order to facilitate the processing and presentation of the SFTSV antigens
116 by dendritic cells (DCs) and enhance antigen-specific T cell responses, these recombinant
117 antigens were fused with Fms-like tyrosine kinase-3 ligand (Flt3L) [15, 16]. Moreover, we
118 generated a recombinant DNA encoding IL-12 α and β in addition to the recombinant viral
119 antigens (pSFTSV-IL12) to further enhance cell-mediated immunity [17]. Vaccination of
120 pSFTSV-IL12 provided complete protection of IFNAR KO mice upon lethal SFTSV
121 challenge, whereas immunization with pSFTSV elicits only partial protection, indicating that
122 antigen-specific cellular immune responses enhanced by co-expression of IL-12 could play a
123 significant role in protection against lethal SFTSV infection. We confirmed significantly
124 higher levels of Gn and NP-specific CD4⁺ and CD8⁺ T cell responses in mice vaccinated with
125 pSFTSV-IL12 when compared to those in mock vector-immunized mice. Therefore, our
126 results indicate that enhanced antigen-specific T cell immunity against multiple SFTSV

127 antigens by DNA vaccination could be a promising direction for developing an effective
128 vaccine against SFTSV infection.

129

130 **Methods**

131 **Ethics statement**

132 Animal experiments were conducted in an Animal Biosafety Level 3 facility at Seoul
133 National University Hospital and International Vaccine Institute. This study was approved by
134 the Seoul National University Hospital and International Vaccine Institute Institutional
135 Animal Care and Use Committee (SNUH IACUC No. 15-0095-C1A0 and IVI IACUC No.
136 2018-018) and conducted in strict accordance with the recommendations in the National
137 Guideline for the care and use of laboratory animals.

138

139 **Preparation of SFTSV DNA plasmid**

140 In order to generate plasmids for DNA vaccination, genes encoding ectodomains of Gn, Gc
141 (from Genebank accession no. AJO16082.1), and NP/NS fusion protein (from Genebank
142 accession no. AJO16088.1 and AKI34298.1, respectively) were synthesized (GenScript,
143 Piscataway, NJ, USA) and cloned into pGX27 vector (Genexine, Seongnam, Republic of
144 Korea). All these genes were fused with signal peptide of tissue Plasminogen Activator (tPA,
145 Uniport no. P00750) and Flt3L (Uniport no. P49771) in their N-terminus [15] (pSFTSV, [Fig](#)
146 [1](#)). In addition, murine IL-12 α and β genes (Uniport no. P43432) [17] were also synthesized
147 (GenScript) and cloned into pSFTSV (pSFTSV-IL-12, [Fig 1](#)).

148

149 **Gene expression of plasmid DNAs**

150 To confirm the expression of cloned genes in pSFTSV and pSFTSV-IL12, HEK 293T cells

151 (ATCC CRL-1573, Manassas, VA, USA) were transfected with pGX27, pSFTSV, or
152 pSFTSV-IL12 plasmid using the polyethylenimine (PEI) transfection method [18]. Briefly,
153 plasmids and PEI were diluted with Opti-MEM media (Gibco, Gaithersburg, MD, USA) and
154 incubated with HEK 293T cells. After 4 h of incubation, media were changed with
155 Dulbecco's Modified Eagle medium (DMEM) (Gibco) containing 10% fetal bovine serum
156 (FBS) and incubated in humidified CO₂ atmosphere at 37°C. After 48 h of culture, cells and
157 culture supernatants were harvested. Cells were washed with phosphate-buffered saline (PBS)
158 and lysed with NP-40 lysis buffer (1% NP-40 in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl)
159 containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Lysates and
160 supernatants were stored at -80°C until use.

161 For quantification of Flt3L-fused proteins and mouse IL-12 in the supernatants of transfected
162 HEK293T cells, human Flt3L and mouse IL-12p70 Quantikine ELISA kits (R&D systems,
163 Minneapolis, MN, USA) were used according to manufacturer's instructions. Expression of
164 Gc, Gn, and NP/NS fusion protein in the culture supernatants and cell lysates were examined
165 by immunoblotting using rabbit anti-Gn, Gc (NBP2-41153 and NBP2-41156, NOVUS
166 Biologicals, Centennial, Colorado, USA), and NP (produced by custom polyclonal antibody
167 production service via Abclon, Seoul, Republic of Korea) antibodies, respectively. Each
168 sample was separated on 8% polyacrylamide gels and transferred onto 0.45 µm PVDF
169 membranes (Merck, Darmstadt, Germany). PVDF membranes were blocked with PBS
170 containing 0.05% Tween 20 (PBST) and 5% skim milk at room temperature for 2 h. The
171 membranes were sequentially incubated with primary and secondary antibodies conjugated
172 with horse radish peroxidase (HRP, Invitrogen, Waltham, MA, USA). The membranes were
173 then visualized using a LAS-4000 system (Fujifilm, Tokyo, Japan).

174

175 **Preparation of recombinant proteins**

176 The gene encoding the NP of the KASJH strain (Genbank Accession No. KP663733) was
177 cloned into the pET28a (+) vector (Novagen, Gibbstown, NJ, USA). NP protein was then
178 purified from *E. coli* strain BL21 (DE3) harboring the recombinant plasmid. Following
179 induction with 0.1 mM isopropyl β -D-thiogalactoside (IPTG) for 18 h at 16°C, the protein
180 was purified using HisTrap HP histidine-tagged protein columns (GE healthcare, Chicago, IL,
181 USA) according to the manufacturer's instruction. Recombinant Gn and Gc glycoproteins
182 fused to Fc region of human immunoglobulin heavy chain were purified as previously
183 described [19]. Briefly, vectors cloned with gene encoding Gn or Gc were transfected into
184 HEK293F cells (Thermo Fisher Scientific, Waltham, MA, USA) using polyethylenimine. The
185 transfected cells were cultured in FreeStyle™ 293 expression medium (Gibco) for 6 days.
186 Overexpressed recombinant proteins in supernatants were purified by AKTA start affinity
187 chromatography system HiTrap Mabselect (GE Healthcare) according to the manufacturer's
188 instructions (S1 Fig).

189

190 **Enzyme-linked immunosorbent assays (ELISA)**

191 To determine the antibody titers specific to Gc, Gn, and NP in sera of immunized mice,
192 immunoassay plates (96-well plates; Nunc, Rochester, NY, USA) were coated with 100
193 ng/well of purified antigens at 4°C overnight. His-tagged Gn and Gc recombinant proteins
194 were purchased from Immune Technology Co. (New York, NY, USA). His-tagged NP
195 recombinant protein was purified as mentioned above. After antigen coating, the
196 immunoassay plates were blocked for 2 h at room temperature with PBST containing 5%
197 skim milk. 100 μ l of serially-diluted serum samples were incubated for 1 h at room
198 temperature and subsequently detected using HRP-conjugated goat anti-mouse IgG (Santa

199 Cruz Biotechnology, Santa Cruz, CA, USA). 3,3',5,5'-tetramethylbenzidine peroxidase
200 substrate solution (KPL, Gaithersburg, MD, USA) was then added to develop color for 7 min,
201 and the reaction was stopped by the addition of 1 M H₃PO₄ solution. Absorbance was
202 measured at 450 nm using a microplate reader (TECAN, Mannedorf, Switzerland).

203

204 **Flow cytometric analysis**

205 Spleen cells were released into RPMI 1640 media (Gibco) by mincing the spleen through a
206 70 µm cell strainer (BD Biosciences, San Jose, CA, USA). After lysis of red blood cell with a
207 Red Blood Cell Lysing Buffer Hybri-Max™ (Sigma, St. Louis, MO, USA), splenocytes were
208 cultured for 18 h in RPMI media containing 10% FBS (Gibco) and 1%
209 penicillin/streptomycin (Gibco) in the presence of 10 µg of purified Gn or NP antigens in 96
210 well V-bottomed culture plates (Nunc, Roskilde, Denmark). For intracellular detection of
211 IFN-γ, splenocytes (2 × 10⁶ cells/well) were treated with 1 µg of Golgiplug (BD Bioscience)
212 for the final 6 h. Cells were then blocked with ultra-block solution (10% rat sera, 10%
213 hamster sera, 10% mouse sera (Sigma), and 10 µg/ml of anti-CD16/32 (2.4G2) (BD
214 Pharmingen, Franklin Lakes, NJ, USA), followed by staining with anti-CD3 (145-2c11) (BD
215 Biosciences), CD4 (RM4-59) (BD Biosciences) and CD8 (53-6.7) (Biolegend, San Diego,
216 CA, USA) antibodies conjugated to different fluorescent dyes. After surface staining,
217 splenocytes were fixed and permeabilized with Cytofix/Cytoperm kit (BD Bioscience) and
218 incubated with anti-IFN-γ antibody (XMG1.2) (BD Pharmingen). The stained cells were
219 analyzed on a CytoFLEX S flow cytometer (Berkman Coulter Inc, Brea, CA, USA). Flow
220 cytometry data were analyzed using FlowJo software version 10.6 (Tree Star, Ashland, OR,
221 USA). Gating strategies for the flow cytometric analyses are summarized in [S2 Fig](#).

222

223 **Preparation of SFTSV**

224 SFTSV (Genbank accession no. MN329148-MN329150) isolated from a Korean SFTS
225 patient was propagated in Vero E6 cells (ATCC CRL-1586). The supernatant of infected cells
226 was harvested at 5 d after infection and stored at - 80°C after filtering with 0.45 µm syringe.
227 Focus-forming unit (FFU) of SFTSV was determined by plaque assay using methylcellulose
228 media [20]. Briefly, the filtered supernatants were serially diluted and added to a monolayer
229 of Vero E6 cells and incubated for 1 h at 37°C. Viral supernatants were removed and cells
230 were incubated under an overlay media (DMEM supplemented with 5% FBS and 1%
231 methylcellulose) at 37°C for 7 days. Cells were fixed with 4% paraformaldehyde (Intron,
232 Seongnam, Republic of Korea) and 100% methanol (Merck, Darmstadt, Germany). The
233 SFTSV foci were detected using rabbit anti-SFTS NP antibody (Abclon) and goat anti-rabbit
234 IgG secondary antibody conjugated with alkaline phosphatase (Thermo Fisher Scientific).
235 Viral plaques were visualized by incubation with NBT/BCIP solution (Roche, Mannheim,
236 Germany).

237

238 **Neutralizing antibody assay**

239 To evaluate the neutralizing activity of immune sera, a focus reduction neutralization titer
240 (FRNT) assay was performed using immunized mice sera [21]. SFTSV (0.0001 multiplicity
241 of infection) was pre-incubated with serially diluted sera from mice at 4°C for 1 h. The
242 mixture of virus and sera was added onto a monolayer of Vero E6 cells in 24-well plate. After
243 incubation for 2 h, supernatant of cells were removed and cells were cultured under an
244 overlay media at 37°C for 7 days. Viral foci were visualized as described above. The
245 percentage of focus reduction was calculated as [(No. of plaques without antibody) – (No. of

246 plaques with antibody)] / (No. of plaques without antibody) x 100. 50% focus reduction
247 neutralization titers (FRNT₅₀) were calculated by a nonlinear regression analysis
248 (log[inhibitor] versus normalized response method) embedded in GraphPad Prism Software
249 v5.01 (GraphPad Software; <https://www.graphpad.com>).

250

251 **Immunization of mice and SFTSV challenge**

252 Six to eight-week-old female Interferon α/β receptor knockout (IFNAR KO, B57BL/6) mice
253 [22] were used for immunization and challenge tests. They were housed and maintained in
254 the specific pathogen-free facility at Seoul National University College of Medicine. Mice
255 were intramuscularly immunized by electroporation using Orbijector EP-I model (SL
256 Vaxigen Inc., Seongnam, Republic Korea) in the hind leg three times at two-week intervals. 4
257 μg of purified pGX27, pSFTSV, or pSFTSV-IL12 in 100 μl of PBS was used for each
258 immunization. Mice were also subcutaneously immunized with 20 μg of Gn-Fc or Gc-Fc
259 protein absorbed in aluminum hydroxychloride (Alhydrogel[®] adjuvant 2%, InvivoGen, Hong
260 Kong). Mice sera were collected from immunized mice at one week after the third
261 immunization to determine the levels of specific antibody titers. Two weeks after the final
262 immunization, mice were subcutaneously challenged with 1×10^5 FFU of SFTSV. Body
263 weight and mice survival were monitored until surviving mice fully recovered. Blood and
264 tissues of mice were collected at the indicated time after viral challenge and applied for viral
265 quantitation using qRT-PCR, or hematological analysis.

266

267 **Quantitative reverse transcript – polymerase chain reaction (qRT-PCR)**

268 Total RNA was extracted from the plasma of SFTSV-infected mice using Trizol LS Reagent
269 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Total

270 RNA was reverse transcribed into cDNA using HiSenScript™ RH (-) RT Premix kit (Intron,
271 Seongnam, Republic of Korea). cDNA was quantified using TaqMan Universal Master Mix 2
272 (Applied Biosystems, Waltham, MA, USA). qRT-PCR was performed on BioRad CFX
273 connect real-time system (Bio-Rad, Hercules, CA, USA) under following conditions: uracil-
274 N-glycosylase incubation at 50°C for 2 min, polymerase activation at 95°C for 10 min,
275 denaturation at 95°C for 15 s, annealing and extension at 53°C for 1 min. Amplification was
276 performed for 45 cycles and the fluorogenic signal was measured during annealing/extension
277 step. Primer set and detecting probe for qRT-PCR was derived from the NP gene of SFTSV:
278 NP forward (5'-CCTTCAGGTCATGACAGCTGG-3'), NP reverse (5'-
279 ACCAGGCTCTCAATCACTCCTGT-3') and detecting probe (5'-6FAM-
280 AGCACATGTCCAAGTGGGAAGGCTCTG-BHQ1-3'). Copy numbers were calculated as
281 a ratio with respect to the standard control.

282

283 **Hematology**

284 To collect hematological data, animals were euthanized and bled by cardiac puncture. Blood
285 was prepared in tubes coated with 0.5 M EDTA (Enzymomics, Daejeon, Republic of Korea).
286 Prior to evaluation of platelet counts, 4% paraformaldehyde was added to EDTA-
287 anticoagulated whole blood samples at a 1:1 ratio to inactivate virus [23]. The platelet counts
288 were analyzed using ADVIA 2012i Hematology System (Siemens Healthineers, Erlangen,
289 Germany).

290

291 **Statistical analysis**

292 Data was analyzed using the Graph Pad Prism 5.01 software (GraphPad Software, La Jolla,
293 CA, USA). Statistical analysis was performed using two-tailed Student's *t*-test with 95%
294 confidence interval or one-way analysis of variance (ANOVA) followed by Newman-Keuls
295 *t*-test for comparisons of values among different groups. Data are expressed as the mean \pm
296 standard deviation (S.D.). Statistical analysis on survival rates were performed using the
297 Mantel-Cox Log Rank test. A *p*-value of < 0.05 was considered statistically significant.

298

299 **Results**

300 **Characterization of SFTSV DNA vaccines and their gene expression**

301 Four viral genes of SFTSV were cloned into pGX27 vector to be expressed in mammalian
302 cells (Fig. 1). Extracellular domains of viral glycoproteins, Gn and Gc, were separately
303 cloned into the plasmid vector under control of CMV promoter and IRES sequence. Full
304 lengths of viral NP and NS genes fused with a linker peptide (GSGSGSGSGSGRA) were
305 also cloned into the vector under control of RSV promoter. All the proteins were fused with
306 the extracellular domain of Flt3L and the signal sequence of tissue plasminogen activator
307 (tPA) in their N-terminus to promote antigen presentation and trafficking of the fusion
308 proteins as previously described [15]. In addition, pSFTSV-IL12 plasmid also encodes
309 murine IL-12 α and β to enhance antigen-specific T cell responses [17]. Expression and
310 secretion of the viral antigens and IL-12 in HEK293 cells transfected with the plasmids were
311 confirmed by ELISA and immunoblot analysis (Fig. 2). All the viral antigens were detected
312 in cell culture supernatants, as well as in cellular lysates (Fig. 2B).

313

314 **Fig 1. Schematic diagrams of SFTSV DNA vaccines used in this study. pSFTSV**

315 was constructed by inserting genes encoding ectodomains of Gn and Gc, and NP-
316 NS fusion protein into the pGX2 vector. The viral antigens are preceded by the
317 secretory signal sequence of tPA and the extracellular domain of Flt3L. pSFTSV-IL-
318 12 includes additional genes encoding murine IL-12 α and β to promote cellular
319 immunity. ColE1, ColE1-type bacterial origin of replication; gIVS, rabbit β -globin
320 intervening sequence; Kan^R, kanamycin resistance gene; IRES, internal ribosome
321 entry site.

322

323 **Fig 2. Characterization of gene expression after SFTSV DNA vaccine**
324 **transfection.** (A) Expression and secretion of the viral antigens and IL-12 in
325 HEK293 cells transfected with the DNAs was confirmed by measuring the
326 concentration of Flt3L and IL-12 in the cell culture supernatants by ELISA. Data are
327 presented as mean + S.D. from triplicated experiments. (B) Expression of the viral
328 antigens, Gn (~ 74 kDa), Gc (~ 71 kDa), and NP-NS fusion (~ 80 kDa) proteins, in
329 cell lysates (left panels) and culture supernatants (right panels) of transfected
330 HEK293 cells was assessed by immunoblot analysis using anti-Gn, Gc, or NP
331 antibodies, respectively. The specific bands of antigens corresponding to their
332 expected sizes are indicated with arrow heads. β -actin was used as loading control.

333

334 **Antibody and T cell responses against the viral antigens in IFNAR KO mice immunized**
335 **with the DNA vaccines**

336 Next, we assessed antigen-specific adaptive immunity against the viral antigens after
337 vaccination in IFNAR KO mice. Antibody responses to NP antigen was significantly elevated

338 (mean titer \pm S.D.: 255 ± 63 , $n = 3$) only in mice immunized with pSFTSV-IL12, and not in
339 mice vaccinated with mock vector or pSFTSV at two weeks after third immunization (Fig.
340 3A). Specific antibodies against Gn and Gc were barely detectable in all the vaccinated
341 groups (data not shown), suggesting that these DNA vaccines may not efficiently induce
342 specific antibodies against Gn or Gc, but produce NP antibody responses in the presence of
343 IL-12 expression.

344 In order to characterize the potential difference in quality of cell-mediated immunity in
345 IFNAR KO mice immunized with the DNA vaccines, we analyzed antigen-specific T cell
346 responses by assessing IFN- γ secreting T cells in an antigen-dependent manner (Fig. 3B and
347 C). Splenocytes collected from immunized mice were stimulated with Gn or NP antigens and
348 cytokine-positive T cell subsets were analyzed by flow cytometry. The frequencies of
349 cytokine-positive CD4 and CD8 T cells induced by splenocytes of pSFTSV-IL12-immunized
350 mice were significantly higher than those of vector-immunized mice. Even though the
351 cellular responses of immune splenocytes from pSFTSV-vaccinated mice were generally
352 increased, the levels were not statistically significant, suggesting a potent role of IL-12 co-
353 expression in enhancing cell-mediated immunity against the viral antigens.

354

355 **Fig 3. Generation of antigen-specific antibodies and T cell responses in IFNAR**
356 **KO mice vaccinated with SFTSV DNA vaccines. (A) Anti-NP IgG response was**
357 **measured by ELISA at two weeks after the third vaccination. The antibody titers of**
358 **anti-NP IgG in sera of mice ($n = 3$) immunized with different DNA vaccines are**
359 **presented. Cut-off titers (dashed line, mean O.D. + 3 x S.D. at 1:100 diluents) was**
360 **determined using sera from vector-immunized mice. Error bar: mean \pm S.D. (B and C)**
361 **Splenocytes were collected from mice at two weeks after the third immunization with**

362 the indicated DNA vaccines. Production of IFN- γ by CD4⁺ T or CD8⁺ T cells were
363 analyzed by flow cytometry after stimulation with the indicated antigens.
364 Representative flow cytometric results are presented (B) and the percentile of
365 cytokine positive cells among CD4⁺ or CD8⁺ T cell subsets are summarized (C).
366 Data shown as mean + S.D. from duplicate assays with three mice per group. *, $p <$
367 0.05; **, $p < 0.01$.

368

369 **Vaccination with pSFTSV-IL12 provides complete protection against lethal infection of** 370 **SFTSV**

371 Since we observed significant elevation of T cell responses specific to the viral antigens in
372 IFNAR KO mice immunized with pSFTSV-IL12 DNA vaccine, we tested whether it could
373 provide protective immunity against lethal SFTSV infection. The susceptibility of IFNAR
374 KO mice to a Korean SFTSV isolate was examined by inoculating the mice with 1×10^1 to
375 1×10^5 FFU (S3 Fig A). Upon infection, all the mice gradually lost body weight and became
376 moribund from the third to fifth day after infection, depending on the infection dose. All the
377 infected mice died at 5 ~ 9 d after infection (S3 Fig A). Similar survival kinetics were
378 previously reported in IFNAR KO mice infected with other SFTSV strains [11, 14],
379 indicating that our Korean SFTSV isolate possesses an equivalent degree of virulence to prior
380 Chinese isolates. The platelet counts in mice infected with 1×10^5 FFU of SFTSV
381 significantly declined up to 4 d after infection, and the mean platelet volumes of the infected
382 mice were significantly higher than those of control animals (S3 Fig B), suggesting that
383 platelet destruction and the activation of platelet production simultaneously occur during
384 lethal infection [14].

385 To assess the protective efficacy of the DNA vaccine, groups of mice were immunized with

386 mock vector, pSFTSV, or pSFTSV-IL12 three times and then subcutaneously challenged
387 with a lethal dose (10^5 FFU/mouse) of SFTSV (Fig. 4). All the mock-immunized mice
388 expired by 5 d after SFTSV infection. In contrast, all the mice immunized with pSFTSV-IL12
389 were protected from lethal viral challenge, and 40% (2 of 5) of mice vaccinated with pSFTSV
390 survived (Fig. 4A). Consistently, pSFTSV-IL12-vaccinated animals lost weight until 4 d after
391 the viral challenge and recovered thereafter, whereas decrease in body weight of pSFTSV-
392 immunized mice was observed up to 8 d after the challenge and the surviving mice gradually
393 recovered. When we examined the progression of thrombocytopenia during the acute phase
394 of infection in the mice, platelet counts in vector-immunized mice rapidly declined until
395 expiration (Fig. 4B, left panel). However, platelet counts only marginally decreased in mice
396 vaccinated with pSFTSV or pSFTSV-IL12 and counts rebounded in pSFTSV-IL12-
397 immunized mice at 4 d after viral infection. In addition, viral loads were significantly reduced
398 in the plasma of pSFTSV or pSFTSV-IL12-vaccinated mice when compared to those of
399 vector-immunized control at 4 d after infection. Notably, despite similar initial viral loads (\sim
400 10^6 copies/ml of plasma) at 2 d after viral challenge among the mice groups, pSFTSV-IL12-
401 vaccinated mice (mean \pm S.D. = $4.2 \times 10^6 \pm 4.6 \times 10^6$) were approximately five and fifty
402 times lower than those of pSFTSV-vaccinated mice (mean \pm S.D. = $2.1 \times 10^7 \pm 1.7 \times 10^7$)
403 and vector-immunized mice (mean \pm S.D. = $2.0 \times 10^8 \pm 1.9 \times 10^8$) at 4 d, respectively.

404

405 **Fig 4. Complete protection of IFNAR KO mice after vaccination with pSFTSV-**
406 **IL-12. (A) Survival rates (left panel) and body weight changes (right panel) of mice (n**
407 **= 5/group) immunized three times with the indicated DNAs and challenged s.c. with**
408 **10^5 FFU of SFTSV at two weeks after vaccination. (B) Platelet counts (left panel)**
409 **and viral loads in plasma (right panel) of mice vaccinated with the indicated DNA**

410 vaccines are presented. Blood samples were collected at 2 (D2) and 4 (D4) days
411 after viral challenge. Uninf, uninfected; $n = 3 \sim 4$ mice/group. *, $p < 0.05$; **, $p < 0.01$;
412 ***, $p < 0.001$.

413

414 **Vaccination of Gn protein provides partial protection against lethal infection of SFTSV**

415 Since antibody responses against the viral glycoproteins, Gn and Gc, were poorly induced in
416 IFNAR KO mice immunized with the DNA vaccines, we further investigate the potential
417 protective role of neutralizing antibodies against Gn or Gc protein by immunization with the
418 protein antigens fused with human Fc fragment (Fig. 5). Vaccination with Gn-Fc or Gc-Fc
419 protein with Alum adjuvant efficiently induced specific antibodies against the immunized
420 antigens in IFNAR KO mice; levels of antibody titers against both antigens were fairly
421 similar (mean titer \pm S.D. = $3,584 \pm 1,024$, $n = 4$). In addition, levels of neutralizing
422 antibodies against SFTSV in immune sera from mice immunized with either protein antigen
423 were significantly higher than those from Fc-immunized controls, when assessed by focus
424 reduction neutralization test (FRNT, Fig. 5B). FRNT₅₀ titers of immune sera from mice
425 immunized with Gc-Fc (mean titer \pm S.D. = 209 ± 140 , $n = 4$) and Gn-Fc (mean titer \pm S.D. =
426 $929 \pm 1,134$, $n = 4$) were approximately five and twenty folds higher than those of Fc-
427 immunized mice (mean titer \pm S.D. = 42 ± 29 , $n = 4$). It is also notable that FRNT₅₀ titers of
428 Gn-Fc immune sera were generally higher than those of Gc-Fc sera, although the difference
429 was not statistically significant. Finally, we examined the protective efficacy of the protein
430 vaccines against lethal infection with SFTSV (Fig. 5C). All the control mice immunized with
431 Fc antigen died at five days after infection, whereas 50% of Gn-Fc immunized mice were
432 protected from lethal challenge and regained their lost body weight. Interestingly, weight loss
433 of Gc-Fc vaccinated mice was delayed and observed from 4 d after infection, whereas the

434 control mice lost weight from 2 d after infection. Even though all the mice immunized with
435 Gc-Fc succumbed to death, they survived three days more than control mice and the
436 extension of median survival time was statistically significant ($p = 0.0046$).

437

438 **Fig 5. Partial protection of IFNAR KO mice after vaccination with Gn-Fc antigen.**

439 (A) Anti-Gc (blue dots) or Gn (red dots) IgG response was measured by ELISA at
440 two weeks after the third immunization with Gn-Fc or Gc-Fc in IFNAR KO mice ($n =$
441 4/group). Cut-off titers (dashed line, mean O.D. + 3 x S.D. at 1:100 diluents) was
442 determined using Fc-immune sera (black dots). The antibody titers of anti-Gc or Gn
443 IgG in sera of immunized mice are summarized in the right panel. Error bar, mean +
444 S.D.; black line, mean. (B) Neutralizing antibody response to SFTSV generated by
445 SFTSV DNA vaccines in IFNAR KO mice. The amount of neutralizing antibody
446 against SFTSV was determined based on FRNT₅₀ (left panel) and summarized (right
447 panel). (C) Survival rates (left panel) and body weight changes (right panel) of mice
448 immunized three times with Fc (black dots, $n = 6$), Gc-Fc (blue dots, $n = 4$), or Gn-Fc
449 (red dots, $n = 6$) antigen and challenged s.c. with 10^5 FFU of SFTSV at two weeks
450 after vaccination. *, $p < 0.05$; **, $p < 0.01$.

451

452 **Discussion**

453 DNA vaccination has been widely investigated for various infectious diseases, especially
454 targeting viral infections, during the last two decades [24, 25]. Although, human clinical trials
455 of DNA vaccines have yielded poor immunogenicity and less than optimal results, the
456 approval of a few veterinary vaccines is a testimony of proof-of-concept and the hope that
457 licensed DNA vaccines for human use may not be too far away [24]. While we were

458 preparing this manuscript, Kwak J.E. *et al.* reported a promising demonstration of DNA
459 vaccination against lethal SFTSV infection in old-aged (> 4-years-old) ferret model [26].
460 They used five plasmid DNAs encoding individual viral gene, Gn, Gc, NP, NS, or RdRp, and
461 found that vaccination of old ferrets with a mixture of the five plasmids completely protected
462 ferrets from lethal SFTSV challenge without developing any clinical symptoms and systemic
463 viremia [26]. In addition, adoptive transfer of immune sera from ferrets immunized with two
464 plasmids encoding Gn and Gc transiently induced systemic viremia but provided complete
465 protection against lethal challenge, suggesting that Gn/Gc may be the most effective antigens
466 for inducing protective immunity. They also found that non-envelop (NP, NS, and RdRp)-
467 specific T cell responses also contribute to protection against SFTSV infection although it
468 failed to induce neutralizing activity [26]. Here we also observed that vaccination with a
469 plasmid encoding Gn, Gc, NP, and NS, together with murine IL-12, could provide protective
470 immunity in IFNAR KO mice against lethal SFTSV challenge. However, our current DNA
471 vaccine failed to suppress initial systemic viremia and weight loss (Fig. 4). This might be due
472 to the lack of type I interferon signaling and/or antibody responses against Gn and Gc
473 glycoproteins, which are required for viral neutralization during the early stage of viral
474 infection. Inefficient generation of antibodies against Gn and Gc might be due to the absence
475 of type I interferon signaling in IFNAR KO mice or fusion of Flt3L with the glycoproteins,
476 resulting in conformation defect of the antigens and/or dysregulation of B cell responses.
477 Given that immunization with Gn-Fc and Gc-Fc proteins can induce specific antibodies in
478 IFNAR KO mice (Fig. 5), the absence of type I IFN signaling might not be the cause of
479 inefficient antibody generation. Instead, Flt3L may suppress specific antibody responses as
480 observed in other studies [16, 27-29]. Flt3L may exert tolerogenic effect on CD4 T cells via
481 dendritic cells, thereby promoting B cell hypo-responsiveness *in vivo*; however, the

482 underlying mechanisms of CD4 T cell dysregulation are still unclear [28]. Nevertheless,
483 Flt3L can significantly enhance CD8 T cell responses when fused with a target antigen and
484 promote persistent maintenance of antigen-specific CD8 T cells *in vivo* [16, 29]. In this study,
485 we also observed significant enhancement of Gn and NP-specific CD8 T cell responses, as
486 well as CD4 T cells, in the presence of IL-12 expression (Fig. 3B and C), which correlated
487 well with vaccine protective efficacy in mice infected with lethal doses of SFTSV. The potent
488 effect of antigen-specific T cell responses in protection against SFTSV infection is consistent
489 with the results of the aforementioned ferret infection model [26].

490 Finally, we evaluated the degree of protective immunity provided by the individual
491 glycoprotein, Gn or Gc, after protein antigen immunization for the first time (Fig. 5), since
492 previous studies showed that vaccination with both antigens retained in a live viral vector [11]
493 or DNA vaccine [26] can provide complete protection against lethal SFTSV challenge in
494 IFNAR KO mice or old ferrets, respectively. Both protein antigens are immunogenic and
495 induce significant levels of neutralizing activity against SFTSV, although anti-Gn antibodies
496 retain relatively higher neutralizing capacity than anti-Gc antibodies. Consistently, the
497 protective efficacy of Gn protein vaccination is relatively higher than Gc protein
498 immunization. While vaccination with either protein antigen failed to provide complete
499 protection against lethal SFTSV challenge, Gc vaccine prolonged the survival time of
500 infected mice and Gn antigen can provide partial protection. Considering that Gn and Gc
501 glycoproteins function as viral ligands for cellular receptor binding and viral membrane
502 fusion in host endosome, respectively [30-32], neutralization of both antigens might be
503 required to completely block viral attachment and entry into host cells.

504 Taken together, we confirmed that antigen-specific T cell responses against SFTSV induced
505 by DNA vaccination can provide complete protection against lethal viral challenge and

506 immunization with each individual glycoprotein of SFTSV can also confer partial protective
507 immunity. Given that the Achilles heel of DNA vaccines remains their poor immunogenicity
508 in human trials when compared to protein vaccines [25], optimal combinations of DNA
509 and/or protein vaccines, proper selection of target antigens, and incorporation of efficient
510 vaccine adjuvant, need to be further investigated for development of the best SFTSV vaccine
511 formulation for human application.

512

513 **Supporting information**

514 **S1 Fig.** Purified Gn-Fc and Gc-Fc proteins were resolved by SDS-PAGE and stained with
515 Coomassie blue.

516 **S2 Fig.** Gating strategies for T cell analysis by flow cytometry.

517 **S3 Fig.** Clinical manifestations in IFNAR KO mice infected with SFTSV. (A) IFNAR KO
518 mice were subcutaneously infected with different doses (0 , 10^1 , 10^3 , or 10^5 FFU/mouse) of
519 SFTSV. The mice were monitored daily to assess survival rate (left) and body weight change
520 (right) up to 10 d after infection when all the infected mice died. (B) The blood from the
521 infected mice were collected and platelet counts and their volumes were examined by
522 hematological analyzer. Red line: mean value, *: $p < 0.05$, **: $p < 0.01$, compared with
523 mock-infected controls.

524

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530

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532

533 **Author Contributions**

534 Conceptualization: JGK KHL NHC.

535 Formal analysis: JGK KJ HC YK HIK HJR NHC.

536 Methodology: JGK KJ HC YK HIK HJR YKJ.

537 Resources: JC KHL.

538 Supervision: YSK KHL NHC.

539 Writing - original draft: JGK KJ NHC.

540 Writing - review & editing: NHC.

541

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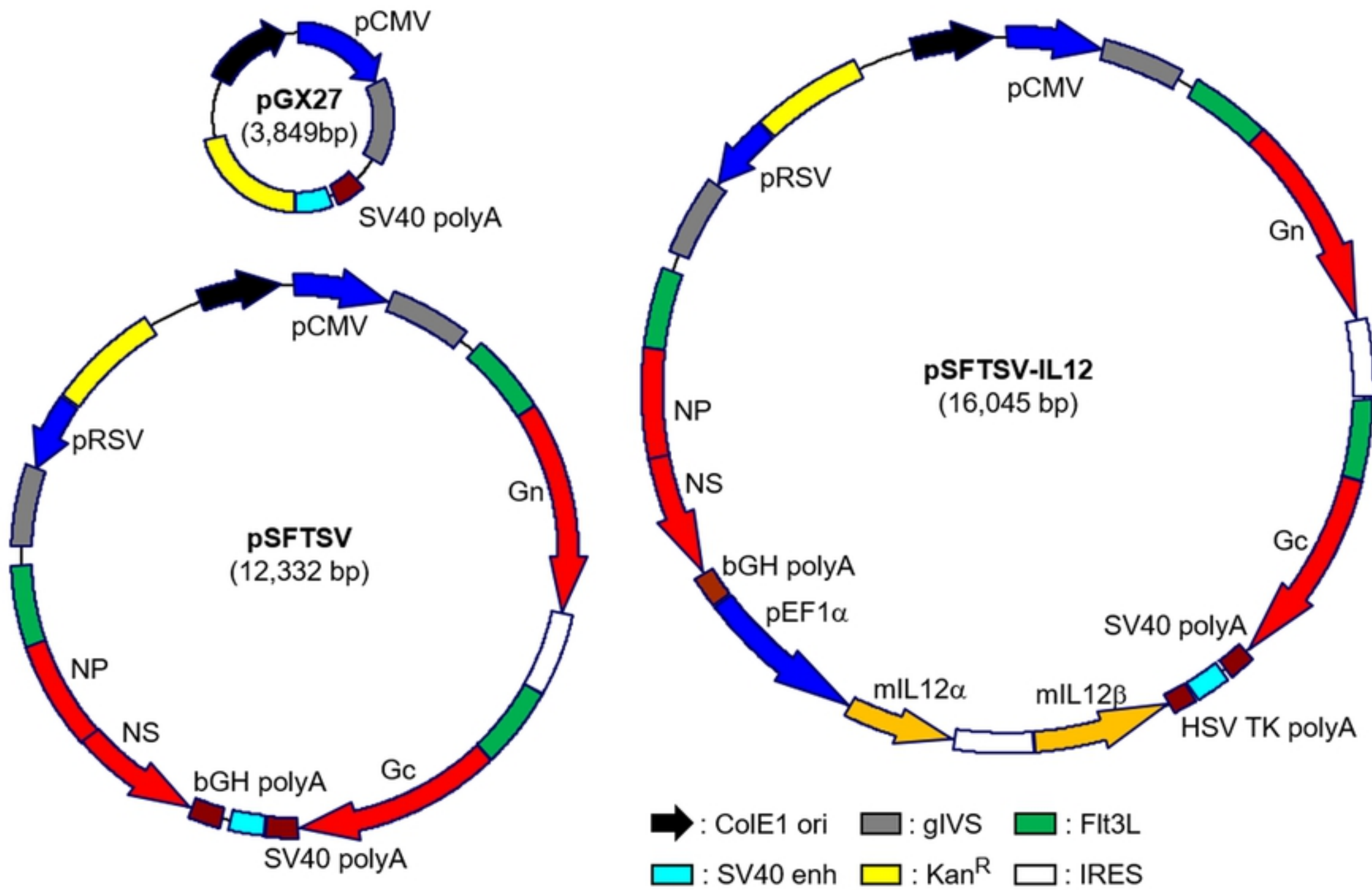


Figure 1

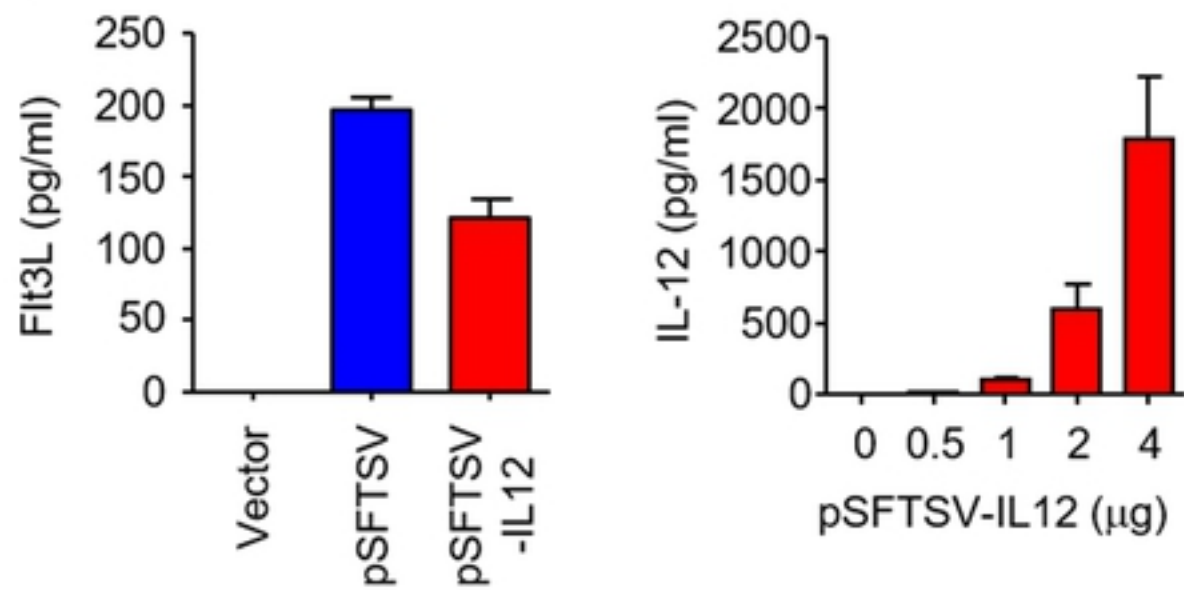
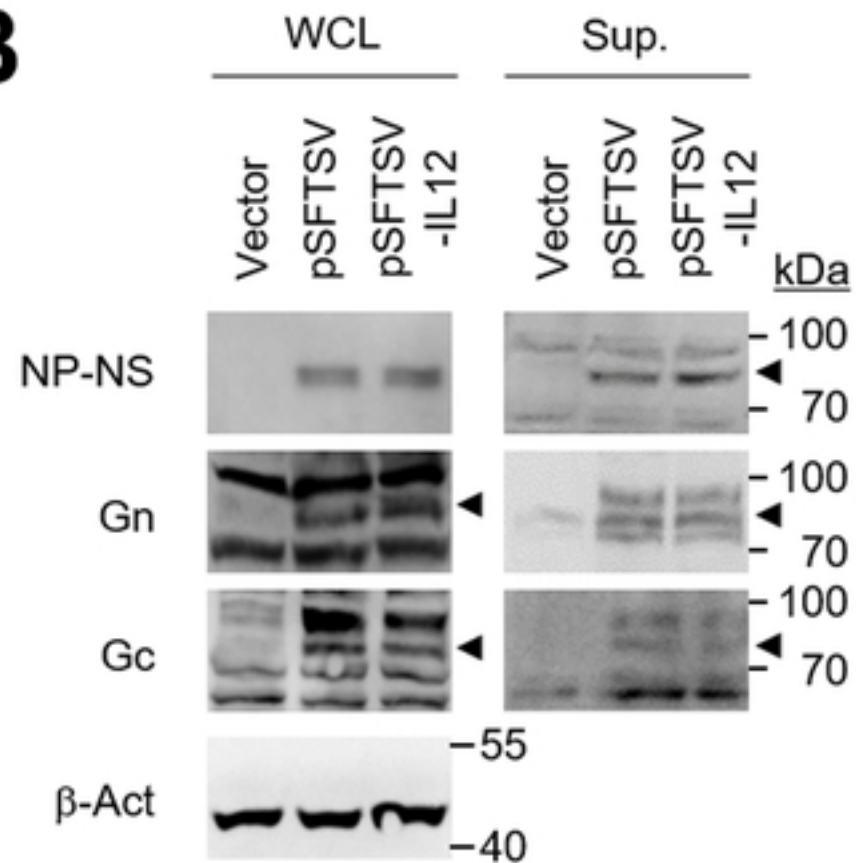
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Figure 2

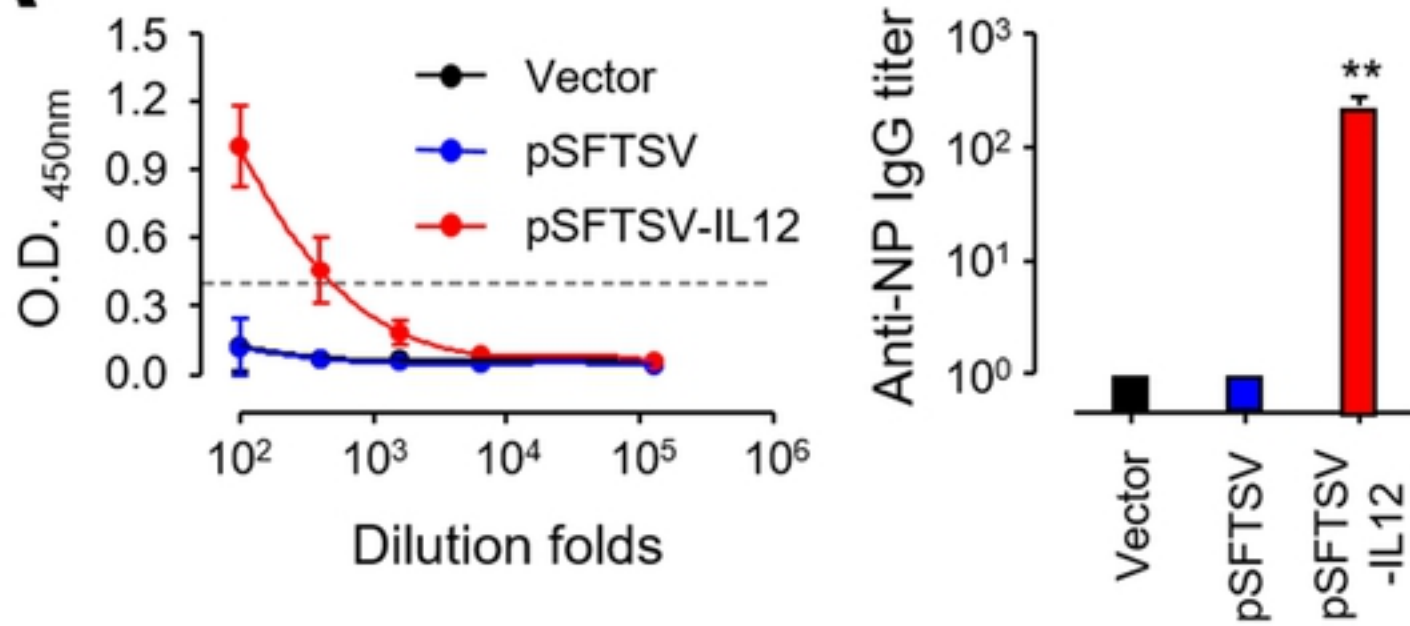
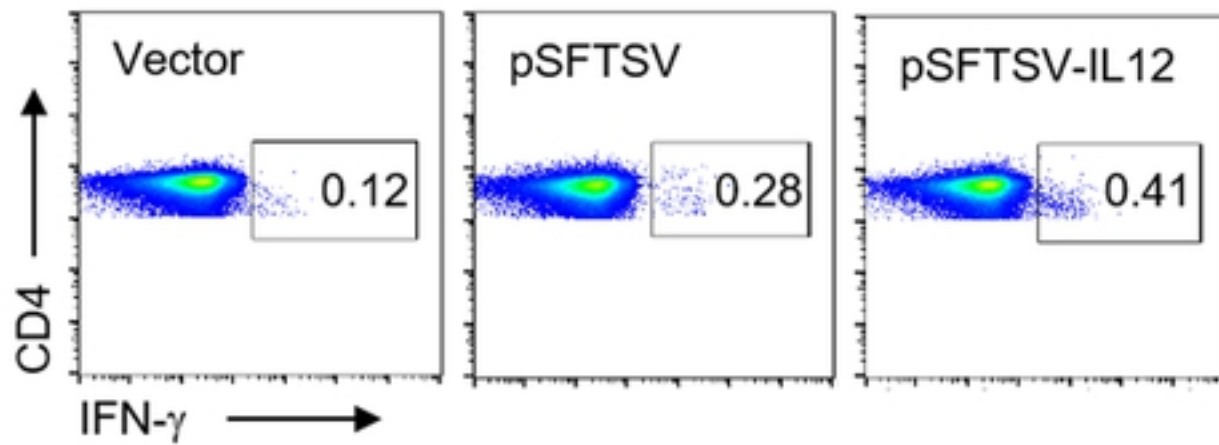
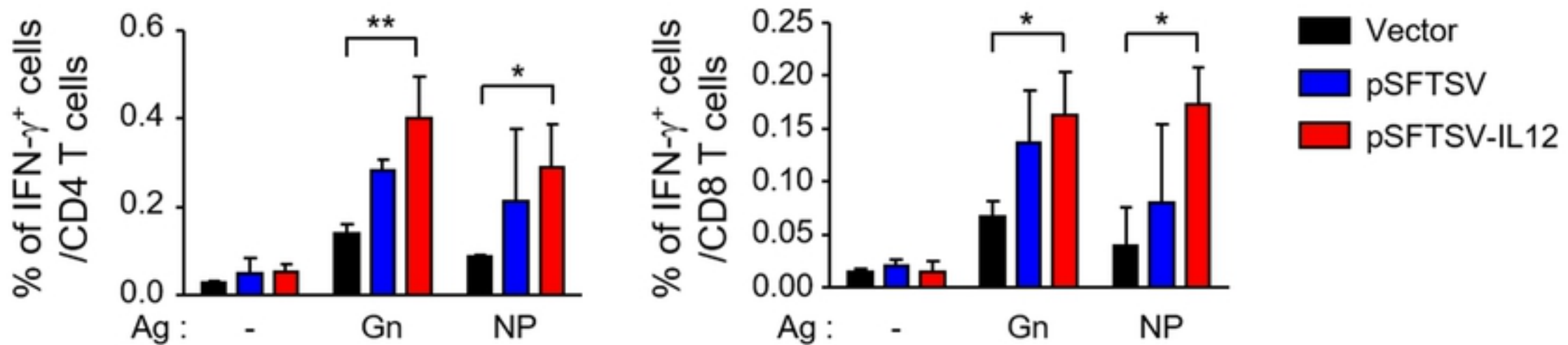
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Figure 3

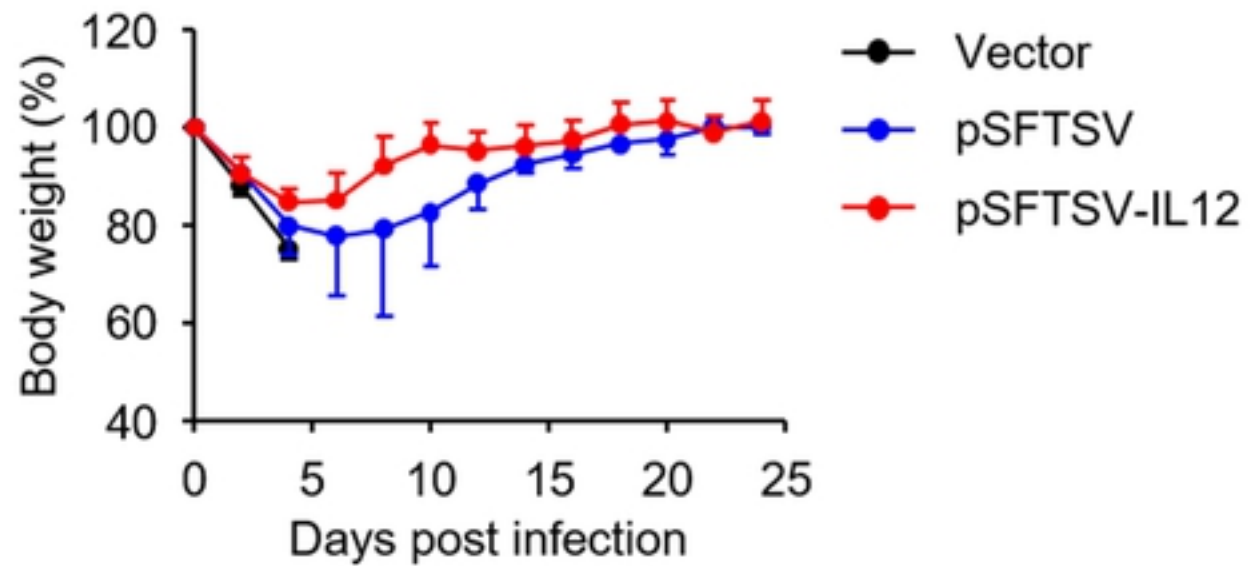
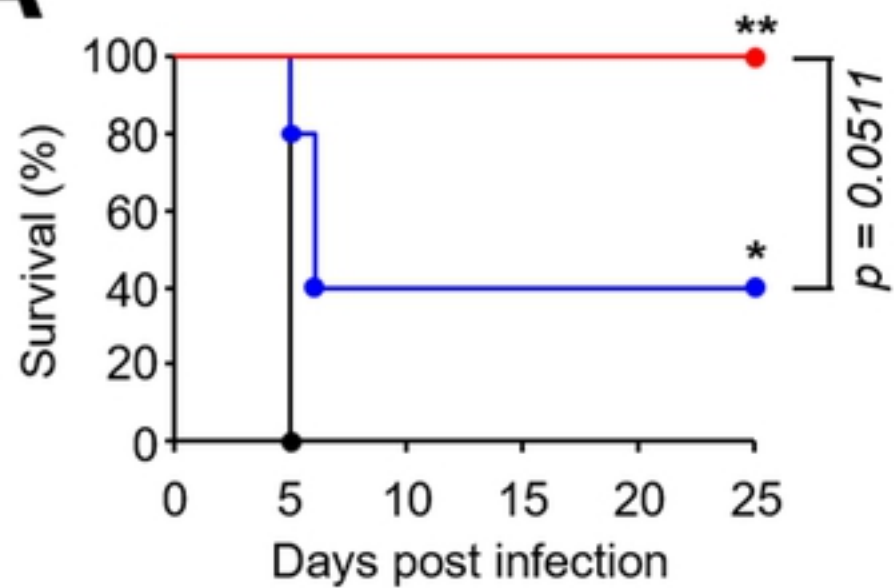
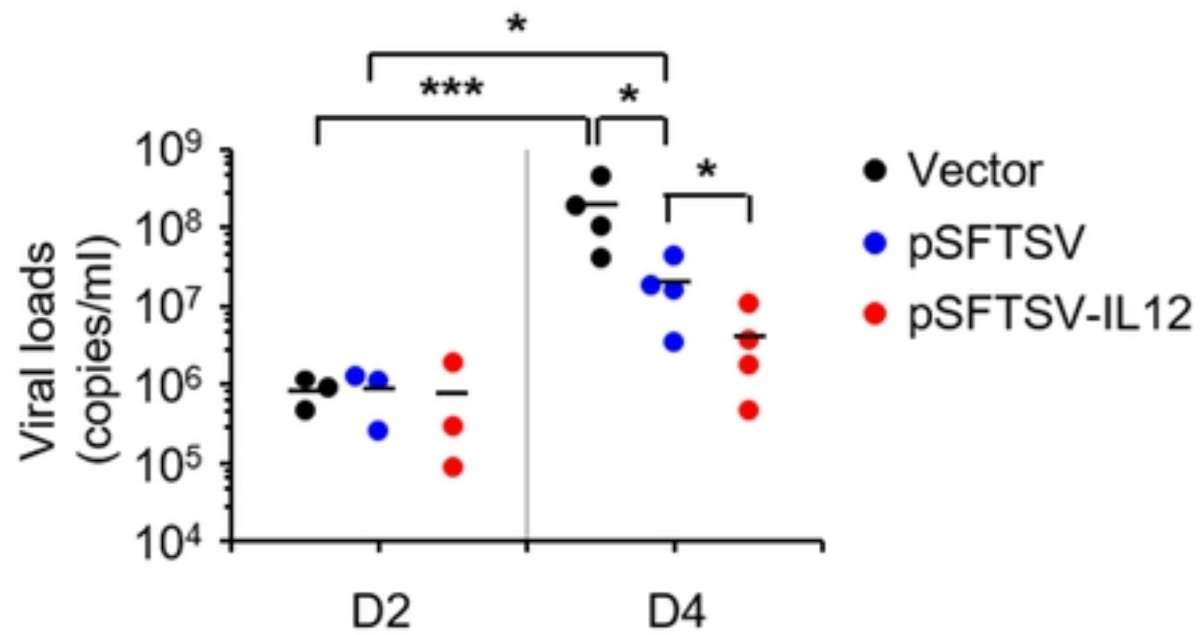
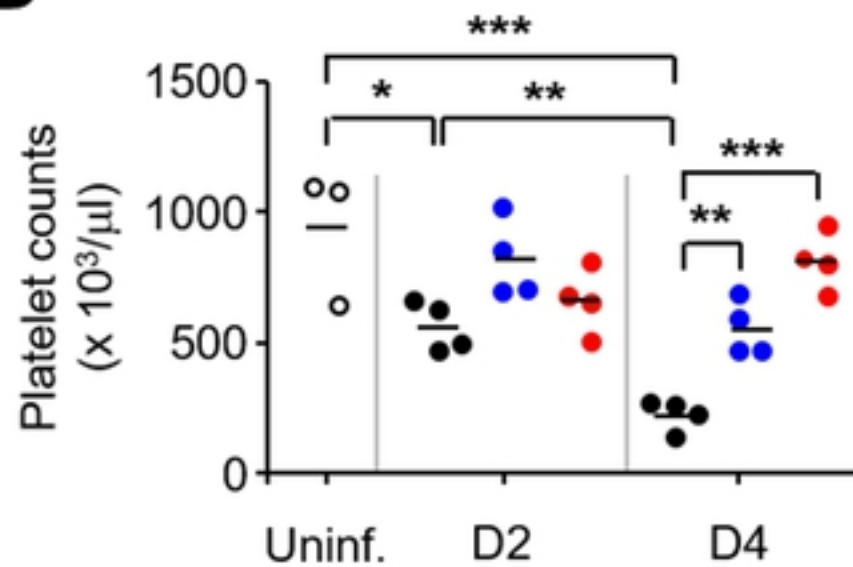
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Figure 4

