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중증열성혈소판감소증 바이러스에 대한 재조합 백신 개발 연구

Development of recombinant vaccine against Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV)

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서울대학교 대학원
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August 2020

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Development of recombinant Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV)

by

Hooncheol Choi

A thesis submitted to the Department of Biomedical Science in partial fulfillment of the requirements for the Degree of Master of Science in Medicine at Seoul National University College of Medicine

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Professor _____Chairman

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ABSTRACT

Severe Fever with Thrombocytopenia Syndrome virus (SFTSV) is an emerging tick-borne virus which causes SFTS disease with a mortality rate of 5-27% in humans. Despite the high mortality rate of SFTS, no effective vaccine or therapy is available yet. Here, I have developed DNA encoding of SFTSV genes fused with FMS-like tyrosine kinase-3 ligand (Flt3L) at N-terminus and IL-12 genes which can stimulate cell-mediated adaptive immunity. Vaccination of the recombinant DNA conferred complete protection of interferon-α/β receptor knock-out (IFNAR K/O) mice against a lethal dose of SFTSV. Although I failed to observe a neutralizing antibody response, antigen-specific T cell responses were significantly increased in the immunized mice. The degree of protective immunity elicited by the immunization of various subunits (Gn, Gc, and NP) of SFTSV in a single subunit form or combinatorial formulation was also investigated. Vaccination of a single recombinant protein, NP or Gn, provided approximately 50% protection in IFNAR K/O mice upon lethal infection with SFTSV, whereas immunization of Gc protein failed to protect the challenged mice. Interestingly, immunization with NP showed enhanced antigen-specific T cell responses and Gc vaccination provided strong neutralizing activity against SFTSV. Finally, IFNAR K/O mice were immunized with a mixture of SFTSV proteins (Gn+NP, Gc+NP, and Gn+Gc+NP). Among these groups, Gc+NP immunization conferred the best (approximately 90%) protection against a lethal SFTSV infection when compared to those of other groups (Gn+NP: 70%, and Gn+Gc+NP: 57%) and this might be due to an enhanced T cell response and increased neutralizing activity against SFTSV. Collectively, vaccination with

DNA encoding SFTSV subunit genes or with combinatorial SFTSV subunits, including NP and Gc, could be a promising candidate for protection against SFTSV infection.

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Key words: Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV), Tick-borne disease, DNA vaccine, Recombinant subunit vaccine. Cellular immunity, Humoral immunity

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Part I.

Part II.

LIST OF ABBREVIATIONS

SFTSV	Severe Fever with Thrombocytopenia Syndrome virus
NP	Nucleocapsid Protein
Gn	Glycoprotein N
Gc	Glycoprotein C
DC	Dendritic Cell
IPTG	Isopropyl β-D Thiogalactoside
PBST	PBS containing 0.05% Tween-20
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme-Linked Immunosorbent Assay
TNF-α	Tumor Necrosis Factor-α
IFN-ɣ	Interferon gamma
WT	Wild Type
IFNAR	Interferon Receptor
K/O	Knock Out
FRNT	Focus reduction neutralization test
IB	Immuno-Blot
IP	Immuno-Precipitation
S.C.	Subcutaneous
Flt3L	FMS-like tyrosine kinase 3 ligand
IL-12	Interleukin 12
qRT-PCR	quantitative real time polymerase chain reaction
LB	Luria-Bertani
OD	Optical Density
Ni-NTA	Nickel-nitrilotriacetic acid

INTRODUCTION

Severe Fever with Thrombocytopenia Syndrome Virus (SFTSV) is an emerging tick-borne arbovirus, included in the *Phenuiviridae* family of *Bunyavirales* order [4, 5]. SFTSV is a spherical shaped virion of about 100nm in diameter [6, 7]. This virus has three different segments of ambisense or negative sense RNAs: Large (L), Medium (M), and Small (S). Each segment encodes the RNA-dependent RNA polymerase (L segment), the envelop glycoproteins: Gn and Gc (M segment), and a nucleocapsid protein N and non-structural protein NSs (S segment) [8]. SFTSV is transmitted by *Haemaphysalis longicornis* which is a major host [9, 10], and by the *Rhipicephalus microplus* tick and others [11]. This virus can be spread through direct contact with blood and body fluids [12, 13]. SFTSV has been mostly reported in China, South Korea, and Japan [14-16], but there are some virus infection cases reported in Vietnam [17]. Of over 7,419 cases of SFTS reported before 2016, around 355 death cases were estimated in China, and the viruses were mainly located in Eastern and Central China [18]. The first SFTS case reported in Japan was in Yamaguchi in 2012. Until the end of 2013, the total positive number of SFTSV cases had increased to 40 in Japan with designating emerging diseases [19]. The first suspected patient of SFTSV was reported on Jeju Island in South Korea in May 2013, followed by SFTSV isolation by Seoul National University Hospital [20, 21]. 90% of SFTSV patients were older than 35 years old and most of them were farmers in these countries [22]. In other Asian countries, SFTS cases have been reported in Vietnam [17], or North Korea [8, 23]. The major clinical symptoms of SFTS are fever, fatigue, vomiting, diarrhea, thrombocytopenia, and leukocytopenia as well as multi-system organ failure [24, 25]. The

cases of infection of these viruses have rapidly increased from 2012 to 2018 [8]. Even though the disease mortality rate of SFTS is approximately 5-27%, there is no licensed vaccine or therapy currently.

There recently are a few of vaccine studies of SFTSV with immunocompromised animal models. Full length of glycoprotein (Gn and Gc), Nucleocapsid protein (Np), Nonstructural protein (NS), and RdRp gene cloned DNA vector which can also express target proteins triggered not only robust IFN-ɣ against five antigens mentioned above, but also humoral immune response in BALB/c mice. In addition, this vector vaccination confers complete protection against lethal SFTSV infection in old ferret (>4 years) decreasing viral load in serum [26]. Recombinant VSV having SFTSV glycoproteins vaccination elicited strong broad-spectrum neutralizing antibodies and complete protection against lethal various SFTSV strain challenge in IFNAR K/O C57BL/6 mice [27]. Glycoproteins (Gn&Gc) of SFTSV are dealt with an important target of vaccination [3, 26, 27]. Injection of selected SFTSV mAb (monoclonal antibody) from convalescent patients against glycoprotein N showed complete protection of IFNAR1 K/O A129 mice with lethal SFTSV challenge [3]. Live attenuated SFTSV having mutation of NS region also provides complete cross-genotype protection to lethal challenge. It could induce a humoral response and reduce viral load in both serum and tissues [56]. However, immunization with SFTSV NSs protein using Freund's adjuvant could not promote virus clearance in C57BL/6J mouse serum, liver, spleen, and kidney, even though it promoted high titer of anti-NS antibodies and IFN-ɣ level [28].

There are lots of vaccine types in these days. Attenuated and inactivated vaccines have abilities of long-term protection, good

immunogenicity [29]. However, inducing disease and recovery virulence of pathogens are the main disadvantages [30]. Returning to natural virulence is a rising problem of this types of vaccine, resulting in the development of subunit, recombinant, and conjugated vaccines. Researchers had used subunits of pathogens, conjugated, and recombinant vaccines [31]. Subunit vaccines includes one or more proteins, protein-peptide or polysaccharide that are natural pathogenic structure [32]. Because of low production costs or increasing bio-safety, scientists have easily used for vaccine study. DNA vaccine is a format of the administration of a plasmid encoding the target antigen of a pathogen. DNA directly injected into a cell can be expressed with the aid of promoter and causes induction of the immune system. The expressed proteins could be the natural form that can trigger innate and adaptive immune response. The advantages of DNA vaccines are producing natural form of antigens, no possibility of recovery virulence of pathogens, and making sustainable immunity [33].

Alum adjuvant which is commonly used in human vaccination may cause a robust Th2 response, but provoke relatively weak Th1-cell-mediated immunity [34]. Alum with antigens stays in the injection site by forming depot and slowly releases antigen to the body, then induces activation of APCs [35]. Recently, it is controversial that NLRP3-inflammasome would be associated with the immune-stimulatory characteristic of aluminum adjuvant [36]. FMS-like tyrosine kinase 3 ligand (Flt-3L) is an endogenous small molecule as a cytokine and growth factor that elevates the number of lymphocytes by activating the hematopoietic progenitors. The development of DC and their activation state is also dependent on Flt-3L cytokine [37]. This cytokine can significantly change the

absolute number of DC and the subset composition of lymphoid tissue and blood-borne DC [38, 39]. IL-12 is also cytokine used for adjuvant of DNA vaccine [40]. IL-12 stimulates protective immune response by interacting with other cytokines and lymphoid cells. Role of IL-12 has been known to elevate proliferation of T cells and NK cells, activities of CD8+ T cells, NK cells, macrophages, and Th1 cells, and induce production of IFN-ɣ and other cytokines [41, 42].

Disadvantage of DNA and recombinant subunit vaccines is lower immunogenic effect [31-33]. To overcome this drawback, recently, heterologous DNA prime-protein boost immunization process has been tired in preclinical and even clinical cases. Recombinant subunit vaccines can trigger a strong antibody immune response and DNA vaccines are able to elicit the T cell immune response [43]. With these advantages, heterologous prime-boost immunization could induce a strong adaptive immunity against antigens [44].

In this study, plasmid encoding ectodomain of SFTSV Gn, Gc, and Np-Ns fusion fused with Flt3L at N-terminus and murine IL-12 cytokine and recombinant SFTSV subunit were evaluated as an SFTSV vaccine candidate. Antibody and T cell immunity were analyzed in SFTSV DNA plasmid and subunit vaccinated IFNAR K/O mice. Then, survival and body weight changes were assessed in the DNA and subunit vaccinated IFNAR K/O mice after challenging a lethal dose of SFTSV. pSFTSV-IL12 provides complete protection against a lethal challenge with high Np and Gn specific IFN-ɣ secreting $CD4^+$ and $CD8^+$ T cells although weak antibody dependent immunity was observed. Immunization of recombinant Np or Gn enhanced the percentage of antigen specific IFN-ɣ secreting $CD4^+$ and $CD8^+$ T cell and elicited high level of antigen specific antibody titer, while they conferred

approximately 50% protection against viruses. Vaccination of recombinant Gc provided a strong neutralizing effect against SFTSV with a high level of antigen specific antibody titer though all mice immunized with the Gc subunit were dead. Lastly, vaccination of recombinant Gc+Np mixed subunits increased the frequency of Np specific IFN-ɣ and TNF-α producing $CD4^+$ and $CD8^+$ T cell and antibody dependent immunity. Besides, approximately 90% of the GcP immunized mice survived a lethal dose of SFTSV. Therefore, these results suggested that pSFTSV-IL12 DNA prime recombinant SFTSV Gc+Np subunits boost vaccine could be a novel vaccine for SFTSV by providing a high level of T cell immunity and strong antibody dependent immunity.

METHODS AND MATERIAL

Ethics statement

All animal experiments were conducted in an Animal Biosafety Level 3 facility at Seoul National University Hospital. These studies were approved by Seoul National University Hospital (SNUH IACUC No.15-0095-C1A0 and IVI IACUC No. 2018-018) and followed by strict regulation with recommendations in the Guideline of care and use of laboratory animals.

Preparation of SFTSV DNA plasmids.

To generate plasmids for DNA vaccination, genes including ectodomains of Gn, Gc (from Genebank accession no. AJO16082.1), and NP-NS fusion protein (from Genebank accession no. AJO16088.1 and AKI34298.1, respectively) were manufactured (GeneScript, Piscataway, NJ, USA) and cloned into pGX27 vector (Genexine, Seongnam, Republic of Korea). All of the genes were fused with the signal sequence of tissue Plasminogen Activator (tPA, Uniport no. P00750) and Flt3L (Uniport no. P49771) at N-terminus [1] (pSFTSV, Part Ⅰ, Fig 1). Furthermore, murine IL-12α and β genes (Uniport no. P43432) were also manufactured (GenScript) and inserted into pSFTSV-IL12 (Part Ⅰ, Fig 1).

For expression of SFTSV recombinant subunits, the SFTSV Nucleocapsid protein gene (from Genebank accession no. KP663733) was cloned into pET28a+ bacterial expression vector (Novagen, Gibbstown, NJ, USA). SFTSV Glycoprotein N or C genes (from Genebank accession no. KF358692) were cloned to pcDNA3

mammalian expression vector (Thermo Fisher Scientific, Waltham, MA, USA). These glycoprotein genes have signal peptide on N-terminal and every gene was fused with a six-histidine tag on C-terminal. The glycoprotein N or C genes (from Genebank accession no. KF358692) having signal peptide on N-terminal and Fc fusion protein on C-terminal were also entered into pCEP4 vector (Thermo Fisher Scientific, Waltham, MA, USA). This vector was thankfully provided by Prof. Chung laboratory (Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine).

Gene expression of plasmid DNAs

To assess the expression of cloned genes in pSFTSV, pSFTSV-IL12, pET28a+ encoding SFTSV nucleocapsid protein gene fused with six histidines at C-terminus, pCEP4 including SFTSV glycoprotein N&C gene with human IgG Fc domain at C-terminus, and pcDNA3 encoding SFTSV glycoprotein N&C fused with six histidines at C-terminus, HEK293T cells (ATCC CRL-1573, Manassas, VA, USA) and BL21(DE3) *E.coli* were used. HEK293T cells were transfected with pGX27, pSFTSV, pSFTSV-IL12, pCEP4 including SFTSV glycoproteins, or pcDNA3 inserting SFTSV glycoproteins plasmids using the polyethylenimine (PEI) transfection method [2]. Briefly, plasmids and PEI mixture was diluted with Opti-MEM media (Gibco, Gaithersburg, MD, USA) and incubated with HEK293T cells for 4hrs. Following incubation, the media was changed with Dulbecco's Modified Eagle medium (DMEM) (Gibco) containing 10% fetal bovine serum (FBS) and incubated in humidified CO2 atmosphere at 37℃ for 48hrs. Cells and supernatant were harvested for confirming expression. Cells were lysed with NP-40

lysis buffer (1% NP-40 in 50mM Tris-HCl, 150mM NaCl, pH8.0) containing protease inhibitor cocktail (Sigma-Aldrich, St.Louis, MO, USA). BL21(DE3) *E.coli* were transformed with pET28a+ vector having SFTSV NP-His through heat shock at 56℃. Transformed *E.coli* were incubated in LB broth (MP biomedical, Irvine, CA, USA) overnight at 37℃ in bacterial incubator. Incubated *E.coli* were lysed with Lysozyme (Thermo Fisher Scientific, Waltham, MA, USA), protease inhibitor cocktail (Sigma-Aldrich, St.Louis, MO, USA) in the buffer (20mM sodium phosphate, 0.15M NaCl, pH 7.2) and then sonicated using Mixonix Sonicator XL2020 (Spectralab, St. Markham, ON, CA).

For quantification of Flt3L and murine IL-12 cytokines in the supernatant of transfected HEK293T cells, human Flt3L and mouse IL-12p70 Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) were used following with manufacturer's instructions. Expression of all proteins in culture supernatant and cell lysates was confirmed by western blot using rabbit anti-Gn, Gc (NBP2-41153 and NBP2-41156, NOVUS Biologicals, Centennial, Colorado, USA), NP (produced by custom polyclonal antibody production service through Abclon, Seoul, Republic of Korea), Histidine (Santa Cruz, California, USA), and human IgG (Promega, Madison, WI, USA) antibodies, respectively.

Production of recombinant proteins

SFTSV nucleocapsid proteins cloned into pET28a+ vector were purified from BL21(DE3) *E.coli* strain having cloned plasmids. After induction using 0.1mM isopropyl β-D-thiogalactoside (IPTG) for 18hrs at 16℃, all of the bacterial cells were lysed. The proteins were

purified using HisTrap HP histidine-tagged protein columns (GE Healthcare, Chicago, IL, USA) following the manufacturer's instruction. And pcDNA3 vector encoding SFTSV glycoprotein N and C fused with His tag was transfected into HEK293F cells (Thermo Fisher Scientific, Waltham, MA, USA) using polyethylenimine (PEI) [2]. Following harvesting supernatant, these proteins were also purified through HisTrap HP histidine-tagged protein column with the manufacturer's instruction. Besides, pCEP4 vector encoded ectodomain of Gn/Gc fused to the human immunoglobulin Fc region was also transfected into HEK293F cells with polyethylenimine (PEI) together. For the over-expression, transfected cells were cultured in the FreeStyle 293 expression medium (Gibco, Waltham, MA, USA). MabSelect (GE Healthcare, Chicago, IL, USA) in AKTA start affinity chromatography (GE Healthcare, Chicago, IL, USA) [3].

Enzyme-linked immunosorbent assays (ELISA)

To determine the titer of antibodies against SFTSV subunits after 3rd immunization, immunoassay 96-well plates (Nunc, Rochester, NY, USA) were used. 100ng of Proteins were plated on each of wells in 96-well plate for 4℃ overnight. Following this process, all of the well plates containing proteins were washed with PBST (PBS including 0.05% Tween 20). Every washed well was blocked using five percentage of skim milk and treated immunized mice sera. Four-folds dilution with 10point starting 1:100 was conducted every mouse serum. Sequentially, Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibodies (Thermo Fisher Scientific, Waltham, MA, USA) were incubated for 1hr at room temperature. Wells were washed with 0.05% PBST again and incubated with

3,3',5,5'-Tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD, USA) for 7min at room temperature and the reaction was stopped with 1M phosphoric acid. Microplate reader (Beckman Coulter Inc, Fullerton, CA, USA) detected plates by using 450nm absorbance.

Viruses and cells

SFTSV (Genbank accession no. MN329148-MN329150) isolated from Korean SFTS patient. For virus preparation, Vero E6 cells (ATCC no. CRL-1586) culturing under 10 passages were plated into T-75 flask (Thermo Fisher Scientific, Waltham, MA, USA) with the confluent monolayer. Viruses were incubated in complete media (DMEM supplemented with 10% FBS, 1% penicillin-streptomycin solution) on Vero(E6) cells plated flask for 5days. After 5 days, the supernatant was harvested and stocked at -80℃. Virus infectivity was confirmed by plaque assay method with 10 points, ten-folds dilution. Viruses supernatant was incubated in complete DMEM media for 1.5hrs at 37℃. Overlay media (DMEM supplemented with 2% FBS and 1% methylcellulose) was plated on confluent Vero(E6) cells after incubating viruses for 5 days. Cells were fixed with 100% methanol (Merck, Darmstadt, Germany) and 4% paraformaldehyde (Intron, Seongnam, Republic of Korea) for 20min at room temperature. Permeabilization step using 0.2% Triton X-100 (Sigma, St. Louis, MO, USA) proceeded for 15min at room temperature. Viral plaques were detected by rabbit anti-SFTSV NP antibodies (Abclon) and HRP conjugated goat anti-rabbit IgG antibodies (Invitrogen, Waltham, MA, USA) using 3,3'-Diaminobenzidine (DAB) substrate (Merck, Darmstadt, Germany).

Focus Reduction Neutralization Test (FRNT)

Mice sera immunized with SFTSV subunits were incubated with 100FFU SFTSV at four dilution points starting from 1:100 for an hour at 4℃. Mixture of SFTSV and serum was placed into confluent Vero(E6) cells monolayer in each well of 24 well cell culture plate (SPL Life Sciences, Pocheon, Republic of Korea) for 1.5hrs at 37℃. Viruses were incubated in overlay media (DMEM supplemented with 2% FBS and 1% methylcellulose) for 7 days at 37℃ in a CO2 incubator. Cells were fixed with 100% methanol (Merck, Darmstadt, Germany) and 4% paraformaldehyde (Intron, Seongnam, Republic of Korea) for 20min at room temperature. Permeabilization step using 0.2% Triton X-100 (Sigma, St. Louis, MO, USA) proceeded for 15min at room temperature. Viral plaques were detected by rabbit anti-SFTSV NP antibodies (Abclon) and HRP conjugated goat anti-rabbit IgG antibodies (Invitrogen, Waltham, MA, USA) using 3,3'-Diaminobenzidine (DAB) substrate (Merck, Darmstadt, Germany).

Quantitative reverse transcript-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the plasma of SFTSV-infected mice after immunizing using Trizol LS reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Extracted RNA was reverse transcribed to cDNA using the HiSenScript RH (-) RT Premix kit (Intron, Seongnam, Republic of Korea). cDNA was quantified using TaqMan Universal Master Mix 2 (Applied Biosystems, Waltham, MA, USA). Viral copy numbers were determined by BioRad CFX to connect real-time system (Bio-Rad, Hercules, CA, USA) under following conditions: uracil-N-glycosylase

incubation at 50℃ for 2 min, polymerase activation at 95℃ for 10 min, denaturation at 95℃ for 15 s, annealing and extension at 53℃ for 1 min for 45 cycles. Primer set and detecting probe was derived from the NP gene of SFTSV: NP forward (5'-CCTTCAGGTCATGACAGCTGG-3'), NP reverse (5'-ACCAGGCTCTCAATCACTCCTGT-3') and detecting probe (5'-6FAM AGCACATGTCCAAGTGGGAAGGCTCTG-BHQ1-3').

Flow cytometry

Mice spleens were ground by 70um cell strainer (BD Biosciences, San Jose, CA, USA) into RPMI 1640 media (Gibco, Waltham, MA, USA). RBC lysing buffer Hybrid-MaxTM (Sigma, St. Louis, MO, USA) was treated into spleen grinding supernatant. Splenocytes were cultured with 3 μg of purified NP, Gn, Gc proteins for 18hrs at 37℃ CO2 incubator in complete RPMI (supplemented with 10% FBS, 1% penicillin& streptomycin solution) on 96-well cell culture plate. To detect intracellular cytokine IFN-γ and TNF-α, Golgi-plug (BD Bioscience) was treated for 5hrs at 37℃ CO2 incubator and then blocked by using ultra-block solution (10% rat sera, 10% hamster sera, 10% mouse sera) (Sigma). Following blocking, anti-CD3 (145-2c11) (BD Bioscience), CD4 (RM4-59) (BD Bioscience), and CD8 (53-6.7) (Biolegend, San Diego, CA, USA) conjugated fluorescent dyes antibodies were treated for 30min, at room temperature. All of splenocytes bound with surface marker were fixed and permeabilized with Cytofix/Cytoperm kit (BD Bioscience). Then, anti-IFN-γ (XMG1.2) antibody (BD pharmingen, Franklin Lakes, NJ, USA) and TNF-α (MF6-XT22, Affymetrix, Cleveland, OH, USA) detected intracellular cytokines. Stained cells were detected through the

CytoFLEX S flow cytometer (Beckman Coulter Inc, Brea, CA, USA). Data were analyzed by FLowjo software (Tree Star, Ashland, OR, USA).

Immunization of mice and SFTSV challenge

8-to 12-week-old female C57BL/6 WT mice (Koatech, Pyeongtaek, Republic of Korea) or C57BL/6 Interferon α/β receptor K/O mice were used for immunization and challenge. They were maintained in the pathogen-free animal facility at Seoul National University College of Medicine. 4 μg of Mock vector (pGX27), pSFTSV, and pSFTSV-IL12 DNAs were intramuscularly immunized to mice by electroporation using Orbijector EP-I model (SL Vaxigen Inc., Seongnam, Republic Korea) in leg three times at two-week intervals. 10 μg of SFTSV NP-His, Gn-Fc, Gc-Fc, Fc subunits with aluminum hydroxy chloride (Alhydrogel® adjuvant 2%, InvivoGen, Hong Kong) were immunized through subcutaneous injection three times at two-week interval during 6weeks. Mice sera were collected at a week after 3rd immunization. A thousand FFU of SFTSV were challenged two weeks later after the final immunization. Survival rate and body weight change were recorded until 14 day post-infection (dpi).

Hematology

To analyze hematological data, lots of blood was prepared through cardiac puncture in euthanized animals. The blood was collected into 0.5M EDTA(Enzynomics, Daejeon, Republic of Korea) coated tubes. Before the assessment of platelet counts, 4% paraformaldehyde was

added to EDTA-anti-coagulated whole blood samples at a 1:1 ratio for inactivating the virus. The platelet counts were analyzed using ADVIA 2012i Hematology System (Siemens Healthimeers, Erlangen, Germany).

Data analysis

Data was analysed by the Graph Pad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA). Statistical analysis was performed using a two-tailed Student's t-test with a 95% confidence interval or one-way analysis of variance (ANOVA) followed by Newman-Keuls t-test for comparisons of values among different groups. Data are expressed as the mean ± standard deviation (S.D.). Data analysis on survival rates was performed using the Mantel-Cox method. A p-value of <0.05 was considered statistically significant.

RESULTS

Part Ⅰ.
Evaluation of single plasmid DNA vaccine encoding IL-12 and SFTSV antigens.

Characterization of SFTSV DNA vaccines and their gene expression.

Four major antigens (Nucleocapsid protein (Np), Nonstructural protein (NS), and Glycoprotein (N&C)) of SFTSV were inserted into the pGX27 vector plasmid (Part Ⅰ, Fig 1). Full lengths of the NP-NS fusion protein gene linked through peptide (GSGSGSGSGSGRA) were under the control of the RSV promoter which provides high levels of expression in many cell types [45, 46]. Extracellular domains of the SFTSV Gn and Gc genes were separately cloned into the vector and under control of the CMV promoter and IRES sequence. All expressed antigens were fused with the extracellular domain of Flt3L and signal sequence of tissue plasminogen activator (tPA) in N-terminus to promote antigen presentation and trafficking of the fusion proteins as previously described [47]. Murine IL-21α and β gene which are growth factors for activated T and NK cells were cloned into the vector to stimulate the production of IFN-γ from antigen-specific T cells [48]. Secretion cytokines and SFTSV antigens from HEK293 cells transfected with indicated DNAs were analyzed by

ELISA and Western blot (Part Ⅰ, Fig 2). All SFTSV antigens were confirmed at whole cell lysate (left panel) and supernatant (right panel) of transfected cells (Part Ⅰ, Fig 2B).

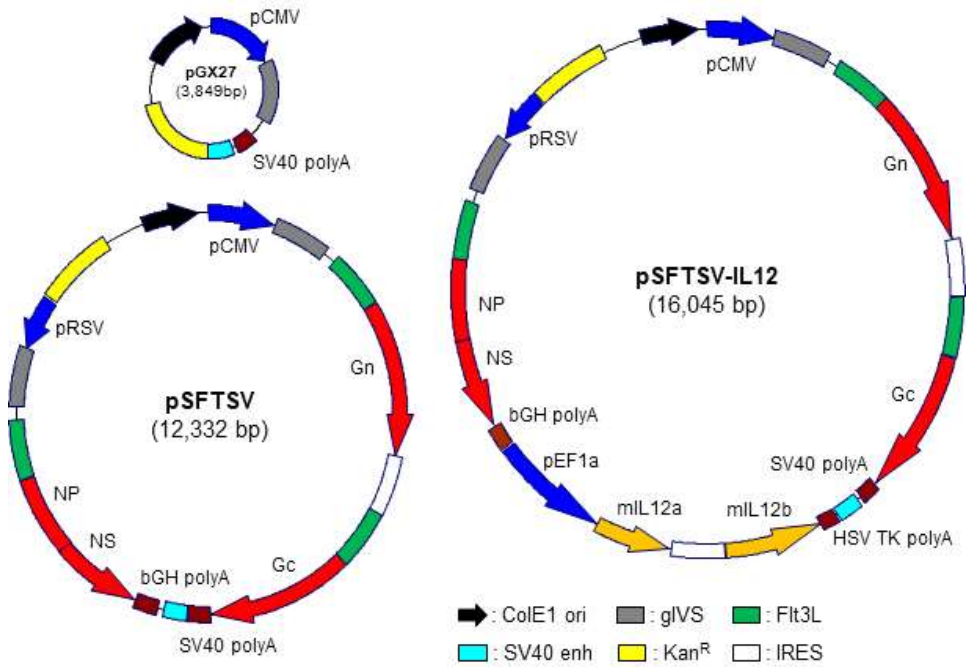


Figure 1. Map of plasmid encoding SFTSV antigen and IL-12.
SFTSV surface protein Gn and Gc, and Np-NS fusion protein were inserted into the pGX27 vector (pSFTSV). pSFTSV-IL-12 vector includes murine IL-12α and β gene to enhance cellular immunity. ColE1, ColE1-type bacterial origin of replication; gIVS, rabbit β -globin intervening sequence; KanR, kanamycin resistance gene; IRES, internal ribosome entry site.

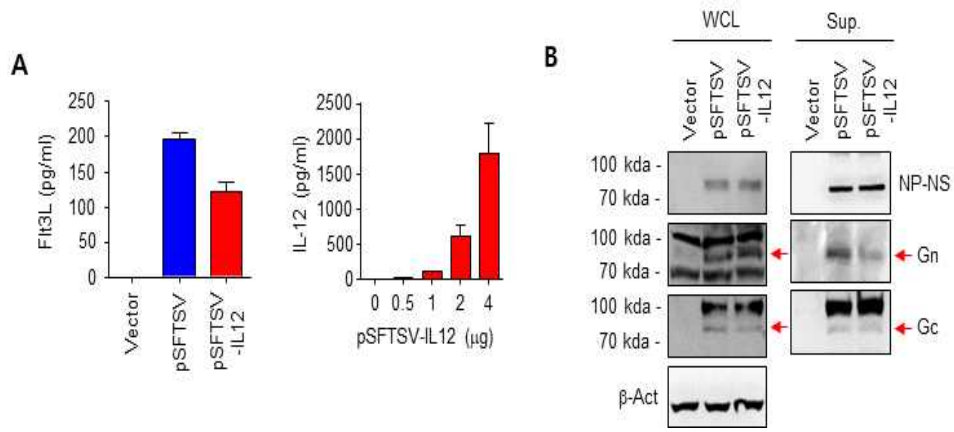


Figure 2. Expression of each of SFTSV antigens and cytokines after SFTSV DNA transfection.

(A) Expression of Flt3L and IL-12 cytokines in supernatant of HEK293 cells transfected with each of DNA was assessed by ELISA measurement. Data are presented as mean + S.D from triplicated experiments. (B) Expression of viral antigens, Np-NS fusion (~80kDa), Gn(~74kDa), and Gc(~71kDa) proteins, in whole-cell lysates (left panels) and culture supernatants (right panel) from transfected HEK293 cells, was confirmed by western blot analysis using anti-Gn, Gc, or NP antibodies. The position of each of the viral proteins is indicated by an arrow. β-actin was used as loading control.

Antibody and T cell responses against the viral antigens by immunization of the DNA vaccines.

Next, antibody and T cell immune responses in IFNAR K/O mice vaccinated mock, pSFTSV, and pSFTSV-IL12 DNA plasmids were evaluated. Antibody response against the Np antigen had considerably increased (mean titer ± S.D.: 255 ± 63, n=3) only in the mice vaccinated with pSFTSV-IL12 one week after the third immunization (Part Ⅰ, Fig 3A). SFTSV Gn and Gc specific antibodies were hardly detectable in all the DNA vaccine groups, indicating that these DNA vaccines may not efficiently induce SFTSV glycoproteins specific antibodies, but SFTSV NP-specific antibodies could be induced in the vaccination of pSFTSV-IL12. Neutralizing assay tests using sera from mice vaccinated with mock, pSFTSV, and pSFTSV-IL12 DNA one week after the third immunization showed neutralizing titer of pSFTSV-IL12 vaccine group, which was higher than both the mock and pSFTSV vaccine groups (Part Ⅰ, Fig 3B).

To assess T cell immunity in IFNAR K/O mice vaccinated with mock, pSFTSV, and pSFTSV-IL12 DNA, a percentage of SFTSV antigens specific IFN- ɣ producing T cells in the spleen of mice immunized with SFTSV DNA vaccines was measured (Part Ⅰ, Fig 3C and D). Splenocytes from vaccinated mice were stimulated with SFTSV NP or Gn antigens and IFN-ɣ secreting T cell subsets were analyzed by flow cytometry. The percentage of NP or Gn specific IFN-ɣ secreting $CD4^+$ and $CD8^+$ T cells of mice vaccinated with pSFTSV-IL12 was considerably higher than those of mice vaccinated with the mock vector. Even though the levels were not statistically significant, the antigen specific IFN-ɣ

producing T cell from pSFTSV vaccinated mice were commonly elevated. All of this data indicated that the expression of IL-12 may increase T cell immunity against the viral antigens.

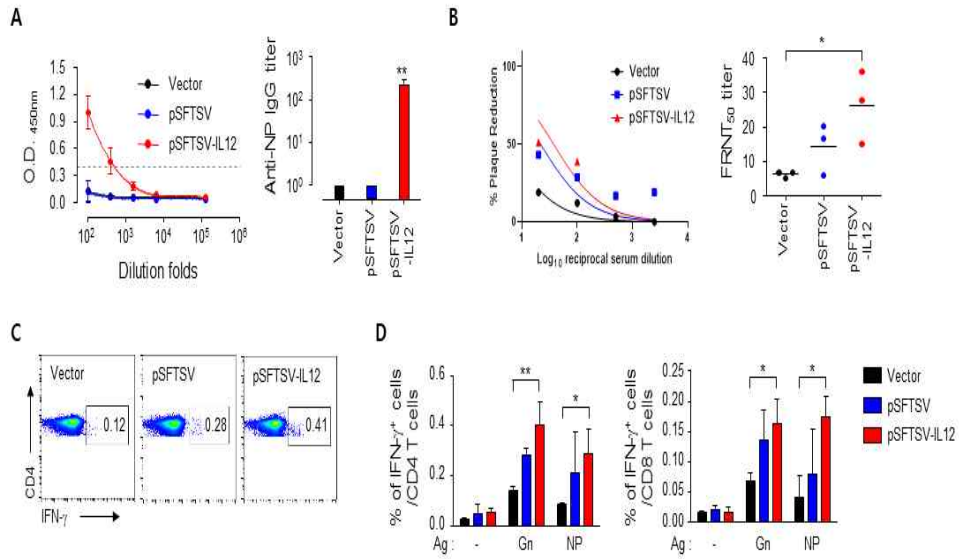


Figure 3. Antibody or T cell immune response of SFTSV DNA vaccine against viruses.

(A) Anti-Np IgG response was measured by ELISA at two weeks after the third immunization of each of DNA are presented. Cut-off titers (dashed line, mean O.D. + 3x S.D. at 1:100 diluents) was determined by sera of vector immunized mice. Error bar: mean ± S.D.

(B) Neutralizing antibody response to SFTSV of each indicated DNA vaccines. The amount of neutralizing antibody was determined based on $FRNT_{50}$. *, $p < 0.05$.

(C and D) Antigen specific IFN-ɣ cytokine-producing $CD4^+$ and $CD8^+$ T cells from immunized mice at two weeks after the third vaccination were analyzed using flow cytometry after stimulation with the indicated antigens. Representative flow cytometric results are displayed (C) and the percentage of IFN-ɣ secreting $CD4^+$ or $CD8^+$ T cells were summarized (D). Data are shown as mean + S.D. from duplicate assays with three mice per group. *, $p < 0.05$; **, $p < 0.01$.

Protection of DNA plasmid vaccine encoding SFTSV antigens and IL-12 anainst .

To assess whether the SFTSV DNA vaccines can provide a protective ability against lethal virus challenge, mock vector, pSFTSV, or pSFTSV-IL12 plasmid DNA were immunized to IFNAR K/O mice three times through electroporation before injection of 10^5 FFU SFTSV (Part Ⅰ, Fig 4). After virus infection, all groups of mice were monitored for survival rate, body weight, platelet counts, and viral titers in plasma. Following the challenge virus, all of the mice (n=5) immunized with mock vector were dead on 5 day post-infection, while 40% (2 of 5) of the pSFTSV vector immunized mice survived, and all mice vaccinated with pSFTSV-IL12 were completely protected from the viruses (Part Ⅰ, Fig 4A, left panel). Consistently, the mean body weight of mock-vaccinated mice rapidly decreased to around 70% of their original body weight on 4 day post-infection, and then all the mice were dead. However, the pSFTSV-IL12 vaccine group lost weight until they reached 82% of their original body weight on day 4 after challenge and recovered thereafter. Surviving mice in the pSFTSV vaccine group lost weight until they reached 78% of their original body weight on 6 day post-infection and then recovered. (Part Ⅰ, Fig 4A, right panel). When I examined the platelet counts of each immunization group during the acute phase of infection, the mock vector injection group dramatically decreased until dead, while the counts of mice vaccinated with pSFTSV or pSFTSV-IL12 slightly declined and the pSFTSV-IL12 group's counts recovered at 4 day post-infection (Part Ⅰ, Fig 4B, left panel). Furthermore, viral titers were significantly reduced in the

plasma of the pSFTSV or pSFTSV-IL12 vaccinated mice compared to the mock vector vaccinated mice at 4 day post-infection (Part Ⅰ, Fig 4B, right panel). In detail, despite similar initial viral titers (~ 10^6 copies/ml of plasma) at 2 day post-infection in all vaccinated groups, the titer of pSFTSV-IL12 vaccinated mice (mean ± S.D. = $4.2 \times 10^6 \pm 4.6 \times 10^6$) was about five to fifty times lower than that of pSFTSV immunized mice (mean ± S.D. = $2.1 \times 10^7 \pm 1.7 \times 10^7$) or mock immunized mice (mean ± S.D. = $2.0 \times 10^8 \pm 1.9 \times 10^8$) at day 4 post-infection, respectively.

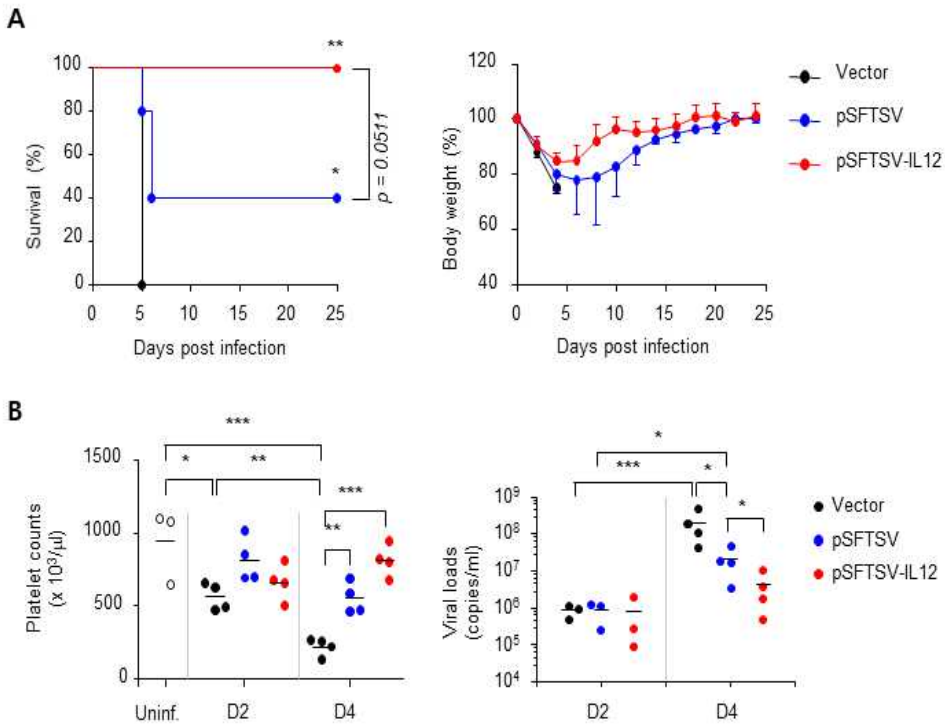


Figure 4. Survival, relative body weight change, hematological analysis, and distribution of viral RNA copy number in plasma of SFTSV DNA plasmid immunized IFNAR K/O mice against lethal SFTSV challenge.

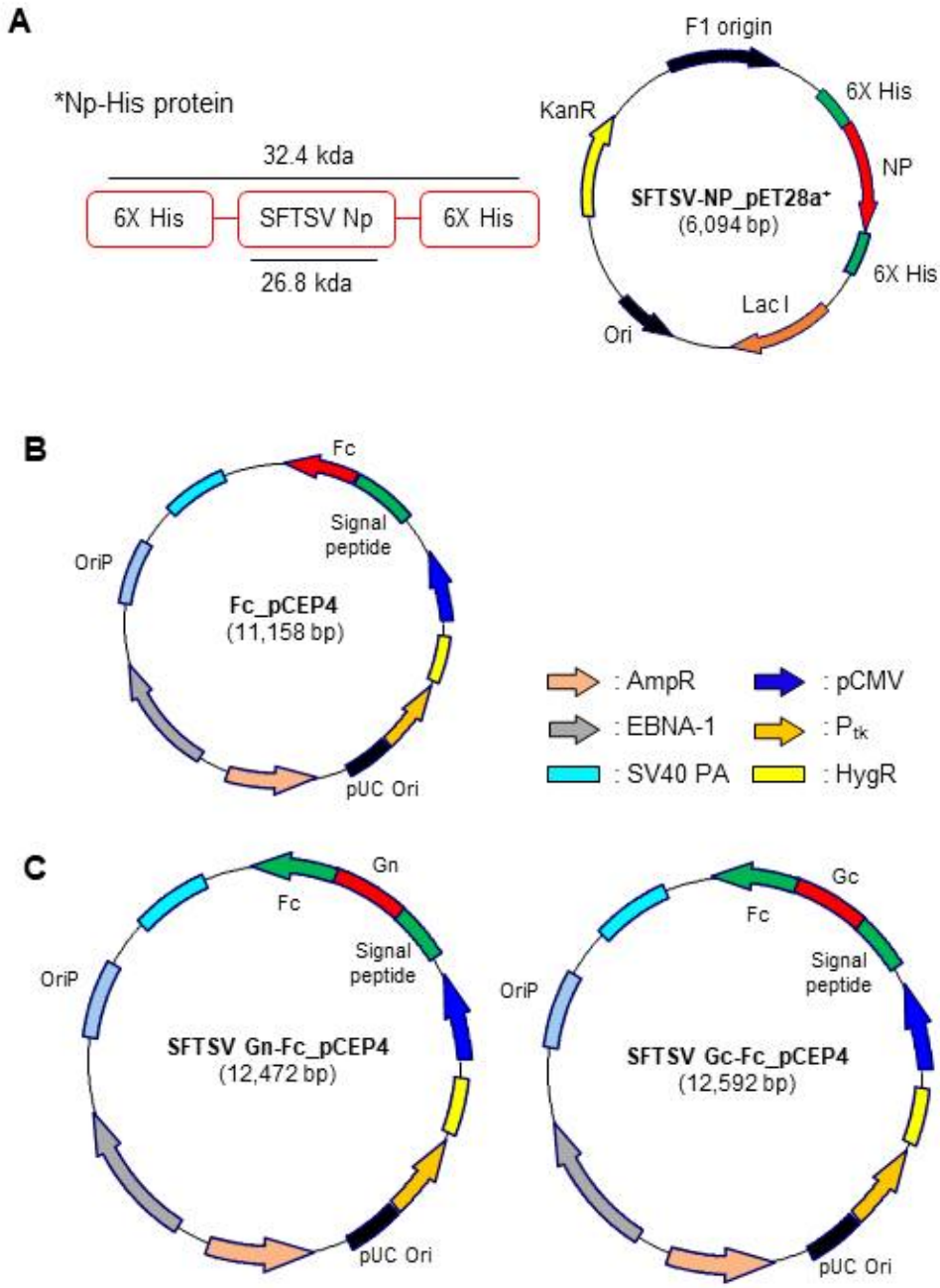
(A) Survival proportion (left panel) and body weight change (right panel) of three times vaccination of indicated DNAs into IFNAR K/O mice (n=5/group) and challenged with 10^5 FFU of SFTSV through S.C. at two weeks after vaccination. (B) Platelet counts (left panel) and viral loads in plasma (right panel) of vaccinated mice on 2 day post-infection (D2) and 4 dpi (D4). Uninf, uninfected; n=3~4 mice/group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Part Ⅱ.
Evaluation of SFTSV subunit vaccine.

Characterization and expression of recombinant SFTSV subunits.

Three major SFTSV antigens (Nucleocapsid protein and Glycoprotein N&C) were cloned into different vectors to be purified with a high rate of purity. Recombinant SFTSV Nucleocapsid proteins (NP) were fused with six histidine peptides at both N and C-terminus in the pET28a^{+} bacterial vector (Part Ⅱ, Fig. 1A). T7 promoter and lac I genes in the pET28a^{+} vector suppress the target protein gene without lactose or IPTG [49]. The expression of recombinant SFTSV NP in BL21(DE3) *E.coli* whole cell lysate was detected using the anti-His HRP antibody (Part Ⅱ, Fig. 1E). Glycoprotein N&C, which are viral surface proteins, were conjugated with signal peptides at N-terminus and human IgG Fc fragment at C-terminus and cloned into the pCEP4 vector to create a specialized protein purification from mammalian cells (Part Ⅱ, Fig.1C). The plasmid vector is under the control of the CMV promoter and enhanced gene expression efficiency and maintenance by the EBNA-1 gene. The recombinant Gn or Gc proteins could be secreted thanks to signal peptides to the extracellular area. Recombinant Gn or Gc-Fc proteins that could be expressed outside of HEK293F cells reacted with the anti-human IgG HRP antibody (Part Ⅱ, Fig 1G). Only human IgG Fc proteins were also cloned into the pCEP4 vector plasmid to be used as a negative control (Part Ⅱ, Fig 1B). Fc proteins were

also confirmed to be expressd outside of HEK293F cells (Part Ⅱ, Fig. 1F). Glycoproteins fused with signal peptides at N-terminus and six histidine at C-terminus were inserted into the pcDNA3 vector (Part II, Fig 1D). This plasmid vector also has the CMV promoter which can strongly express the genes. The recombinant Gn and Gc proteins expressed in HEK293F cells were confirmed with the anti-His HRP antibody (Part II, Fig 1H).


A
*Np-His protein
32.4 kda
6X His
SFTSV Np
6X His
26.8 kda
F1 origin
6X His
NP
6X His
Lac I
Ori
KanR
SFTSV-NP_pET28a+
(6,094 bp)
B
Fc
Signal peptide
OriP
Fc_pCEP4
(11,158 bp)
pUC Ori
: AmpR
: pCMV
: EBNA-1
: Ptk
: SV40 PA
: HygR
C
Gn
Fc
Signal peptide
OriP
SFTSV Gn-Fc_pCEP4
(12,472 bp)
pUC Ori
Gc
Fc
Signal peptide
OriP
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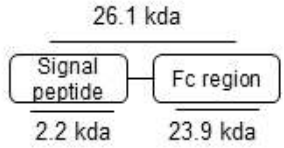
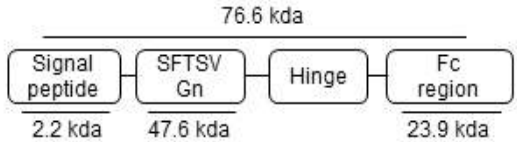
pUC Ori

*Fc protein

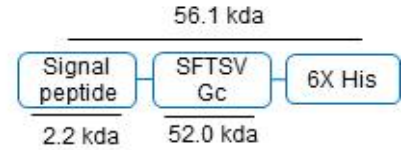


*Gn-His protein

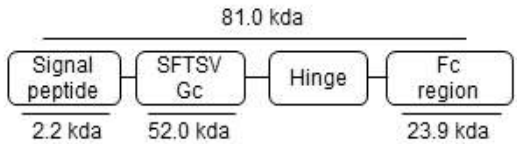
*Gn-Fc protein



*Gc-His protein



*Gc-Fc protein



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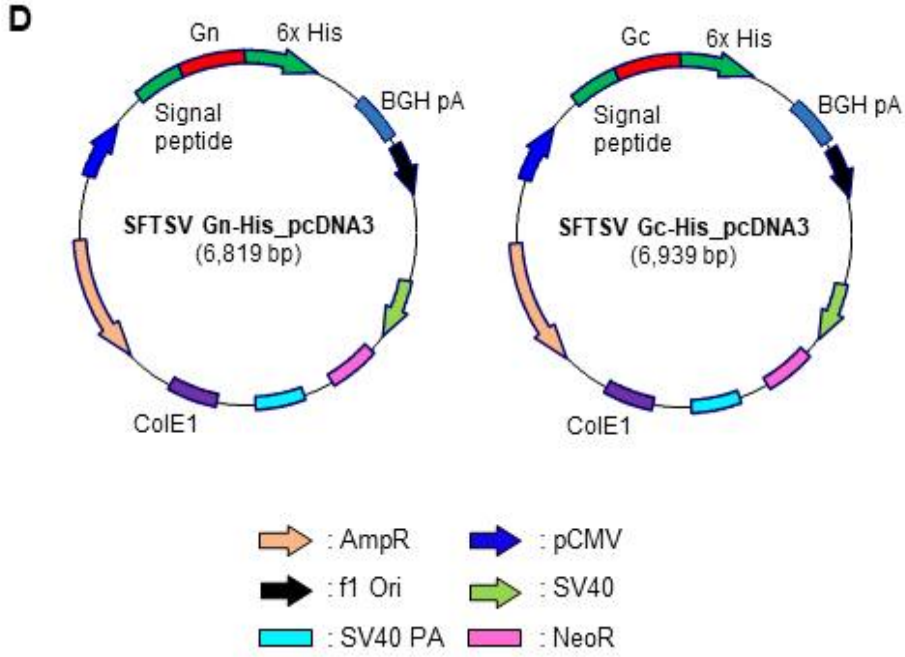


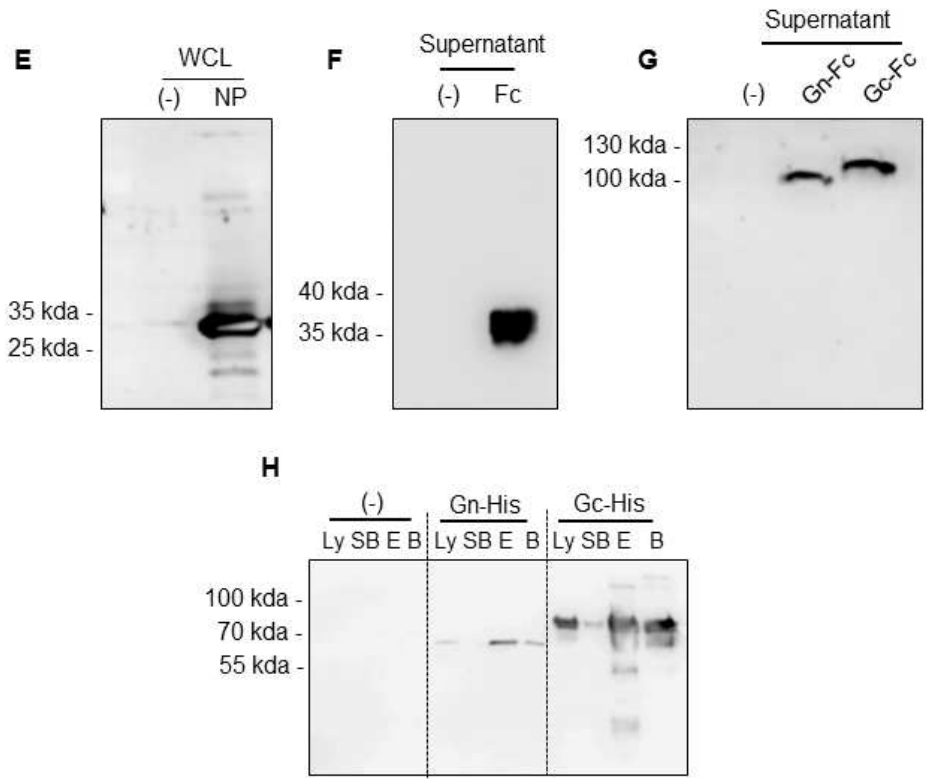


Figure 1. Map of SFTSV subunits expression vector and recombinant proteins expression test.

(A-D). Each of protein expression plasmid and schematic diagram showing recombinant SFTSV proteins size and composition.

(A) Recombinant SFTSV N protein fused with six histidine gene was inserted into pET28a^{+} vector. Ori; KanR, Kanamycin resistance gene; Iac I, regulatory gene for Iac operon.

(B and C) Human IgG Fc fragment or recombinant SFTSV fused with Fc fragment gene was inserted into pCEP4 vector plasmid. Ori, pUC origin; ApmR, Ampicillin resistance gene; HygR; Hygromycin resistance gene; CMV promoter; SV40 PA, SV40 poly A; EBNA-1,

EBV origin of nuclear antigen.
(D) Recombinant SFTSV Glycoprotein N&C (Gn&Gc) with six histidine peptides gene was inserted into pcDNA3 vector. Ori; SV40 PA, SV40 poly A; NeoR, Neomycin resistance gene; KanR, Kanamycin resistance gene; CMV promoter.
(E-H) Confirmation of recombinant SFTSV nucleocapsid protein (Np) (E), Fc protein (F), Glycoprotein N&C(Gn&Gc) fused with Fc fragment (G) or His peptides (H) expression by immunoblot analysis using anti-His-HRP or anti-human IgG-HRP antibodies.

Production of recombinant SFTSV nucleocapsid protein-tagged His peptides from *E.coli*

Isopropyl-β-thiogalactopyranoside(IPTG) has been used for the overexpression of recombinant proteins in BL21(DE3) *E.coli* system. I used this overexpression system to purify recombinant SFTSV Nucleocapsid proteins (rSFTSV NP). To determine the high-level expression condition, I tested several induction processes based on two different temperatures (16℃ and 37℃) and four various IPTG concentrations (0.0, 0.1, 0.5, and 1.0mM). I also checked whole bacterial cell lysate, supernatant after centrifuge of lysate, and pellets after centrifuge of lysate to confirm whether recombinant NPs were soluble or insoluble proteins. Bacterial cells were induced with each of the different IPTG concentrations at an optical density (OD) at 600nm of 0.6-0.8, and they were cultured overnight at 16℃ or for 5hrs at 37℃. Overall SDS-PAGE results showed 16℃ induction could produce more recombinant NPs in all of the whole cell lysate, supernatant, and pellets (Part Ⅱ, Fig 2A). Although N proteins were placed in both supernatant and pellets, these proteins were usually found in the supernatant, suggesting that recombinant SFTSV NP may be soluble proteins. Also, NP could be induced with a high level at over 0.1mM IPTG condition. Immunoblot analysis of recombinant NPs of 16℃ and 37℃ with 0.1mM IPTG induction showed that 0.1mM IPTG induction at 16℃ would be the best process of SFTSV NP overexpression (Part Ⅱ, Fig 2B).

Next, I aimed to develop a step to further purify the high purity of recombinant SFTSV N proteins. Because lots of other non-target proteins in bacterial cell lysate could bind to Ni-NTA

bead, I had to remove non-target proteins for the high purity SFTSV NP using a certain concentration of imidazole. The washing step was processed with various concentrations of imidazole (0-200mM) and recombinant SFTSV NPs were eluted by 250mM imidazole, then SDS-PAGE analysis showed the purity of recombinant target proteins (Part Ⅱ, Fig 3A-E). 10mL of BL21(DE3) *E.coli* transformed rSFTSV NP plasmid vector was induced in 16℃ overnight with 0.1mM of IPTG, then the *E.coli* lysate was incubated with Ni-NTA bead. First, I washed rSFTSV NP binding to Ni-NTA bead with an increasing imidazole concentration from 0mM to 200mM. After treating with 100mM of imidazole, other non-target proteins were cleared (Part Ⅱ, Fig 3A). Next, I tested washing two times with a lower concentration (10mM) imidazole (Part Ⅱ, Fig 3B) and washing two times with a higher concentration (70 and 100mM) imidazole (Part Ⅱ, Fig 3C). Higher concentration washing was more efficient to remove non-specific proteins. Then, washing two times with 70mM imidazole and three times with 100mM imidazole showed gradual removal of non-specific proteins (Part Ⅱ, Fig 3D). Finally, the process of washing five times with 1mL of 100mM imidazole and 1mL of 250mM imidazole for elution provided a high purity of rSFTSV NP, furthermore, target proteins were no longer bound to bead after elution (Part Ⅱ, Fig 3E). These recombinant N proteins were confirmed by immunoblot using anti-histidine or anti-NP antibodies (Part Ⅱ, Fig 4).

To sum up, BL21(DE3) *E.coli* transformed rSFTSV-NP vector plasmid could be overexpressed at 16℃ overnight with 0.1mM IPTG, then washing 5 times with 100mM imidazole and 2-3mL

250mM imidazole elution per 1L *E.coli* culture was the best condition for rSFTSV-NP purification.

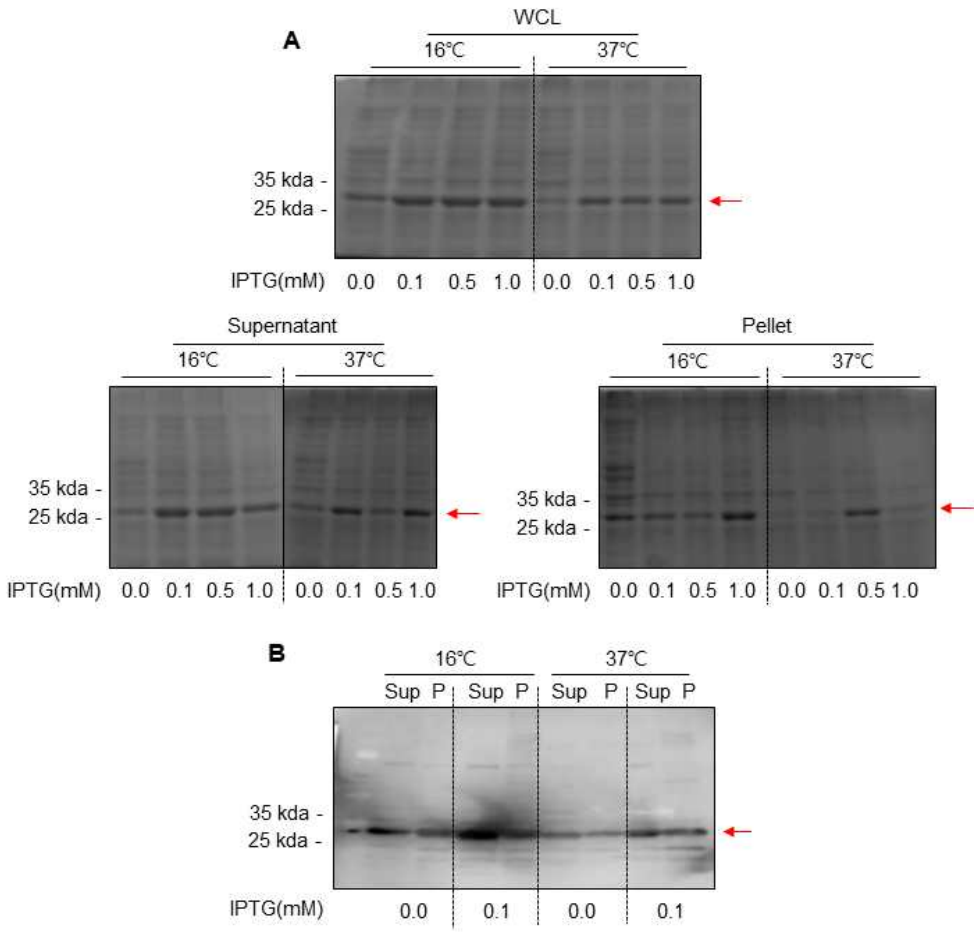


Figure 2. Recombinant SFTSV nucleocapsid protein expression test depends on Isopropyl-β-thiogalactopyranoside (IPTG) concentration or temperature.

(A) SDS-PAGE of whole cell lysate (WCL), supernatant after centrifuge of cell lysate (Supernatant), and pellet after centrifuge of cell lysate (Pellet) from BL21 *E.coli* induced by various IPTG

concentration (0.0, 0.1, 0.5, and 1.0mM) in 16℃ for 18hrs or 37℃ for 5hrs. (B) Western blot analysis of recombinant N proteins in some induction conditions. The position of recombinant SFTSV N proteins is indicated by an arrow. Sup: Supernatant of lysate after centrifuge, P: Pellet of lysate after centrifuge.

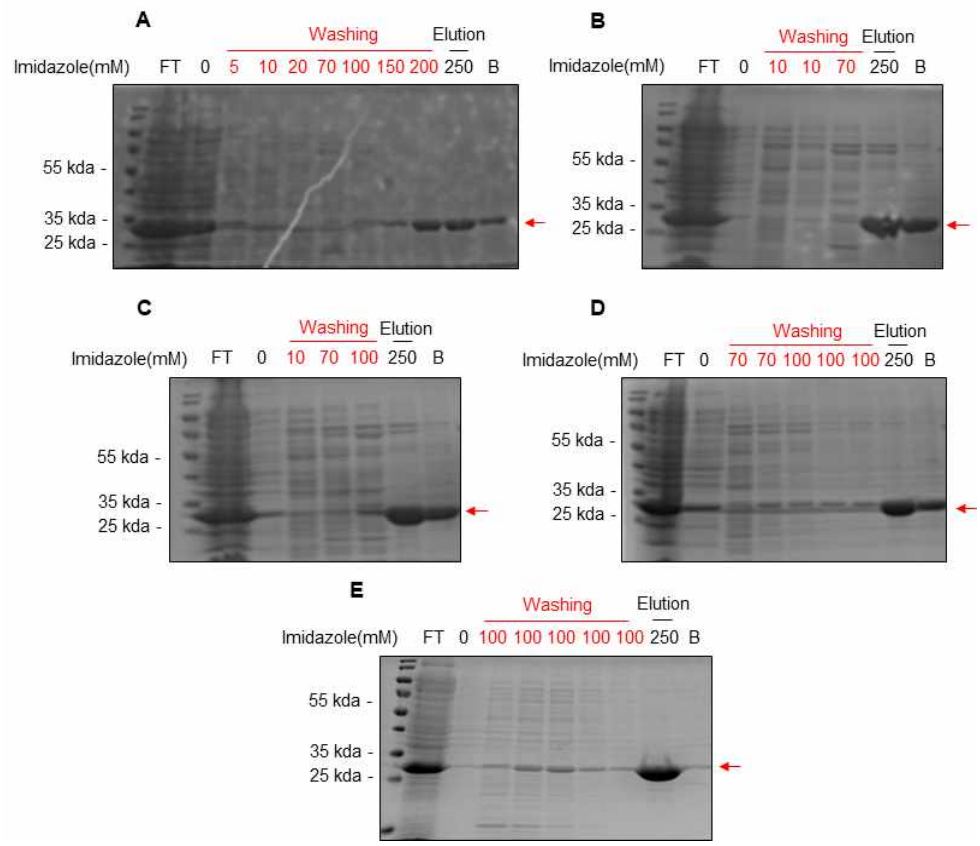


Figure 3. Washing condition of recombinant SFTSV N protein using various imidazole concentration.

(A-E). SDS-PAGE of washing conditions of recombinant SFTSV N proteins using a variety of imidazole concentration. FT, Flow-through after Ni-NTA bead binding; Washing, washing step of bead binding rSFTSV Np-His; Elution, elution of rSFTSV Np-His from bead using 250mM imidazole; B, Ni-NTA bead after eluting of rSFTSV Np-His. The position of recombinant SFTSV N proteins is indicated by an arrow.

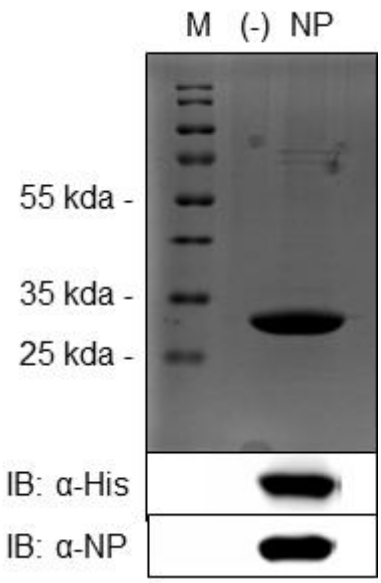


Figure 4. High purity of recombinant SFTSV N protein-tagged His peptides.

SDS-PAGE and Western-blot analysis high purity of recombinant SFTSV N protein expressed in BL21 *E.coli.* SDS-PAGE shows Protein marker (M), protein purified from BL21 *E.coli* transformed empty pET28^{+} vector as a negative control (-), and recombinant SFTSV N protein expressed from BL21 *E.coli* and washed with imidazole (NP). Anti-Histidine-HRP antibody and rabbit polyclonal anti-N protein-specific antibody detected target recombinant protein in Western blot.

Purification strategies of recombinant SFTSV glycoproteins using mammalian cells.

Glycoproteins N&C which are membrane proteins of SFTSV are important for entering the host cell to infect [50, 51]. For this reason, these proteins have been used for vaccine targets [3, 26, 27]. Recombinant SFTSV glycoprotein N&C (Gn&Gc) fused with Fc region a C-terminus cloned vector plasmid was thankfully provided by Prof. Chung. HEK293T and HEK293F cells were used for expression and purification of glycoproteins, respectively. First, I tested whether glycoproteins fused with Fc fragments bound to protein A/G bead. The supernatant of HEK293T cells transfected was treated with protein A/G bead for an hour at 4℃. The supernatant of HEK293F cells without transfection was the negative control. Immuno-blot analysis of supernatant or bead after centrifuge of bead-bound with glycoproteins was showed using anti-human IgG Fc fragment HRP antibody (Part Ⅱ, Fig 5). Reactivity of Gn or Gc-Fc proteins with antibodies at Bead lane indicated that Gn or Gc-Fc proteins could be expressed in mammalian cells and bind with protein A/G bead stably.

To obtain a huge amount of rSFTSV Gn or Gc proteins, I tested four different transfection conditions with rSFTSV glycoproteins. Polyethylenimine (PEI) was used for transfection and the DNA to PEI ratio was 1:4. A million cells per mL were transfected with 0, 1.0, 1.5, and 2.0 μg DNA per ml, respectively, then cells were analyzed by immunofluorescence confocal microscopy during 1 day post-transfection (dpt). The percentage of transfected cells showed 1.0 μg per mL DNA was the most efficient condition (Part Ⅱ, Fig 6A). Next, I tested translation efficiency after transfection

with the rSFTSV glycoproteins plasmids. The supernatant of transfected HEK cells was bound to protein A/G bead at each 2-5 days post-transfection. Each day's protein amount was analyzed based on the BSA amount (Part Ⅱ, Fig 6B, bottom panel). The protein amount was the highest at 2 day post-transfection and gradually decreased until day 5 (Part Ⅱ, Fig 6B). It is important to maintain cell viability for protein expression after transfection.

The viability of HEK293F cells was also tested during 7 day post-transfection. Transfected HEK cells without media change gradually decreased, and finally all cells were dead at 7 day post-transfection, while the viability of HEK cells with a media change at dpt 2 and 5 increased until dpt 7 (Part Ⅱ, Fig 6C). With these conditions, the rSFTSV Gn and Gc-Fc proteins were purified using the AKTA system. The supernatant of HEK293F cells without transfection did not display any proteins in the first lane (-). The flow-through of transfected HEK293F cells supernatant after protein A/G bead binding showed other non-specific proteins, while any protein was not shown in the washing solution lane with buffer A (20mM sodium phosphate, 0.15M NaCl, PH 7.2) and the elution lane showed a high purity of rSFTSV Gn or Gc-Fc proteins (Part Ⅱ, Fig 7A and B). rSFTSV glycoproteins were confirmed by immunoblot analysis using anti-human IgG Fc region antibodies.

Collectively, these results indicated that HEK293F cells transfected with 1 μg per mL of rSFTSV glycoproteins and 4 μg per mL PEI during 6 days post-transfection could express a high level of rSFTSV Gn and Gc-Fc, and the proteins were washed with buffer A (20mM sodium phosphate, 0.15M NaCl, PH 7.2), then eluted with buffer B (0.1M sodium citrate, pH 3.0).

To acquire various recombinant SFTSV glycoproteins form, I designed rSFTSV glycoproteins fused with six histidine at C-terminus. His fused glycoproteins were also tested at a high expression condition and washing step. The transfection condition of rSFTSV glycoprotein fused Fc were used for purifying SFTSV Gn and Gc-His proteins. When I measured the translation efficiency of rSFTSV Gn-His protein, the protein amount was the highest on 5 day post-transfection based on the BSA amount (Part Ⅱ, Fig 8). Furthermore, due to the removal of non-specific proteins from the Ni-NTA bead, rSFTSV Gn and Gc-His proteins also needed the washing process. Recombinant Gn-His proteins were washed with 500ul of 0 to 150mM imidazole concentration and eluted with 250mM imidazole. Non-target proteins were gradually removed by increasing the imidazole concentration from 20mM to 150mM. However, over 70mM imidazole could elute rSFTSV Gn-His proteins from bead (Part Ⅱ, Fig 9A). For this reason, I designed a washing step using 500ul of 50mM imidazole five times. Beads were gradually washed from the non-target proteins without eluting target Gn-His proteins. Finally, a high purity of Gn-His protein was obtained at 250mM imidazole (Part Ⅱ, Fig 9B). rSFTSV Gn-His proteins were assessed by western blot using an anti-histidine antibody (Part Ⅱ, Fig 9A and B). Recombinant Gc-His proteins were tried with a rinse of 500ul of 50, 40, 30, 25, and 20mM imidazole five or six times. The 40 and 50mM washing steps were efficient for removing non-specific proteins, but, lots of Gc-His proteins were eluted at every stage during the 40 and 50mM washing step (Part Ⅱ, Fig 9C and D). A lower imidazole concentration of 25 and 30mM washing steps showed a small amount of SFTSV Gc-His proteins were detached

from the bead in every washing stage (Part Ⅱ, Fig 9E and F). A high purity of Gc-His protein without losing was obtained in 20mM imidazole washing (Part Ⅱ, Fig 9G). A high purity of rSFTSV Gn and Gc-His proteins were assessed by SDS-PAGE and immuno-blot using anti-histidine antibodies (Part Ⅱ, Fig 10).

Altogether, data showed that washing with 500ul of 50mM imidazole five times may be the best purification process for rSFTSV Gn-His proteins and washing with 1mL of 20mM imidazole six times could be the best purification method for rSFTSV Gc-His proteins.

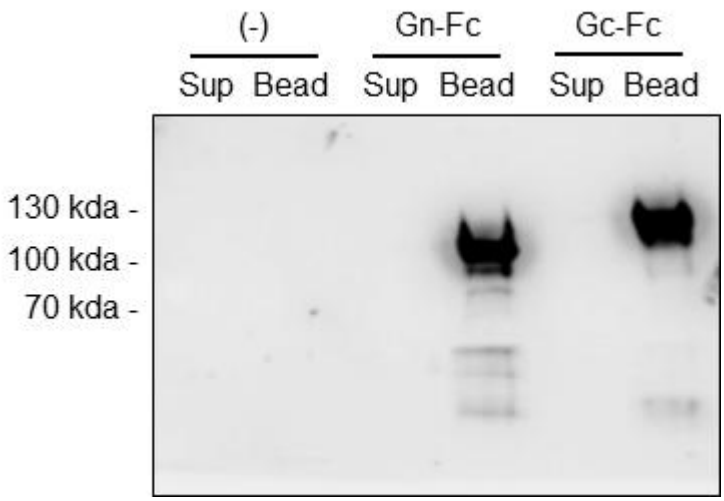
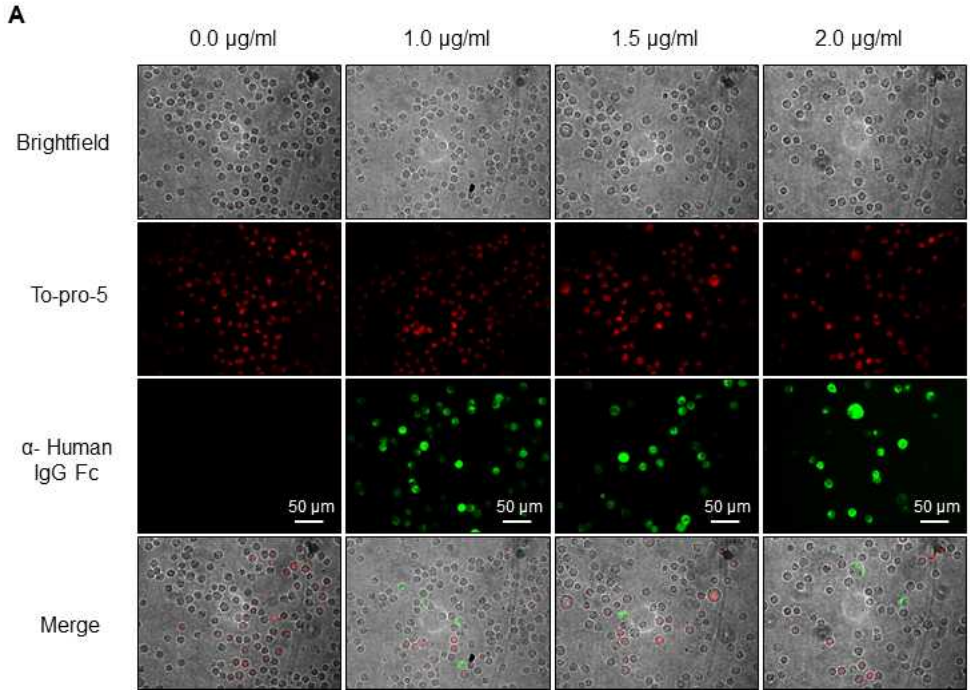
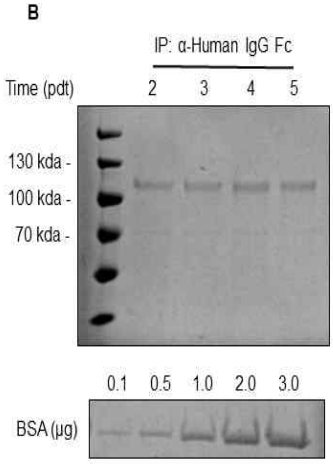


Figure 5. Bead binding ability of recombinant SFTSV glycoprotein N&C fused with human IgG Fc fragment.

Western-blot analysis of binding ability of recombinant SFTSV glycoprotein N&C fused human IgG Fc domain to protein A/G bead. Supernatant of empty pCEP4 vector transfected HEK293T cells (-), supernatant of Gn-Fc plasmid transfected HEK293T cells (Gn-Fc), or supernatant of Gc-Fc plasmid transfected HEK293T cells (Gc-Fc) were bound by protein A/G bead. Sup: Supernatant after centrifuge of bead, Bead: Bead after centrifuge of bead. Reactivity of supernatant of recombinant Gn or Gc-Fc after centrifuge post bead binding, and bead to anti-human IgG HRP antibody.





Day post-transfection (dpt)	Protein amount (μg)
2 dpt	15.236
3 dpt	9.376
4 dpt	9.561
5 dpt	7.276

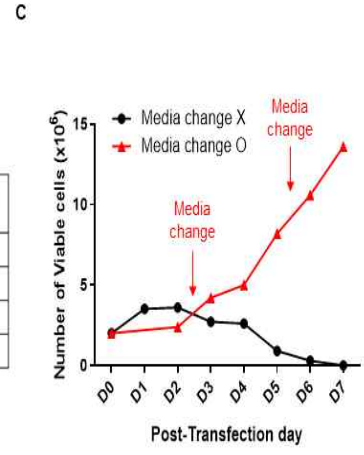


Figure 6. Expression condition of SFTSV glycoprotein N&C fused with human IgG Fc fragment.
(A) Immunofluorescence confocal microscopy using To-pro-5 antibody (α-nucleus) and anti-IgG Fc antibody (α-human IgG Fc) showed nucleus and recombinant SFTSV glycoprotein C (Gc) fused with human IgG Fc fragment in HEK293F cells after transfection, repectively. Transfection DNA amount was indicated with 0.0, 1.0, 1.5, and 2.0 μg per ml. Transfection rate(%), 0.0 μg DNA/ml: 0.0%, 1.0 μg DNA/ml: 42.7%, 1.5 μg/ml: 39.1%, 2.0 μg/ml: 33.3%. Transfection percentage = transfected cells / total HEK293F cells X 100.
(B) SDS-PAGE of protein A/G bead binding recombinant SFTSV Gc-Fc proteins 2-5 days post-transfection (dpt). Protein amount was determined by the BSA amount displayed in lower SDS-PAGE gel using the Image J program.
(C) Viability of HEK293F cells after transfection with changing media (Redline) or without changing media (Blackline) during 7 days post-transfection (dpt).

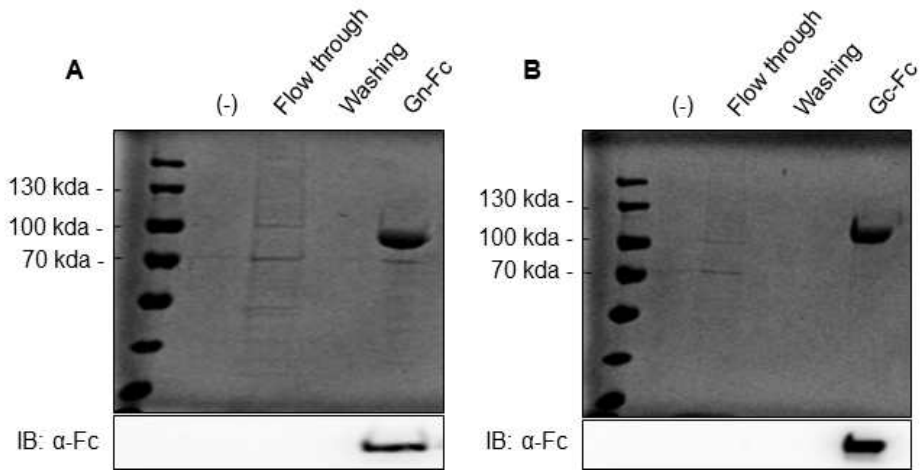
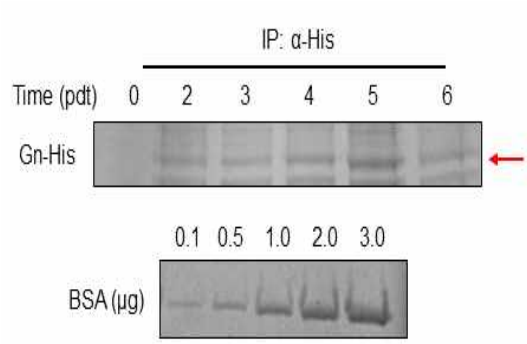


Figure 7. Purification of recombinant glycoprotein N&C fused with human IgG Fc fragment.

(A and B) SDS-PAGE and Western-blot analysis of high purity purification of recombinant SFTSV glycoprotein N&C fused with Fc fragment using AKTA start. Flow through, Flow-through after protein A/G bead binding; Washing, washing step of bead binding rSFTSV Gn or Gc-Fc. Recombinant proteins (Gn-Fc and Gc-Fc) were assessed by immunoblot analysis using the anti-human IgG Fc antibody.



Day post-transfection (dpt)	Protein amount (μg)
0 dpt	0.00
2 dpt	0.10
3 dpt	0.09
4 dpt	0.31
5 dpt	0.76
6 dpt	0.22

Figure 8. Expression condition of SFTSV glycoprotein N&C fused with His peptides.

SDS-PAGE analysis of translation efficiency of recombinant glycoprotein N protein in the supernatant of transfected HEK293F cells. Recombinant Gn-His proteins binding with Ni-NTA bead during 0-6 day post-transfection (dpt) were assessed. The protein amount was determined by the BSA amount displayed in lower SDS-PAGE gel using the Image J program.

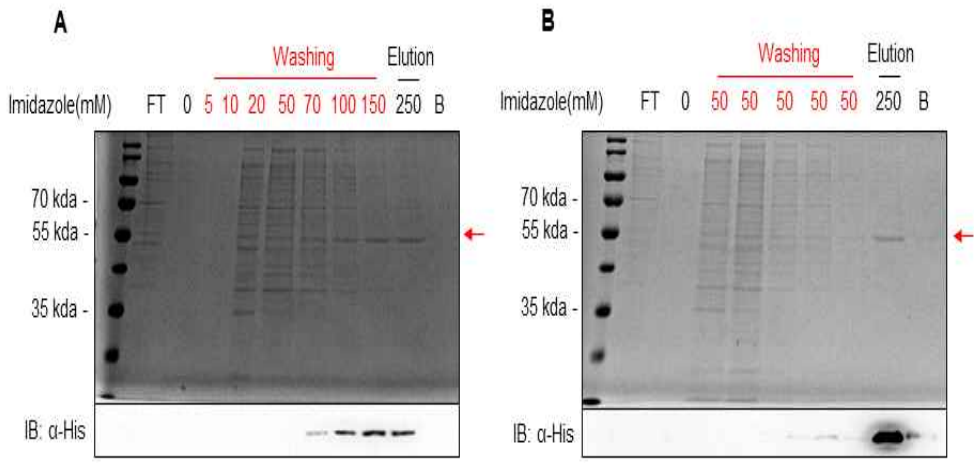
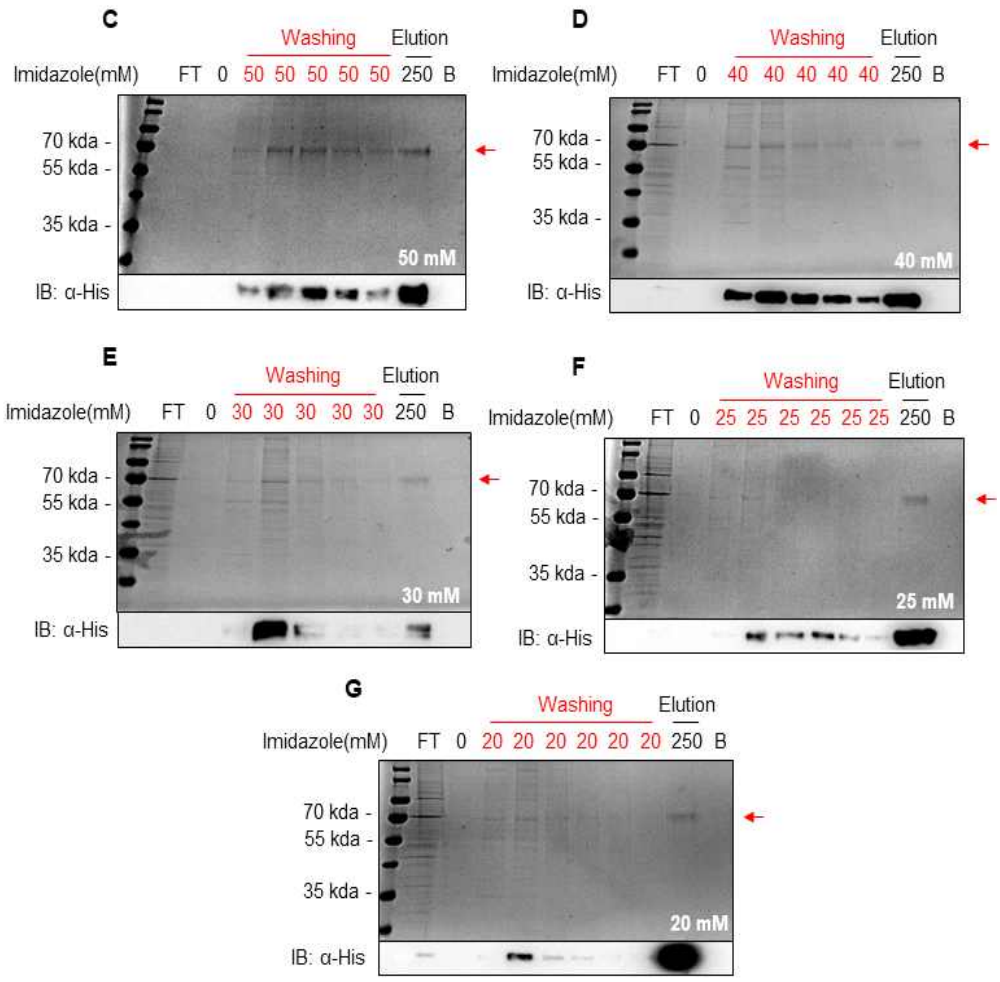


Figure 9. Washing conditions of recombinant SFTSV glycoprotein N&C fused with His peptides using various imidazole concentration.
(A and B) SDS-PAGE and Immunoblot analysis of recombinant SFTSV glycoprotein N (Gn) fused His peptides washing condition using various imidazole concentrations. FT, Flow-through after Ni-NTA bead binding; Washing, washing step of bead binding rSFTSV Gn-His; Elution, elution of rSFTSV Gn-His from bead using 250mM imidazole; B, Ni-NTA bead after eluting of rSFTSV Gn-His. Position of recombinant SFTSV Gn is indicated by arrow. The recombinant proteins (Gn-His) were assessed by immunoblot analysis using the anti-Histidine-HRP antibody.


C
Washing
Elution
Imidazole(mM)
FT 0 50 50 50 50 50 250 B
70 kda -
55 kda -
35 kda -
50 mM
IB: α-His
D
Washing
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Imidazole(mM)
FT 0 40 40 40 40 40 250 B
70 kda -
55 kda -
35 kda -
40 mM
IB: α-His
E
Washing
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35 kda -
30 mM
IB: α-His
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Washing
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Imidazole(mM)
FT 0 25 25 25 25 25 25 250 B
70 kda -
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25 mM
IB: α-His
G
Washing
Elution
Imidazole(mM)
FT 0 20 20 20 20 20 20 250 B
70 kda -
55 kda -
35 kda -
20 mM
IB: α-His

Figure 9. A variety of imidazole concentration washing conditions of recombinant SFTSV glycoprotein N&C fused with His peptides.
(C-G) SDS-PAGE and Immunoblot analysis of recombinant SFTSV glycoprotein C (Gc) fused His peptides washing condition using various imidazole concentrations. FT, Flow-through after Ni-NTA bead binding; Washing, washing step of bead binding rSFTSV Gc-His; Elution, elution of rSFTSV Gc-His from bead using 250mM imidazole; B, Ni-NTA bead after eluting of rSFTSV Gc-His. Position of recombinant SFTSV Gc is indicated by arrow. The recombinant proteins (Gc-His) were assessed by immunoblot analysis using the anti-Histidine-HRP antibody. (C-G) The right lower white letter displayed at each of SDS-PAGE panel showed washing imidazole concentration of recombinant SFTSV Gc proteins.

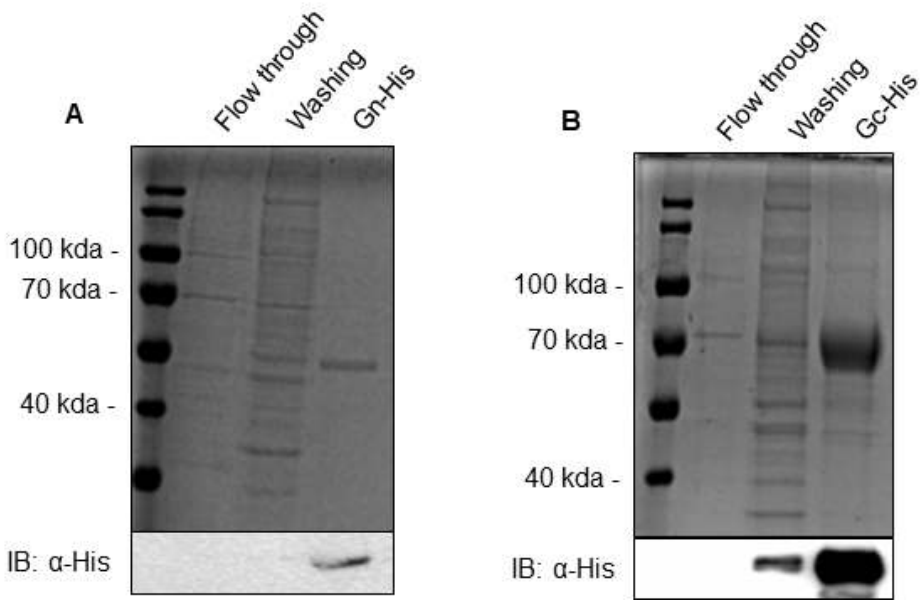


Figure 10. Purification of recombinant glycoprotein N&C fused with His peptides.

(A and B) SDS-PAGE and Immunoblot analysis of high purity purification of recombinant SFTSV glycoprotein N&C fused with Histidine peptides using AKTA start.

Flow through, Flow-through after Ni-NTA bead binding; Washing, washing step of bead binding rSFTSV Gn or Gc-His. Recombinant proteins (Gn-His and Gc-His) were assessed by immunoblot analysis using the anti-Histidine-HRP antibody.

Protection of single subunit vaccine against a lethal dose of SFTSV in IFNAR K/O mice.

Antibody dependent immunity and T cell immune responses in IFNAR K/O mice vaccinated with a single SFTSV subunit vaccine were evaluated. Vaccination with a single SFTSV subunit with alum adjuvant significantly increased antigen specific antibody titer for immunized antigens in IFNAR K/O mice (Np: mean ± S.D. = 102,400 ± 0.0, *n*=5, Gn: mean ± S.D. = 15,040 ± 5,903, *n*=5, Gc: mean ± S.D. = 56,320 ± 42,065, *n*=5) (Part Ⅱ, Fig 11B). The neutralizing antibody titer of mice immunized with recombinant subunit SFTSV Gn-Fc (mean ± S.D. = 135.8 ± 83.15, *n*=5) and recombinant subunit SFTSV Gc-Fc (mean ± S.D. = 639.9 ± 181.0, *n*=5) against SFTSV were approximately sixteen times and eighty times higher than those of the control (Fc) (mean ± S.D. = 8.78 ± 7.36, *n*=5) group. But, the recombinant subunit SFTSV Np-His (mean ± S.D. = 23.19 ± 8.09, *n*=5) immunization group barely had neutralizing antibodies against viruses compared to the control (PBS) (mean ± S.D. = 24.14 ± 15.88, *n*=5). (Part Ⅱ, Fig 11C).

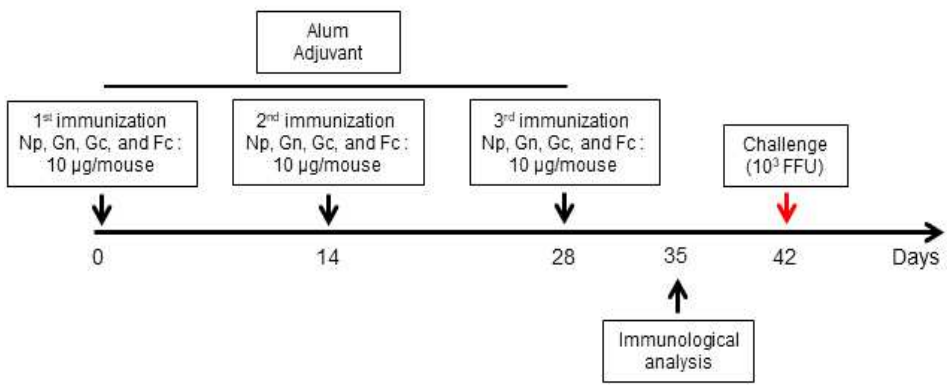
The T cell-mediated immunity in IFNAR K/O mice immunized with the recombinant SFTSV Np, Gn, and Gc subunits were also analyzed. Antigen-specific $CD4^+$ and $CD8^+$ T cell responses producing IFN- ɣ, and TNF-α in the spleen of mice were evaluated by flow cytometry. Antigen-specific $CD4^+$ and $CD8^+$ T cells from immunized mice were stimulated with immunized antigens (Np, Gn, or Gc) (Part Ⅱ, Fig 11D). The frequency of antigen-specific TNF-α producing $CD4^+$ and $CD8^+$ T cells stimulated with immunized soluble antigens for all immunized

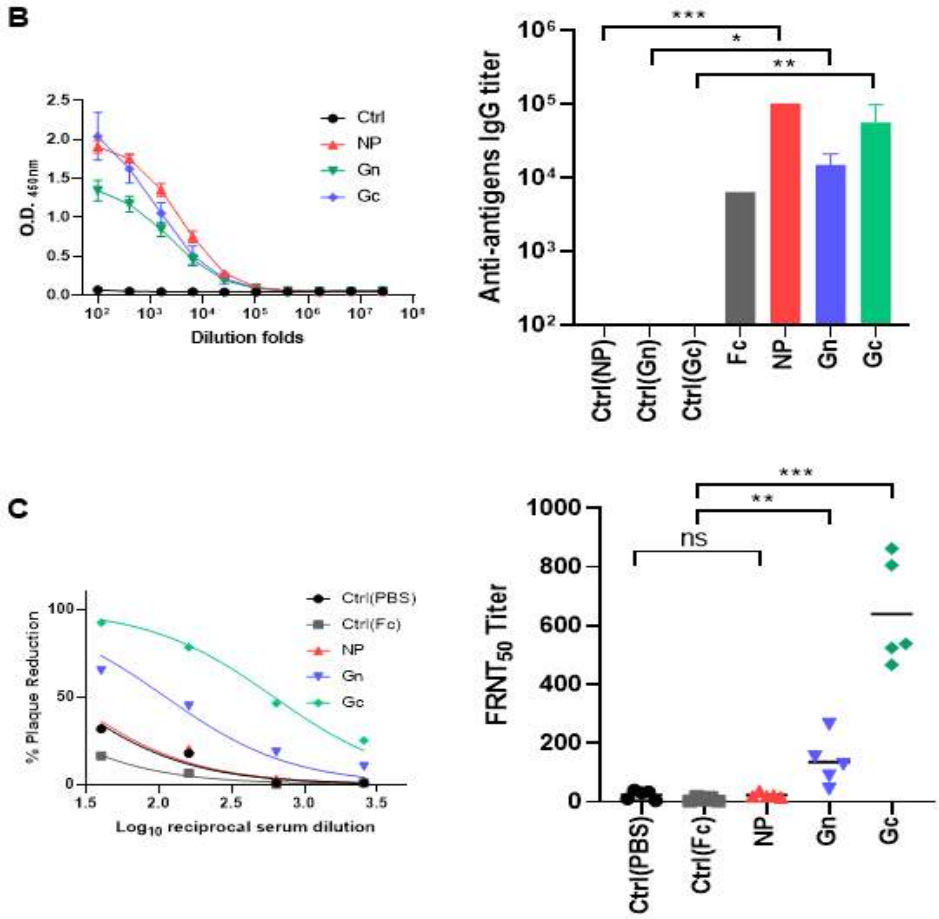
groups was elevated compared to those of unstimulated T cells. The percentage of IFN- ɣ and TNF-α double-positive cells was extremely low for all immunized groups. Interestingly, the level of antigen specific $CD4^+$ and $CD8^+$ T cells secreting IFN- ɣ was increased only in Np and Gn immunized mice after antigen stimulation compared them to unstimulated T cells (Part Ⅱ, Fig 11D).

Next, to assess the protective ability of the single SFTSV subunit vaccine against a lethal dose of SFTSV, 10^3 FFU SFTSV were tested on mice vaccinated with a single SFTSV subunit (Np, Gn, and Gc) and control (PBS or Fc fragment) with alum adjuvant (Part Ⅱ, Fig 12). All vaccinated groups had been monitored for survival rate and body weight change until the recovery of the surviving mice after the challenge (Part Ⅱ, Fig 12A and B). After virus infection, all mice from control (PBS), control (Fc), and Gc-Fc groups were dead on 5, 6, and 8 day post-infection, respectively. However, 60% (3 of 5) of the Np-His immunized mice survived and 50% (2 of 4) of the Gn-Fc immunized mice survived (Part Ⅱ, Fig 12A). PBS, Fc, and Gc-Fc with alum immunized animals significantly lost weight until death, whereas surviving mice vaccinated with NP lost weight until reaching 81% of their original weight on 7 day post-infection, and mice immunized with Gn-Fc lost weight until reaching 80% of their original weight on 8 day post-infection, then both gradually recovered (Part Ⅱ, Fig 12B).

A

Groups (n=4~5/group)	Subunit Amount (per mouse)
Ctrl (PBS)	N/A
Ctrl (Fc)	10 μg
NP	10 μg
Gn	10 μg
Gc	10 μg




B
O.D. 450nm
Dilution folds
Ctrl
NP
Gn
Gc
Anti-antigens IgG titer
Ctrl(NP)
Ctrl(Gn)
Ctrl(Gc)
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% Plaque Reduction
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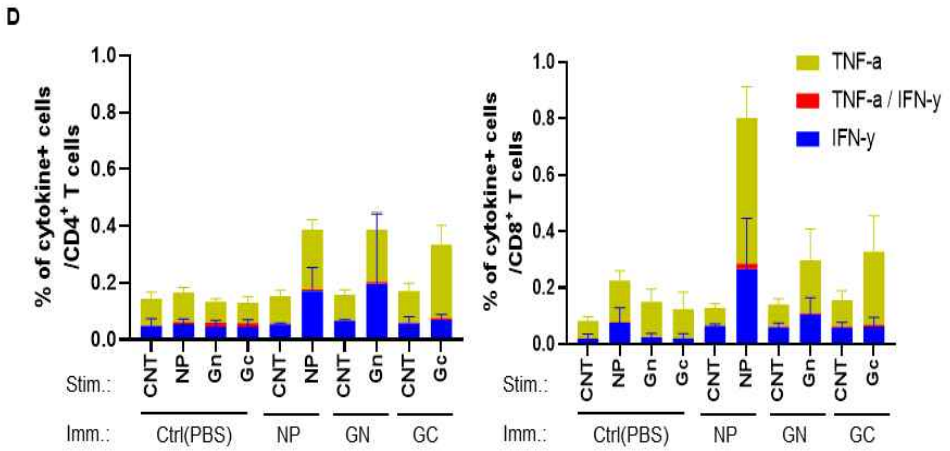


Figure 11. Antibody and T cell immune response of recombinant SFTSV single subunit vaccine against viruses.

(A) A schematic schedule of single subunit immunization and SFTSV challenge. 10 μg of single subunit of SFTSV with 100 μg of Alum adjuvant were immunized to the IFNAR K/O C57BL/6 mice. Four mice were used for Ctrl(Fc), Gn, and Gc-Fc and five mice were used for Ctrl(PBS), and Np-His groups. Total three times immunization interval two weeks, then, immunological analysis and challenge of 10^3 FFU viruses were conducted at a week and two weeks after the third immunization, respectively. (B) Anti-specific antigens (Fc, Np, Gn, or Gc) IgG response was monitored by ELISA at a week after the third immunization. The antibody titers of anti-Fc, Np, Gn, or Gc IgG in sera of mice (n=5) vaccinated with each subunit vaccine are presented. Cut-off titers (mean O.D. + 3x S.D. at 1:100 diluents) were measured using sera from Ctrl(PBS) immunized mice. IgG titers of

ctrl(PBS) serum was determined against Np, Gn, and Gc proteins. (C) The neutralizing antibody titration ($FRNT_{50}$) against SFTSV was measured by sera from Ctrl(PBS), Fc, Np, Gn, or Gc immunized mice (n=5) before virus challenge. Each serum was serially diluted from 1:100 to 1:25,600 and $FRNT_{50}$ was performed in 24-well plates. (D) Splenocytes were collected from mice immunized with each recombinant SFTSV antigen (Ctrl(PBS), Np, GN, or Gc) at a week after the third immunization. Antigen dependent production of IFN-γ, TNF-α, and double-positive by $CD4^+$ T (left panel) or $CD8^+$ T cells (right panel) were evaluated using flow cytometry after stimulation with the indicated antigens. Data are shown as mean ± S.D. from duplicate assays with five mice per group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

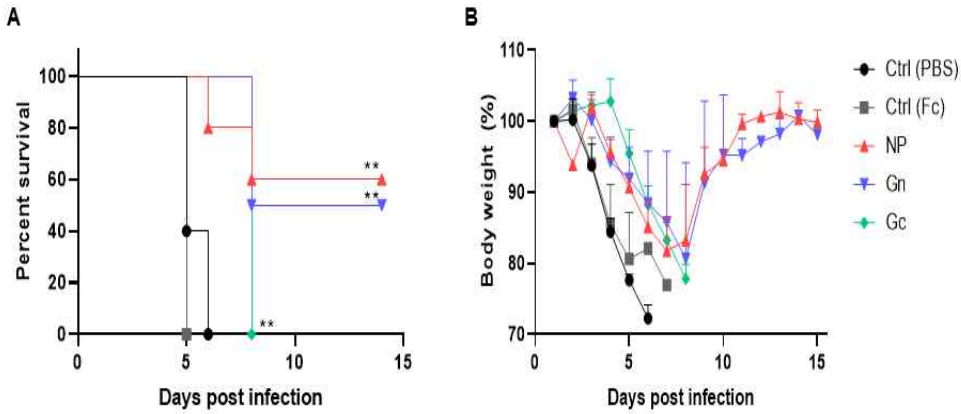


Figure 12. Survival and body weight change of IFNAR K/O mice vaccinated SFTSV single subunit against a lethal dose of SFTSV.
(A and B) Survival curves and body weight chagne of immunized IFNAR K/O mice after lethal challenge with SFTSV. Their survival rate and body weight change were monitored until all surviving mice recovered from the disease. **, $p< 0.01$ when compared with non-immunized group (PBS).

Protection of mixed subunits vaccine against a lethal dose of SFTSV in IFNAR K/O mice.

To assess the antibody dependent immune response in IFNAR K/O mice immunized with a mixed SFTSV subunit vaccine (Gc+Np (GNP), Gc+Np (GcP), and Gc+Gc+Np (NCP)), the titer of antigen-specific antibodies and neutralizing antibodies was examined (Part Ⅱ, Fig 13B). Vaccination with the mixed SFTSV subunits with alum adjuvant significantly increased the titer of antigen-specific antibodies against immunized antigens (Np, Gn, and Gc) in IFNAR K/O mice. Especially, the titer of the anti-Np specific IgG in GcP (mean ± S.D. = $1.1x10^7$ ± $1.3x10^7$, n=7) group was approximately two hundred times higher than the titer of those in the GNP (mean ± S.D. = $4.3x10^4$ ± $4.1x10^4$, n=7) and NCP (mean ± S.D. = $4.8x10^4$ ± $3.7x10^4$, n=7) groups. The $FRNT_{50}$ titer of mice vaccinated with NCP was highest (mean ± S.D. = 678.4 ± 1268, n=7), though the individual titer of NCP had a variation, while the titers of GNP (mean ± S.D. = 143.7 ± 185.4, n=7) and GcP (mean ± S.D. = 143.5 ± 118.2, n=7) were similar to each other.

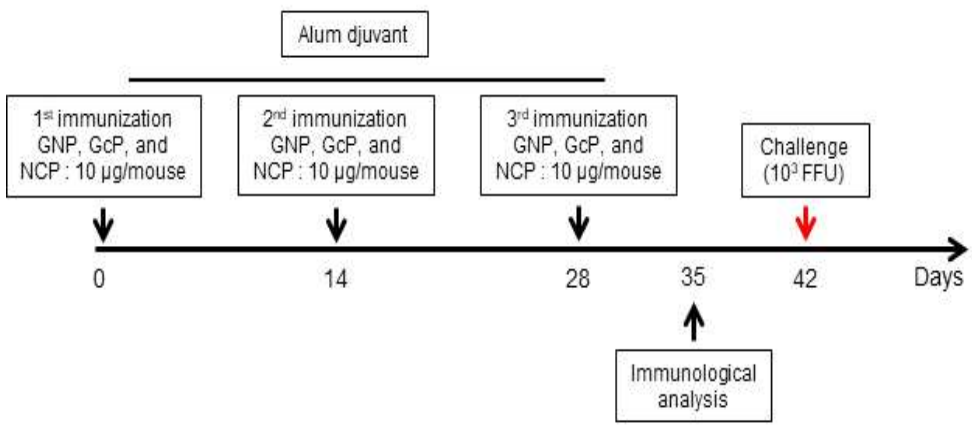
To characterize the T cell-mediated immune response in the IFNAR K/O mice vaccinated with the recombinant mixed SFTSV subunits, antigen-specific $CD4^+$ and $CD8^+$ T cell responses producing IFN- ɣ and TNF-α in the spleen of immunized mice were evaluated by flow cytometry (Part Ⅱ, Fig 13D). The percentage of antigen specific TNF-α secreting $CD4^+$ and $CD8^+$ T cells stimulated with immunized antigens for all immunized groups was dramatically expanded compared to those with unstimulated cells. And the frequency of IFN- ɣ and TNF-α double positive cells was significantly low for all vaccinated groups. Interestingly,

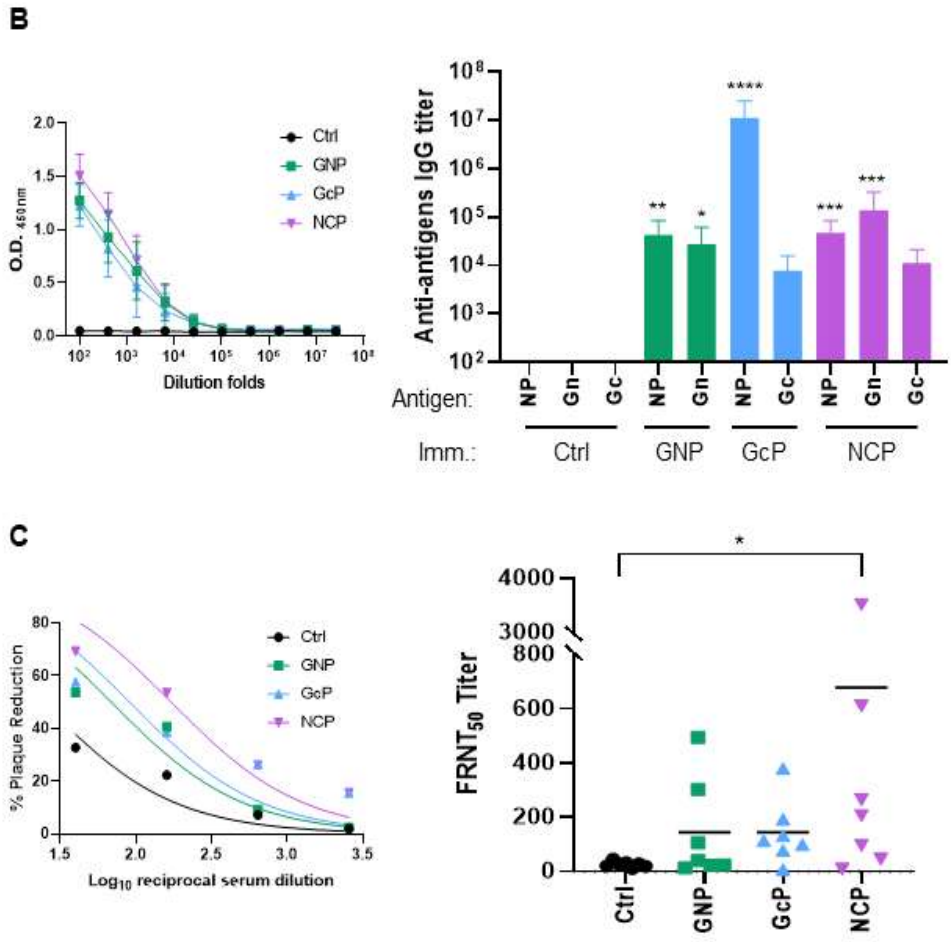
the percentage of Np specific IFN- ɣ secreting $CD4^+$ and $CD8^+$ T cells increased in all mixed vaccine groups compared to those with Gn or Gc antigen-stimulated cells. Noticeably, the frequency of NP dependent IFN- ɣ producing $CD4^+$ and $CD8^+$ T cells in the GcP group significantly increased compared to those of other groups (GNP and NCP) (Part Ⅱ, Fig 13D).

The protective effect of the mixed subunits vaccines against SFTSV was further investigated in vivo by challenging mice with 10^3 FFU SFTSV (Part Ⅱ, Fig 14). All vaccinated groups (Gn+NP (GNP), Gc+NP (GcP), and Gn+Gc+NP (NCP)) had been monitored for survival rate and body weight change until the recovery of the surviving mice after the virus challenge (Part Ⅱ, Fig 14A and B). All of the control (PBS) immunized mice died by 6 day post-infection (dpi). On the other hand, around 70% (5 of 7) of GNP, 86% (6 of 7) of GcP, and 57% (4 of 7) of NCP immunized mice survived (Part Ⅱ, Fig 14A). PBS with alum adjuvant immunized animals rapidly decreased in weight until death. However, surviving mice with the immunizations of GNP, GcP, and NCP lost weight until reaching 86%, 89%, and 82% of their original body weight on 7, 7, and 8 day post-infection (dpi), respectively, and then recovered completely (Part Ⅱ, Fig 14B).

A

Groups (*n*=7/group)	Subunit Amount (per mouse)
Ctrl	N/A
Gn+NP (GNP)	10 μg/each
Gc+NP (GcP)	10 μg/each
Gn+Gc+NP (NCP)	10 μg/each

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Dilution folds
Ctrl
GNP
GcP
NCP
Anti-antigens IgG titer
Antigen:
NP
Gn
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Imm.:
Ctrl
GNP
GcP
NCP
C
% Plaque Reduction
Log10 reciprocal serum dilution
FRNT50 Titer

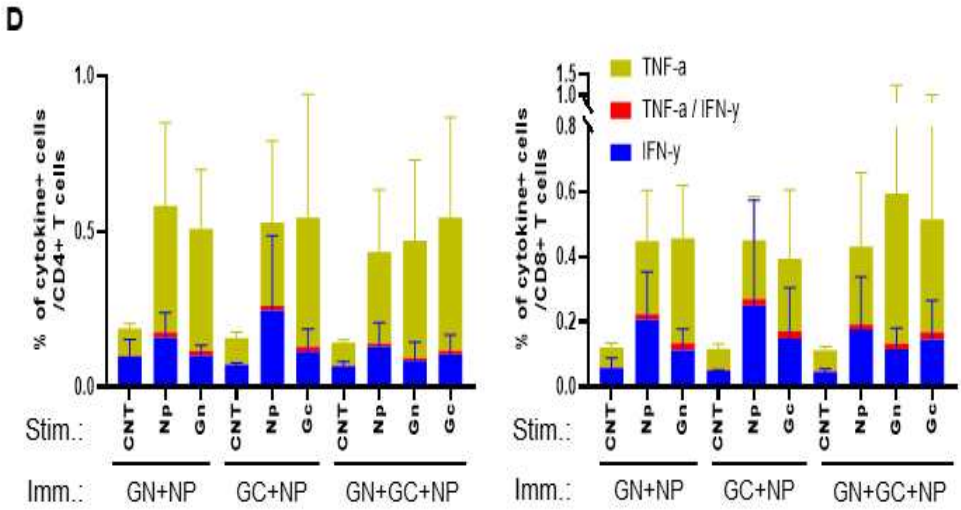

Figure 13. Antibody and T cell immune response of recombinant SFTSV mixed subunits vaccine against viruses.

(A) A schematic schedule of mixed subunits immunization and SFTSV challenge. 10 μg of each subunit used for mixed vaccines with 100 μg of Alum adjuvant were immunized to the IFNAR K/O C57BL/6 mice (n=7 per group). Three times vaccination interval two weeks, then, immunological analysis and challenge of 10^3 FFU viruses were conducted at a week and two weeks after the third immunization, respectively. (B) Anti-specific antigens (Np, Gn, or Gc) IgG response in each mixed vaccine group was monitored by ELISA at a week after the third immunization. The antibody titers of anti-Np, Gn, or Gc IgG in sera of mice (n=7) vaccinated with each of mixed subunits vaccine are presented. Cut-off titers (mean O.D. + 3x S.D. at 1:100 diluents) were measured using sera from Ctrl(PBS) immunized mice. IgG titers of ctrl(PBS) serum was determined

against Np, Gn, and Gc proteins. The asterisks indicate significance compared to the respective control(NP), control(Gn), and cotrol(Gc). (C) The neutralizing antibody titration ($FRNT_{50}$) against SFTSV was measured by sera from Ctrl(PBS), GNP, GcP, and NCP immunized mice (n=7) before virus challenge. Each serum was serially diluted from 1:100 to 1:25,600 and $FRNT_{50}$ was performed in 24-well plates. (D) Splenocytes were collected from mice immunized with each recombinant SFTSV antigens (Ctrl(PBS), GNP, GcP, or NCP) at a week after the third immunization. Antigen specific production of IFN-ɣ, TNF-α, and double-positive by $CD4^+$ T (left panel) or $CD8^+$ T cells (right panel) were evaluated using flow cytometry after stimulation with the indicated antigens. Data are shown as mean ± S.D. from duplicate assays with five mice per group.*, $p < 0.05$; **, $p < 0.01$, *** $p < 0.0003$; ****, $p < 0.0001$.

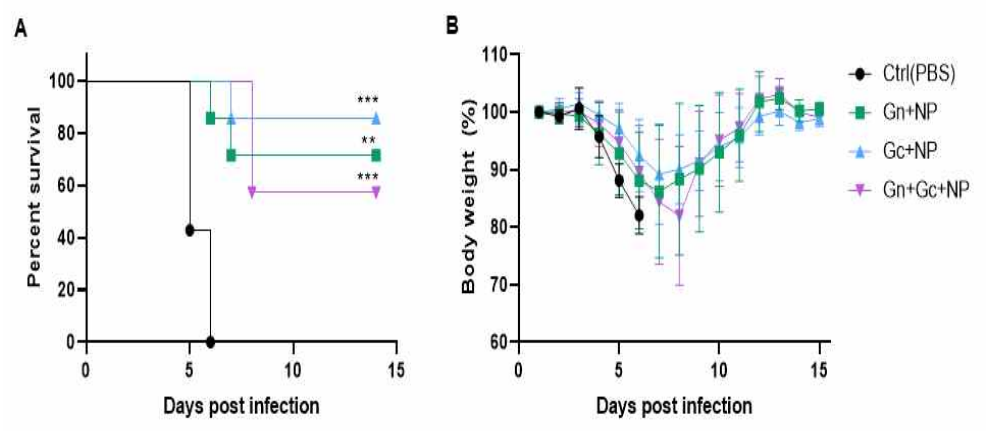

Figure 14. Survival and body weight change of IFNAR K/O mice vaccinated SFTSV mixed subunits against a lethal dose of SFTSV.
(A and B) Survival curves and body weight change of immunized mice after lethal challenge with SFTSV. Their survival rate and body weight change was monitored until all surviving mice recovered from the disease. **, $p < 0.01$, ***, $p < 0.0003$ when compared with non-immunized group (PBS).

DISCUSSION

SFTSV which is an emerging virus has mainly been distributed throughout eastern Asian countries: South Korea [16], China [14], and Japan [19]. SFTS cases have been also reported in other Asian countries: Vietnam [17] and North Korea [8, 23], indicating that SFTSV has gradually been spreading through the world. The mean mortality of SFTS cases in three countries (South Korea, China, and Japan) is approximately 5-27% [18, 52, 53]. Due to the increasing number of SFTS cases and mortality, several research groups have studied in order to develop vaccines protecting against SFTSV.

According to Kim K.H. et al. report, recovered SFTS patients' SFTSV glycoprotein N-specific antibodies might be used for therapies or vaccines against a lethal dose of SFTSV in IFNAR1 K/O mice. Gn-specific antibodies have a neutralizing effect on SFTSV in vitro and offer complete protection in immuno-compromised mice [3]. DNA vaccines encoding the full length of SFTSV Gn, Gc, N, NS, and RdRp genes were also developed in the ferret model [26]. These vaccines have a high $FRNT_{50}$ titer against SFTSV and may elicit a robust T cell response secreting IFN- ɣ about five SFTSV antigens in ferrets as well as evoke long-lasting CD8+ T cells. With these immunological responses, this DNA vaccine successfully confers perfect protection in the old ferret model (>4 years). Furthermore, the transfer of sera from Gn and Gc plasmid immunized ferrets provides complete protection after challenging SFTSV [26]. The vaccination of recombinant SFTSV nonstructural proteins (NS) could elicit strong anti-Ns specific antibodies, but all mice immunized with rSFTSV NS proteins were dead with a high number of viruses in the serum, liver, spleen, and kidneys [28]. Another research team suggested that recombinant viruses encoding the SFTSV glycoprotein N&C gave

100% protection against lethal challenge and have neutralizing antibodies (titer of $FRNT_{50}$: 1:500) [27].

As seen in many vaccine studies about SFTSV, the animals relatively close to humans were considered a good standard animal model for infectious diseases [64]. Among many animal models, the mice have been used for study of various infectious pathogens because the immune systems of mice and humans are very similar [65]. Unfortunately, wild mice are not susceptible to many other pathogens, so IFN receptor knock out mice were a great alternative for vaccine study. Type I IFN receptor knock out mice were created in 1994 [66]. This transgenic mice challenged with Semliki Forest virus, Vesicular stomatitis virus, or vaccinia virus showed increased susceptibility resulting in high viral load in organs and death at lower challenge doses compared to wild-type mice. Among *Bunyaviridae* order, type I IFN K/O mice were generally used for Crimean-Congo hemorrhagic fever virus (CCHFV) [67, 68], Severe fever with thrombocytopenia virus (SFTSV) [69, 70] infectious disease study.

Even though there are issues with DNA vaccines inducing tumors or autoimmune diseases [54], DNA vaccines could produce a natural form of antigens that trigger innate and adaptive immune responses [33]. Here, I demonstrate that DNA vaccines encoding SFTSV Np-NS, Gn, and Gc fused with Flt3L at N-terminus and IL-12 cytokine could stimulate IFN-ɣ secreting lymphoid cells and then confer complete protection in IFNAR K/O mice against a lethal SFTSV challenge (Part Ⅰ, Fig 4A). The increased frequency of IFN-ɣ secreting $CD4^+$ and $CD8^+$ T cells from pSFTSV-IL12 vaccination under SFTSV NP and Gn antigens may be because of an IL-12 cytokine response for adjuvant (Part Ⅰ, Fig 3D) [40, 55]. The viral

copy numbers and platelet counts of both pSFTSV and pSFTSV-IL-12 vaccines were similar with the mock vector plasmid immunization and lower than the uninfected group at the early stage of infection, respectively (Part Ⅰ, Fig 4B). As shown in Part Ⅰ, Fig 3A and B, pSFTSV and pSFTSV-IL12 DNA vaccines elicited a lower antigen-specific antibody production, besides, neutralizing antibody titer was low. Early-stage viremia and thrombocytopenia may be correlated with humoral immune responses [26, 56]. Especially, as shown by Kwak J.E. et al. report, the formation of antibodies against SFTSV glycoprotein Gn and Gc may be important to block viremia and thrombocytopenia [26]. For these reasons, I speculated the reason of failure to reduce increasing SFTSV at an early stage was due to extremely low SFTSV Gn and Gc antibodies (data not shown).

SFTSV subunit vaccines with a single subunit or multiple mixed subunits were also evaluated. Subunit vaccines have generally been used for vaccine studies because of low production costs and increasing biosafety [32]. Four kinds of recombinant SFTSV subunits (SFTSV Np-His, Gn-Fc, and Gc-Fc) were prepared. The human IgG Fc domain in recombinant proteins allows the formation of dimerization to increase avidity and increases plasma half-life using neonatal Fc receptor (FcRn) [1, 57, 58], furthermore, human IgG Fc domain can interact with murine FcγR [59]. For these reasons, Fc proteins have been used for many vaccine studies and this vaccine study [60-62]. 60% (3 of 5) of mice vaccinated with recombinant SFTSV Np and 50% (2 of 4) of mice immunized with recombinant SFTSV Gn proteins survived against a lethal dose of SFTSV. SFTSV Gc protein vaccination could extend the life of mice until 8 day post-infection (dpi) (Part Ⅱ, Fig 12A). Antigen-specific IgG and $FRNT_{50}$ titer of all single subunit vaccine groups was higher than

that of DNA vaccines (Part Ⅱ, Fig 11B). Interestingly, $FRNT_{50}$ titer of the Gc immunized group was higher than the Np and Gn groups, though all of the Gc subunit vaccinated mice were dead. The titer is similar with the $FRNT_{50}$ titer of rVSV encoding SFTSV glycoproteins vaccine that confers complete protection against a SFTSV lethal challenge [27]. Also, as shown in body weight change, the body weight of the Gc vaccination group started to decrease from 4 day post-infection (dpi) even though those of the other groups started to decrease from day 2 or 3 (Part Ⅱ, Fig 12B), indicating that anti-Gc specific antibodies may have a strong neutralizing effect against SFTSV at an early infection period. However, the percentage of antigen specific IFN-ɣ producing $CD4^+$ and $CD8^+$ T cells of mice immunized with Gc proteins did not increase. Only those of mice vaccinated with NP or Gn proteins were elevated (Part Ⅱ, Fig11D). I speculated that NP or Gn proteins would be a strong factor for stimulating antigen specific IFN-ɣ producing $CD4^+$ and $CD8^+$ T cells which may be important for protection against SFTSV. Furthermore, SFTSV Gc immunization could make neutralizing antibodies for SFTSV that may prolong the life expectancy of IFNAR K/O mice.

To develop a strong protective vaccine against SFTSV, I tried to use mixed SFTSV subunits (Gn+NP (GNP), Gc+NP (GcP), and Gn+Gc+NP (NCP)) for the vaccination of IFNAR K/O mice based on single protein vaccine data. 70% (5 of 7) of mice of GNP, 86% (6 of 7) of mice of GcP, and 57% (4 of 7) of mice of NCP groups survived more than the single subunits Np (60%) and Gn (50%) of the vaccine groups (Part Ⅱ, Fig 14A). The GcP group triggered a high level of anti-NP specific antibody titer and the neutralizing antibody titer ($FRNT_{50}$) was similar with the single subunit Gn vaccine group (Part Ⅱ, Fig 13B, and C). These results showed anti-Np specific antibody

titer and neutralizing titer ($FRNT_{50}$) of serum from the GcP vaccinated mice was significantly higher than that of the pSFTSV-IL12 vaccine (Part Ⅰ, Fig 3A and B, Part Ⅱ, Fig 13B and C). Antigen specific TNF-α secreting CD4+ and CD8+ T cells in all mixed subunit groups were significantly increased compared to all groups with a single protein vaccination. In addition, the percentage of IFN-ɣ secreting both CD4+ and CD8+ T cells against N proteins in the GcP immunized mice was more increased than those of other groups (GNP and NCP) (Part Ⅱ, Fig 13D). However, the percentage of IFN-ɣ secreting CD4+ T cell was about two times lower than that of pSFTSV-IL12 vaccinated mice (Part Ⅰ, Fig 3D, Part Ⅱ, Fig 13D). Taken together, I found that DNA vaccine confers complete protection by enhancing T cell immune response against SFTSV, however, this vaccine has the limitation of low titer of Ag-specific antibodies and neutralizing antibodies. Recombinant SFTSV Np and Gn provide marginally increased CD4+ T cell immune response and an approximately 50% survival rate against a lethal dose of SFTSV. However, the serum of recombinant Gc subunit vaccinated mice has a strong neutralizing effect against SFTSV. Mixed Gc and Np (GcP) subunit vaccinations elevated the neutralizing effect against SFTSV even though CD4+ T cell response was slightly increased. Besides, approximately 90% of GcP vaccinated mice survived against a lethal dose of SFTSV. While pSFTSV-IL12 DNA vaccine presents a high level of antigen specific IFN-ɣ secreting T cells, it has a poor antibody dependent immune response. On the other hand, the Gc+Np (GcP) subunit vaccine provides great antibody dependent immune response despite increasing T cell immunity a bit. Generally, the disadvantage of DNA vaccines is a weak immune response in human trials [63] and they may induce tumors or autoimmune diseases [54].

A subunit vaccine has the defect of low T cell response induction and low immunogenicity. Consistently, in this study, the pSFTSV-IL12 DNA vaccine elicited a high antigen specific IFN-ɣ secreting T cell response and the GcP subunit vaccine provided a high antibody dependent immune response. So, a DNA prime/protein boost vaccine may be the new direction of the SFTSV vaccine as it increases both T cell and antibody dependent immunity with biosafety [71-73]. Further studies on pSFTSV-IL12 DNA prime/GcP subunit boost vaccine evaluation and understanding mechanism of immunological pathogenesis in mice and higher animals should be followed to develop the perfect SFTSV vaccine.

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국문 초록

중증열성혈소판감소증 바이러스(SFTSV)는 한국, 중국, 일본등지에서 중증열성혈소판감소증을 일으키는 진드기 매개 바이러스로, 최근 대만과 베트남 등 동남아시아 지역에서도 발생이 보고되고 있다. 이 바이러스 감염증은 인간에서 5-27%의 높은 치사율을 보이고 있으나, 효과적인 바이러스 백신이나 치료제는 아직 없다. 본 연구에서는 SFTSV에 대한 백신을 개발하기 위하여 DNA 백신과 아단위 백신의 효능을 검증하는 실험을 수행하였다. 먼저 SFTSV의 구조 단백질인 Gn 과 Gc 그리고 Np-NS 융합 단백질을 암호화하는 유전자들과 인터루킨-12 (IL-12)를 발현할 수 있는 DNA plasmid를 백신 후보물질로 선정하여 제1형 인터페론 수용체 돌연변이(IFNAR K/O) 마우스에 면역하고, 치사량의 SFTSV를 감염시켰을 때, 치사율과 증상 유무를 조사하였다. 이 DNA 백신이 투여된 쥐는 치사량의 SFTSV 감염에 대해 100% 보호되는 것을 확인하였다. 이 DNA 백신이 바이러스에 대한 중화 능력을 유도하지는 못하였으나, 항원 특이적인 T 세포 반응을 유의하게 유도하는 것으로 보아 세포 매개 면역반응이 보호능을 제공하고 있음을 알 수 있었다. 다음으로 SFTSV의 구조 단백질들(Np, Gn, 그리고 Gc)을 각각 대장균과 진핵세포에서 발현/정제하고, 이를 아단위 백신으로 사용하여 그 효과를 시험하였다. 개별 아단위 면역시험에서는 NP와 Gn 면역그룹에서 치사량 감염에 대해 약 50% 이상의 보호 효과를 나타내었으나, Gc 재조합 단백질을 면역한 생쥐들은 모두 사망하였다. SFTSV의 NP 단백질은 바이러스에 대한 세포 매개성 면역반응을 잘 유도하였으며, Gc 단백질은 체액성 면역반응을 유의하게 증가시키는 것으로 확인되었다. 마지막으로, IFNAR K/O 생쥐들에게 재조합 항원들을 혼합하여 면역을 하고 백신의 효능을 분석한 결과(Gn+NP, Gc+NP, 그리고 Gn+Gc+NP), Gc+NP 면역그룹은 치사량의 SFTSV 감염에 대하여 약 90%의 생존율을 보였으며,

유의한 T 세포 반응과 바이러스 중화항체 증가가 관찰되었다. Gn+NP 그리고 Gn+Gc+NP 면역 그룹은 각각 70%와 57%의 생존율이 관찰되어 Gc+NP 그룹이 가장 좋은 백신 효능을 보였다. 이 결과들을 종합하였을 때, SFTSV의 유전자들을 암호화하는 DNA 백신이나 그 구조 단백질들의 조합을 면역하는 백신 방법은 세포매개 면역반응 또는 중화항체 생성을 통해 중증열성혈소판감소증에 대한 보호 면역반응을 제공할 수 있다는 것을 확인하였으며, 이를 통해 SFTS의 백신 개발에 활용될 수 있을 것으로 판단된다.

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주요어: 중증열성혈소판감소증 바이러스, 진드기 매개 질병, DNA 백신, 재조합 단백질 백신, 세포 매개성 면역, 체액성 면역

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