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Development of a recombinant vaccine containing a spike S1-Fc fusion protein induced protection against MERS-CoV in human DPP4 knockin transgenic mice

Bo-Kyoung Jung [a], YongHee An [a], Jung-Eun Park [b], Kyung-Soo Chang [c], Hyun Jang [a,*]

[a] Libentech Co., Ltd., Daejeon, Republic of Korea
[b] Department of Veterinary Medicine, Chungnam National University, Daejeon, Republic of Korea
[c] Department of Clinical Laboratory Science, Catholic University of Pusan, Busan, Republic of Korea

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ABSTRACT

The Middle East respiratory syndrome coronavirus (MERS-CoV), belonging to the family Coronaviridae and genus *Betacoronavirus*, has been recognized as a highly pathogenic virus. Due to the lack of therapeutic or preventive agents against MERS-CoV, developing an effective vaccine is essential for preventing a viral outbreak. To address this, we developed a recombinant S1 subunit of MERS-CoV spike protein fused with the human IgG4 Fc fragment (LV-MS1-Fc) in Chinese hamster ovary (CHO) cells. Thereafter, we identified the baculovirus gp64 signal peptide-directed secretion of LV-MS1-Fc protein in the extracellular fluid. To demonstrate the immunogenicity of the recombinant LV-MS1-Fc proteins, BALB/c mice were inoculated with 2.5 μg of LV-MS1-Fc. The inoculated mice demonstrated a significant humoral immune response, measured via total IgG and neutralizing antibodies. In addition, human dipeptidyl peptidase-4 (DPP4) transgenic mice vaccinated with LV-MS1-Fc showed the protective capacity of LV-MS1-Fc against MERS-CoV with no inflammatory cell infiltration. These data showed that the S1 and Fc fusion protein induced potent humoral immunity and antigen-specific neutralizing antibodies in mice, and conferred protection against coronavirus viral challenge, indicating that LV-MS1-Fc is an effective vaccine candidate against MERS-CoV infection.

1. Introduction

Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified in Saudi Arabia in 2012; it has the potential to cause large epidemics. To date, over 2400 MERS-CoV infections have been reported from 27 countries, and 580 Middle East respiratory syndrome (MERS)-associated deaths have been reported (WHO, 2020). A vaccine is required to prevent epidemics. Humans or camels may transmit MERS-CoV to humans. MERS-CoV is an enveloped single-stranded RNA genome virus (Alsolamy and Arabi, 2015) belonging to the family *Coronaviridae* and genus *Betacoronavirus*; its genome encodes ORF1a, 1ab (by frame shifting), 3, 4a, 4b, 5, and 8b, with genes for spike (S), envelope (E), matrix (M), and nucleocapsid (N) proteins (Scobey et al., 2013).

The MERS-CoV spike (S) protein is divided into two subunits, the S1 subunit containing the receptor-binding domain (RBD) and the S2 subunit. The S1 subunit has four domains designated $S1^A$ through $S1^D$ responsible for host receptor-binding, membrane fusion, and cell entry (Du and Jiang, 2015; Du et al., 2016; Li, 2015; Li et al., 2017a,b). $S1^B$ subunit has an important role in the internalization of the virus infection into host cells (Li et al., 2017a,b). The S2 subunit mediates the fusion between viral and cellular membranes and entry of the viral genetic materials into the host cells (Du et al., 2013a,b; Gao et al., 2013; Lu et al., 2014; Raj et al., 2013). These properties make the S protein an important target for MERS-CoV vaccine candidates (Wang et al., 2016).

The Fc domain of immunoglobulin G (IgG) is used as an important

Abbreviations: BSL, biosafety level; CEF, chicken embryo fibroblast; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; Fc, fragment of crystalline; MERS, Middle East respiratory syndrome; MERS-CoV, Middle East respiratory syndrome coronavirus; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline in 0.05 % Tween 20; PCR, polymerase chain reaction; PFU, plaque-forming unit; RBD, receptor-binding domain; RLU, relative light unit; SARS-CoV, severe acute respiratory syndrome coronavirus; SFM, serum-free medium.

* Corresponding author at: Libentech Co., Ltd., C-722 Daedeok BIZ Center, Techno 4-ro, 17 Yuseong-gu, Daejeon, 34013, Republic of Korea.
E-mail address: vacchyun@naver.com (H. Jang).

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fusion partner with several viral proteins. Fc domain depends on their subtype class has an Fc receptor on several kinds of immune cells. Several studies tried using antigen fused with human Fc fusion protein to prevent viral disease. Loureiro research group reported influenza virus HN protein and human Fc fusion protein used an antigen to make the vaccine (Loureiro et al., 2011). Herpes simplex virus type-2 (HSV-2) causes sexually transmitted diseases. Ye et al. reported that glycoprotein D (gD) of HSV-2, which fused with the neonatal Fc (gD-Fc) was enhanced efficient mucosal and systemic antibodies (Ye et al., 2011). Ebola virus-caused hemorrhagic fever in humans fused to the Fc fragment of human IgG1 (ZEBOVGPFc) induced sufficient protective immunity against ZEBOV in mice (Konduru et al., 2011). The human immunodeficiency virus (HIV) attacks the body's immune system. Lu et al. studied that HIV Gag (p24) fused to the Fc domain of IgG (GagFc) vaccine protected at a distal mucosal site (Lu et al., 2011). *Mycobacterium tuberculosis* is a cause of Tuberculosis. Soleimanpour et al. reported that a dormant immunogenic protein (ESAT6 and HspX) fused to Fcγ2a fragment (ESAT6: HspX: Fc) were vaccinated in C57BL/6 mice. The results showed that ESAT6: HspX: Fc protein induced a high level of IFN-γ and IL-12 (Soleimanpour et al., 2015). Recently, a previous study to develop a vaccine to prevent the COVID-19 pandemic demonstrated the efficacy of RBD protein fused with Fc protein (Ren et al., 2020). Overall, Fc proteins have an adjuvant effect of vaccines for the prevention of various diseases.

The RBD of severe acute respiratory syndrome coronavirus (SARS-CoV)-Fc fusion protein using vaccine elect an antibody response was reported (He et al., 2004, 2005). Fc domain is a good immune responses enhancing molecule to use a fusion partner with antigen protein. Because it stimulates Th1-mediated immune responses and increase neutralizing antibody production. The S1 subunit includes the RBD, which is a critical antigen for vaccine development, and a principal target of virus neutralizations (Wang et al., 2017)

Chinese hamster ovary (CHO) cells are widely used for the production of recombinant protein (Fischer et al., 2015; Jayapal et al., 2007). Using CHO-cell based systems easily defined scale-up capacities and, CHO cells have the ability to grow in suspension cultures, which enables fast scale-up of production in bioreactors. (Genzel, 2015).

In this study, we evaluated the recombinant LV-MS1-Fc protein expressed in CHO cells that protects human DPP4 knockin transgenic mice from MERS-CoV infection.

2. Materials and methods

2.1. Construction of expression plasmids

The spike protein of MERS-CoV sequence (Gene Bank No: KT029139.1) fused with the gp64 signal peptide (Gene bank No: AIU56980.1) at N-terminus and human IgG Fc (Gene bank No: AEV43323.1) at C-terminus, were synthesized and cloned into the pcDNA3.4 TOPO vector (Invitrogen, Carlsbad, CA) (pLV-MS1-Fc).The PCR products were gel purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and subsequently cloned into the plasmid, followed by *Escherichia coli* DH5α transformation following general *E. coli* transformation protocol. pLV-MS1-Fc plasmid transformed *E. coli* colonies on Luria-Bertani (LB) agar plates containing 100 μg/mL ampicillin picked and cultured in LB broth containing 100 μg/mL ampicillin for pLV-MS1-Fc plasmid precipitation.

2.2. Establihment of a CHO cell line expressing LV-MS1-Fc protein

Before the transfection, CHO-S cells (Invitrogen) density was adjusted to 6×10^6 cells/mL according to experimental requirements. Transfections were conducted in 250 mL Erlenmeyer tissue culture flasks for 50 mL cultures. Transfection complexes were prepared by adding ExpiFectamine CHO reagent (Invitrogen) in 1.84 mL of OptiPRO serum-free medium (SFM) (Invitrogen) and the plasmid DNA (30–50 μg) diluted in 2 mL of OptiPRO SFM. The complex solution was incubated at room temperature for 5 min and slowly added to the suspension culture while slowly swirling the flasks. The cells were incubated on an orbital shaker for 8 days.

2.3. Purification of LV-MS1-Fc protein

The CHO cell culture supernatant was separated by centrifugation at 5000 rpm for 10 min at 4 °C. Ammonium sulfate was added to the supernatant up to a concentration of 90 %; the solution was incubated for 5 h and centrifuged at 13,000 rpm for 30 min at 4 °C. The supernatant was discarded and the precipitate was redissolved in 20 mM sodium phosphate buffer (pH 7.4) and added into a dialysis bag. Dialysis was performed in 20 mM sodium phosphate buffer for 2 h at 4 °C with gentle stirring, two times. After dialysis, recombinant LV-MS1-Fc was loaded onto a HiTrap Protein-A HP column (GE) at a flow rate of 5 mL/min. The column was washed with 1× phosphate-buffered saline (PBS) (pH 7.5) at 20 column volumes and eluted with 1.2 column volumes of 20 %–80 % elution buffer (0.1 M citric buffer pH 3.0), followed by 18.8 column volumes of 100 % elution buffer. The elution fractions (6.0 mL) were collected in tubes containing 0.2 mL of 1 M Tris−HCl (pH 9.0) to increase the pH of the eluted protein to 7.0. After purifying by affinity chromatography, the samples were purified by size exclusion chromatography (Superdex 200 increase 10/300 G L; GE Healthcare). The purified samples were analyzed by SDS-PAGE, and western blotting. Western blotting was performed with Rabbit-anti-MERS-CoV spike (1:2000) (Loureiro et al., 2011) and rabbit anti-bovine (Fab) 2-biotin (1:4000, Sigma) antibodies to determine the LV-MS1-Fc protein. The LV-MS1-Fc protein were lowered and concentrated to 1 mg/mL with a Vivaspin Turbo 4 Ultrafiltration Unit (MWCO 10 kDa) and buffer-exchanged with 20 mM Tris−HCl and 20 mM NaCl (pH 7.4).

2.4. Animals

All mice experimental procedures approved and followed the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Chugnam National University. Viral Challenge was performed in animal biosafety level 3 (BSL-3) laboratories. The hDPP4-knockin transgenic mice were provided by Seoul National University; they were mated in-house, and then genetically stable hDPP4-knockin was checked.

2.5. Vaccine preparation and mouse immunization

Purified LV-MS1-Fc antigen proteins were mixed with an equal volume of aluminum hydroxide (alum) adjuvant (Imject Alum, Thermo). 10 mL of antigen protein (0.05 mg/mL) and 10 mL of alum adjuvant (0.5 mg/mL) solution were mixed for 20 min under mild stirring at 4 °C. The antigen protein and alum mixture were separated by centrifugation at 10,000 × g for 10 min. The pellet was re-suspended by 1.0 mL of PBS buffer. The sample was stored at 4 °C before inoculation. Three groups of 5-week-old female SPF BALB/c mice (4 per group) were immunized with 100 μl of PBS (G1), 100 μl of LV-MS1-Fc (25 μg/mL, G2), and 100 μl of LV-MS1-Fc with alum adjuvant (25 μg/mL, G3). Immunization was performed intramuscularly at 2-week intervals. Blood samples were collected from the mouse at 14 and 28 days after primary immunization.

2.6. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) test was performed after vaccination and detected the antibody specific MERS-CoV S1 subunit protein. 96-well ELISA plates were pre-coated overnight at 4 °C with a replication-defective recombinant vesicular stomatitis virus expressing the MERS-CoV spike (S) protein (VSV-MERS-S). The plate blocked with 5% skim milk at 37 °C for 1 h. Serial 100-fold diluted serum (50 μL/well) was added to the plates, which was then incubated

at 37 °C for 1 h. The plates followed by three washes with PBS in 0.05 % Tween-20 (PBST). Subsequently, the plates were incubated with HRP-conjugated goat anti-mouse IgG (1:10000) at 37 °C for 1 h. After three washes with PBST, the antibodies in sera were visualized by adding the colorogenic substrate tetramethylbenzidine (TMB) as the chromogen. The reaction was terminated after 10 min by adding 2 M H_2SO_4 as a stop solution. The absorbance (450 nm) was measured on an ELISA plate reader (Bio-Rad).

2.7. Neutralizing antibody assays

To heat inactivation, the sera were initially incubated at 56 °C for 30 min and serially diluted two-fold (1:10 to 1:20480). VSV-MERS-S (5×10^3 RLU/50 μL) was incubated with diluted serum (50 μL) at 37 °C for 1 h. The mixture was added to Huh 7 cells in 96-well plates and incubated at 37 °C for 48 h. The RLU was detected using Beetle Lysis-Juice (PJK GmbH, Cat No. 102512) and a luminescence counter (PerkinElmer, USA) according to the manufacturer's instructions.

2.8. Evaluation of immunogenicity and efficacy of LV-MS1-Fc in hDPP4-Knockin transgenic mice

Two groups of hDPP4-knockin transgenic mice (4 per group) were intramuscularly inoculated with PBS (G1) and 2.5 μg of LV-MS1-Fc with alum adjuvant (G2) at 2-week intervals. Blood samples were collected from the mouse cheek 14 days after each vaccination (Days 14 and 28). Two groups of hDPP4-knockin transgenic mice (4 per group) were intramuscularly inoculated with PBS (G1) and 2.5 μg of LV-MS1-Fc with alum adjuvant (G2) at 2-week intervals. The mice were challenged with 10^4 PFU/50 μL Mouse-Adapted MERS-CoV (HCoV-EMC/2012 strain) intranasally (IN) (Li et al., 2017a,b). After challenge, the mice were checked daily for body weight loss and survival for 1 week and sacrificed at 7 days after challenge to determine clinical symptoms and prepare lung tissue sections for histopathologic assessment. Weight loss of over 20 % of their starting body weight checked as an endpoint, the criteria for humane euthanasia.

2.9. Histopathology

The lung tissues were fixed in 10 % formalin, embedded in paraffin, and sectioned. The sections were stained with hematoxylin-eosin (H&E) to assess the extent of pathologic damage and the eosinophilic part of the inflammatory infiltrates.

2.10. Statistical analysis

Statistical significance was measured using Student's *t*-test or one-way ANOVA. SPSS Version 23 (IBM Corp., Armonk, NY, USA) was used for the analysis. Results with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Establishment of recombinant CHO cell expressing the MERS-CoV S1 subunit

The spike protein gene was synthesized based on the MERS-CoV strain that was isolated from the patents 2015 MERS epidemic in Korea. The synthesized genes were directly linked by ligation of the human IgG4 class Fc protein gene (GenBank AAB59394.1) and a gp64 gene for extracellular releasing signal peptide gene at the N-terminal end as shown in Fig. 1A. The plasmid was transfected into CHO cells. Transformed CHO cells expressing the LV-MS1-Fc proteins were identified by western blotting using anti-spike protein antibodies (Fig. 1B).

3.2. Production and purification of LV-MS1-Fc soluble proteins

LV-MS1-Fc protein was precipitated with 90 % ammonium sulfate, re-suspended in PBS, and then purified with a HiTrap Protein-A HP column (Fig. 1C). The protein fraction was then purified by size exclusion chromatography (Superdex 200 10/300 G L) (Fig. 1D). The LV-MS1-Fc protein in the peak fractions were concentrated to 1 mg/mL with Vivaspin (MWCO 10 kDa). Western blotting was performed with

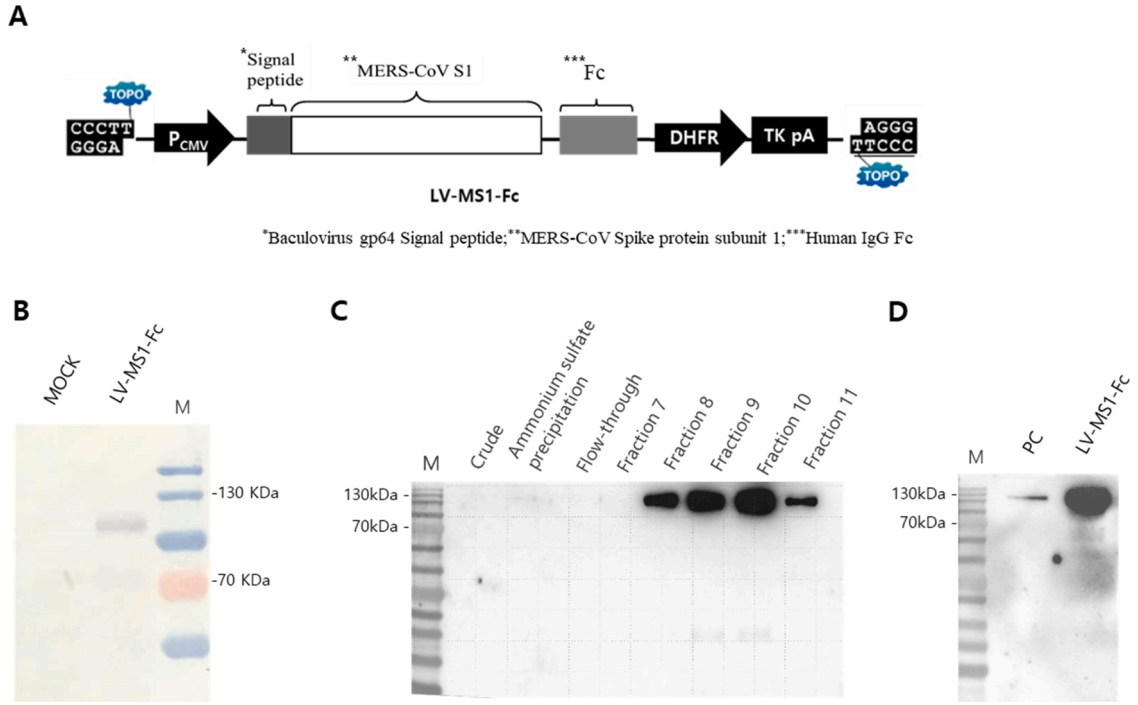


Fig. 1. Characterization of LV-MS1-Fc in CHO cells. (A) Schematic representation of LV-MS1-Fc. The S1 subunit is fused to signal peptide and the human IgG4 Fc fragment. (B) Western blotting of the clarified supernatant of the recombinant CHO cell line expressing LV-MS1-Fc. Western blotting of purified LV-MS1-Fc by (C) affinity chromatography and (D) size exclusion chromatography using anti-MERS antibodies.

Rabbit-anti-MERS-CoV spike (1:2,000) to measure LV-MS1-Fc in the elution fractions. Finally, the purified LV-MS1-Fc protein was concentrated to 4 mg/mL of recombinant cell culture.

3.3. Immunogenicity of LV-MS1-Fc

The mice were inoculated intramuscularly with 2.5 μg of LV-MS1-Fc antigen and 2.5 μg of LV-MS1-Fc antigen mixed with an equal amount of alum twice, at 2-week intervals (Fig. 2A). The LV-MS1-Fc antigen concentration-dependent humoral immune response was determined by ELISA. At 14 and 28 days after the first inoculation, the sample serum was prepared and total IgG was measured (Fig. 2B). The total IgG serum antibody titer was higher in the mice inoculated with antigen mixed with alum adjuvant than in the mice inoculated with antigen alone. The serum neutralizing antibody titers were measured using a luciferase assay at 28 days after the first inoculation. Serum samples diluted 10-fold were further diluted with an equal volume of PBS two-fold serial diluted, mixed with an equal volume of VSV-MERS-S virus, incubated, and then inoculated into pre-prepared Huh 7 cells in a 96-well plate. The arithmetic mean serum neutralizing antibody titer was significantly higher in the mice inoculated with 2.5 μg of LV-MS1-Fc with alum than in the mice inoculated with 2.5 μg of LV-MS1-Fc alone (Fig. 2C).

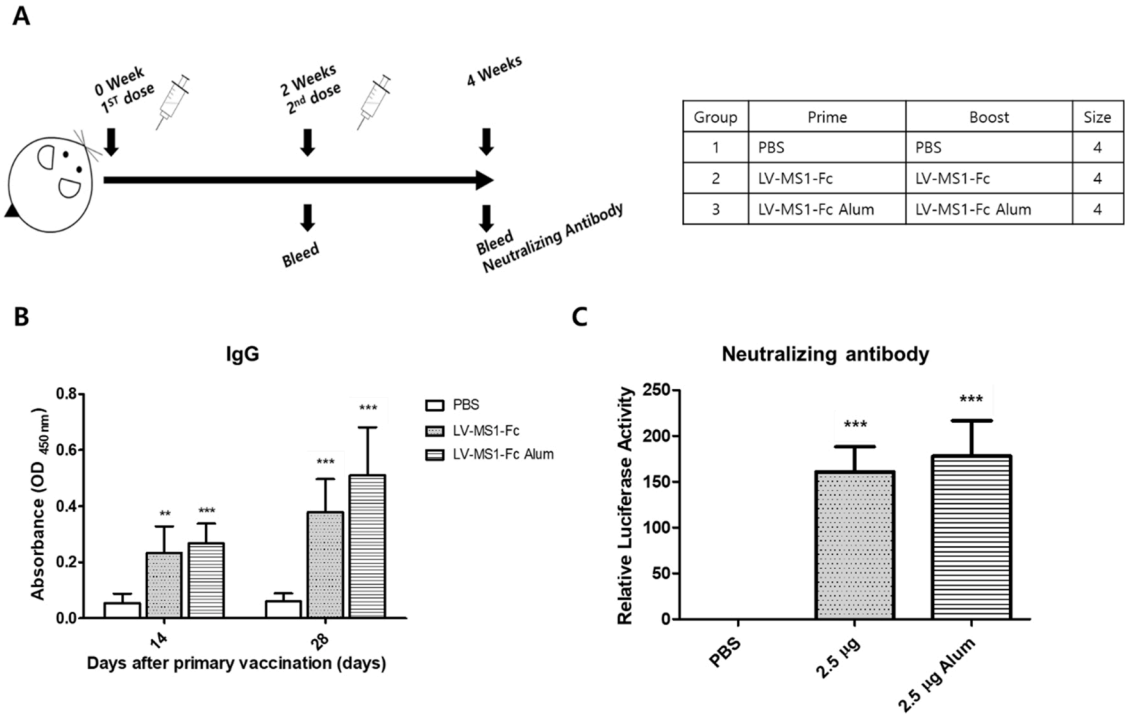
3.4. Protection after pseudovirus challenge using the DPP4 knockin mouse model

The survival rate was measured to determine the protective efficacy against MERS-CoV infection. Histopathological examinations were conducted 1 week after a lethal challenge of hDPP4 knockin mice with 10^4 PFU of MERS-CoV (Fig. 3A). In the mice inoculated with LV-MS1-Fc with alum adjuvant, the 1-week survival rate was 100 % (Fig. 3B) and there was no significant change in the body weight (Fig. 3C). Histopathological analysis showed inflammatory cell infiltration, edema, and exudate in the lung tissue of mice in the PBS group, but no lung lesions were detected in the vaccinated group (Fig. 3D).

4. Discussion

Strong protective immunity inducible epitopes or epitope containing protein are key elements for effective and safe vaccine development. Current MERS vaccine development research uses MERS spike protein or a specific part of the spike protein. Especially RBD is an important domain of the spike protein, which has a receptor for host cell infection. MERS-CoV glycoprotein (RBD, and the S1 and S2 subunits) (Du et al., 2013a,b; Guo et al., 2015; Kim et al., 2014; Ma et al., 2014; Song et al., 2013; Wang et al., 2015), which is the major peptide domain that elicits neutralizing antibodies. MERS-CoV spike protein consists of S1 and S2 subunits. RBD domain exists in the S1 subunit and RBD elect virus neutralization antibody production as a major humoral immune response. Nevertheless, the cellular immune response is also very important to the protection of virus infection because the cellular immune response was more effective against endogenous antigens such as a viral infection. In this study, we try to use some part of the S2 subunit, which has several epitopes that stimulate the cellular immune response against MERS-CoV. But between S1 and S2 cleavage by protease was not prevented by furin cleavage site modification. Total amino acid sequence change of the furin cleavage site reduces cleavage reaction not completely preventing cleavage reaction (data not shown). Therefore, future studies about furin cleavage sites are needed for sequence and structure relationships in the Spike protein cleavage site of MERS-CoV.

The human Fc molecule of the IgG has an immune-enhancing effect when it is used as a fusion protein with an epitope or immunogenic domain of the antigen protein. Several subunit vaccines containing Fc molecule covalent bond-linked antigens confer effective protection against specific diseases. However, the use of the Fc molecule as an immune-enhancing partner needs extensive research, such as immunological and physiological studies. Because Fc molecules have an Fc receptor on the immune cells, which were not clearly identified. Another



Group	Prime	Boost	Size
1	PBS	PBS	4
2	LV-MS1-Fc	LV-MS1-Fc	4
3	LV-MS1-Fc Alum	LV-MS1-Fc Alum	4

Fig. 2. Measurement of serum total IgG titer and serum neutralizing antibody titer. (A) Groups of BLAL/c mice were inoculated intramuscularly with LV-MS1-Fc with or without formulated alum adjuvant, and were administered two doses, 2 weeks apart. (B) The total IgG titers were measured using ELISA. (C) Serum neutralization titers were measured using a luciferase assay. The bars represent arithmetic mean ± SD of the antibody titers. Significant differences from the control are indicated as ***$P < 0.001$.

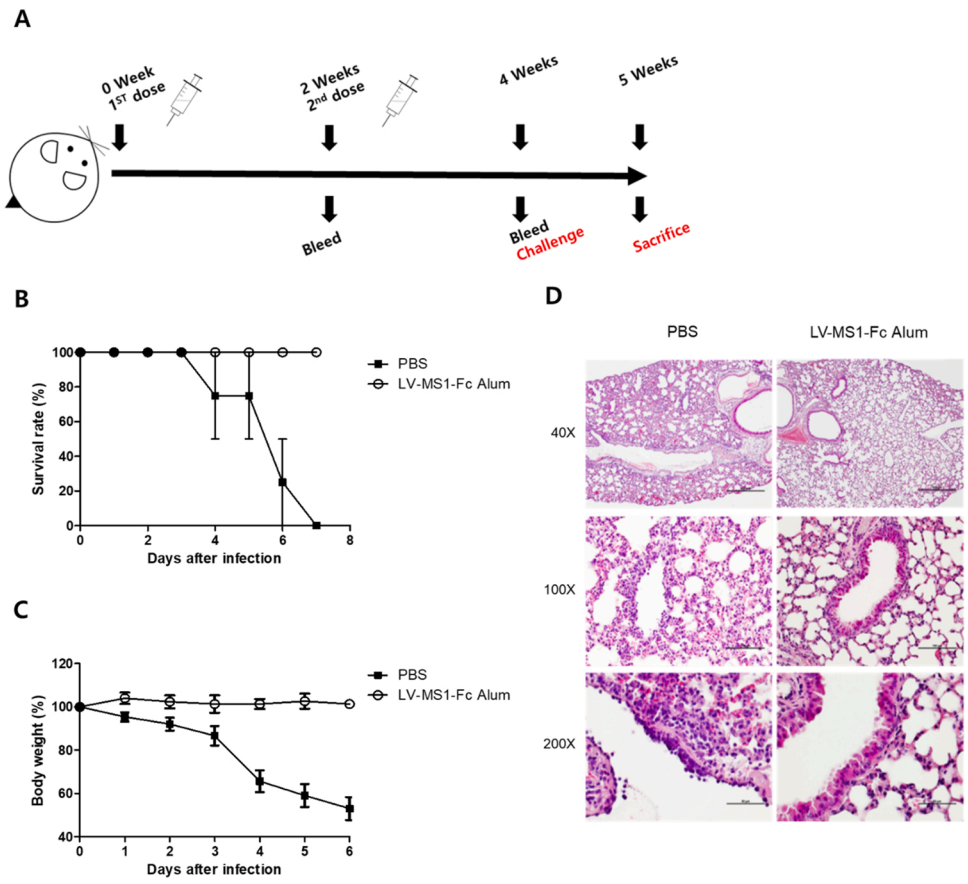


Fig. 3. Protective immunity against MERS-CoV. (A) Groups of hDPP4-knockin transgenic mice were inoculated intramuscularly with LV-MS1-Fc with formulated alum adjuvant, two doses 2 weeks apart. The mice were intranasally inoculated with 10^4 PFU/50 μL MERS-CoV. Serum was collected at 4 weeks after primary vaccination. Body weight was monitored as an indicator of disease progression. At 1 week, the lung tissue was collected for histopathological examination. Survival rate (B) and body weight change (C) during the 7 days after viral challenge were monitored. The mice inoculated with LV-MS1-Fc with alum adjuvant had a 100 % survival rate. Mice in the PBS group whose body weight decrease by more than 25 % were sacrificed. The bars represent arithmetic mean ± SD of the antibody titers. (D) Histopathological examination of the lung tissue of mice at 1 week after the viral challenge. The mice inoculated with PBS presented inflammatory cell infiltration and edema of the lung tissue (left). The mice inoculated with LV-MS1-Fc with alum adjuvant had normal lung tissue (right).

consideration is the structural change of the antigen protein by fusion of the Fc molecule. The immunogenicity of LV-MS1-Fc and LV-MS1 was compared using ELISA titer (data not shown), the Fc molecules increased the immunogenicity of the antigen. In contrast to this, a previous study showed that baculovirus-expressed S1-Fc did not enhance the immunogenicity of the S1 antigen of the MERS-CoV spike protein (data not shown) (Chun et al., 2019).

In this study, a neutralizing antibody test revealed that the LV-MS1-Fc (2.5 μg and 2.5 μg alum)-inoculated groups presented neutralization titers of 160 and 176, respectively. However, we were unable to determine the minimum antigen concentration required for protection. Another important effect of alum adjuvant on antibody titer and immunogenicity was not completely compared. Therefore, adjuvant effect to immunogenicity of the subunit antigen of LV-MS1-Fc must be tested in future study and also protective antibody titer also determined by repeat VN test with a challenge test.

Bacteria and yeast expression systems are suitable for the cost-effective production of antigens to produce vaccines. However, it is difficult to produce a soluble RBD protein and no glycosylation occurred in the bacterial expression system (Nyon et al., 2018). And another problem is using a bacterial system to remove LPS(endotoxin) during the purification. Yeast expression systems have been used antigen protein production and have commercialization cases but sometimes yeast expression systems have different glycosylation patterns with viral protein. MERS-CoV spike protein is a viral glycoprotein therefore, some glycosylation moieties are critical in terms of immunogenicity. In this study, LV-MS1-Fc was constructed in transfected CHO cells. Transiently transfected cell lines produce low concentrations of target proteins and lose the expression efficiency over time if they continuously produce recombinant proteins (Sou et al., 2014). In contrast to this, DNA is incorporated into cells long-term via transfection. Even though establishing foreign protein production in a stable CHO cell line is difficult and time-consuming, sustainable and high-concentration production of foreign protein using CHO cells is essential. Recombinant CHO cell cloning and cell line development are the starting point of vaccine production. In this study, the S1-Fc gene in the pcDNA3.4 TOPO plasmid vector was transfected into CHO-S cells and the transfected CHO cells producing purified by affinity chromatography and size exclusion chromatography. The recombinant CHO cells that were selected depending on the LV-MS1-Fc production capability were determined by quantification using sandwich ELISA.

This study demonstrated that the S1 and Fc fusion proteins are good subunit antigens for vaccine candidates against MERS-CoV infection. In particular, protein-based subunit vaccines have been commercialized and have relatively reliable efficacy and safety. Further studies are required to determine the optimal concentration of the antigen and whether alum is needed as an adjuvant. Further study is also required to the identification of the immune response mechanism by the Fc molecules when it expressed fusion protein with antigen.

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Author contributions

BK, JE, KS, H: Conceived and designed the experiments.
BK, YH, JE: Performed the experiments.
BK, YH, JE, KS, H: Analyzed the data.
BK, H: Wrote the paper.
BK, H: Read and approved the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2021.114347.

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