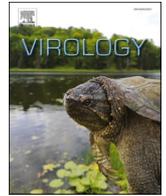




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## Brief Communication

# Immunogenicity of candidate SARS-CoV-2 DNA vaccines based on the spike protein

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## ABSTRACT

Coronavirus disease 2019 caused by the novel human severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is currently a major threat to public health worldwide. To deal with the needs of vaccine, we developed four DNA vaccine candidates against SARS-CoV-2, based on the full-length spike (S) or truncated S protein. Following mice vaccination, we measured T-cell response and antigen-specific neutralizing antibody (NAb) titer. All four candidates induced humoral immune responses, including elevated levels of total IgG and NABs, and cell-mediated immune responses, including multiple cytokine expression. However, the full-length S DNA vaccine enhanced the immune responses most significantly. We then evaluated its appropriate antigen dose and vaccination schedule. Although all immunized groups showed higher immune response than the control group, inoculation with 50 µg antigen led to the highest NAb titer. Immunity was significantly increased after the third inoculation. Thus, the full-length S DNA vaccine can potentially prevent SARS-CoV-2 infection.

## 1. Introduction

Coronavirus disease 2019 (COVID-19) was declared a pandemic by the World Health Organization in 2020. COVID-19 is related to other highly pathogenic coronavirus-associated diseases, such as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS). Infection with SARS coronavirus 2 (SARS-CoV-2), the causative agent of COVID-19, is characterized by severe respiratory symptoms accompanied by fever, dry cough, fatigue, myalgia, dyspnea, anorexia, and acute pneumonia (Wang et al., 2020). Senior people and those with underlying medical conditions, such as respiratory disease, are at higher risk for severe illness and even death due to COVID-19 (Zhou, 2020). SARS-CoV-2 is transmitted via aerosols, respiratory droplets, and environmental contamination by infected patients. Most of the reported COVID-19 outbreaks are due to local transmission by close contact with infected patients, continuing with the emergence of new variants.

As a member of genus *Betacoronavirus*, family *Coronaviridae* SARS-CoV-2 is closely related to SARS-CoV, the causative agent of SARS. The genome of this single-stranded, positive-sense RNA virus encodes four structural proteins, namely spike (S), envelope, membrane, and

nucleocapsid proteins and 16 non-structural proteins. The S protein plays an essential role for virus entry and infection of host cells by binding to the human angiotensin-converting enzyme 2 receptor (Hoffmann et al., 2020). The S protein also contains the major neutralizing epitopes for developing vaccine antigens against coronaviruses, including SARS and MERS. Both the neutralizing antibody response and T-cell immune response play critical roles in vaccination against SARS-CoV-2. Vaccine candidates based on full or truncated S protein using diverse platforms such as DNA- and mRNA-based, subunit, viral vectored, and inactivated virus are under development and clinical trials have been initiated. Recently, diverse vaccines against SARS-CoV-2, including mRNA-1273 from Moderna, BNT162b2 from Pfizer/BioNTech, ChAdOx1 nCoV-19 (AZD1222) from Oxford-AstraZeneca, Ad26.COV2 from Janssen, and NVX-CoV2373 from Novavax, were approved. The vaccination programs are in operation using emergency-approved vaccines in several countries, including South Korea.

For further vaccine development, we have selected the DNA vaccine platform for constructing and evaluating a DNA-based vaccine candidate targeting the S protein of SARS-CoV-2. DNA vaccines are safe and

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easy to design and product on large scale. The present study aimed to evaluate the potential of DNA-based vaccine candidates and to determine their appropriate antigen and dosing regimen for protection against SARS-CoV-2 infection.

## 2. Materials and methods

### 2.1. Cell and virus cultures

Vero cells were grown in Dulbecco's modified Eagle's medium (Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco™) and 1% penicillin/streptomycin. They were maintained in a humidified 5% CO<sub>2</sub> incubator at 37 °C. The first human-isolated SARS-CoV-2 strain in Korea (BetaCoV/Korea/KCDC03/2020, NCCP43326, GISAID accession ID: EPI\_ISL\_407193) was passaged and titrated as plaque forming units (PFU) in Vero cells. All experiments involving live virus were conducted in biosafety level 3 facility following the recommended safety precautions and measures.

### 2.2. COVID-19 DNA vaccine construction and expression

The gene sequence encoding the S protein (1–1273 nucleotides) of the SARS-CoV-2 virus (BetaCoV/Korea/KCDC03/2020) was optimized using the Optimum Gene™ algorithm to enhance its expression and synthesized by GenScript Biotech (Piscataway, NJ, USA). The synthetic full-length S protein,  $\Delta$ CD (S without the cytoplasmic domain),  $\Delta$ TM (S without the transmembrane domain), and S1 fragment only were respectively digested with BamH1 and Xho1 and subcloned into the mammalian expression vector pVax1 (Invitrogen™, Thermo Fisher Scientific). The N-terminal tissue plasminogen activator (tPA) leader sequence was added to improve *in vivo* expression. The recombinant plasmid was purified using the EndoFree Plasmid Giga Kit (QIAGEN GmbH, Hilden, Germany).

HEK 293A cells were transfected with DNA vaccine candidates (2  $\mu$ g) using lipofectamine 2000 (Invitrogen™), and cell lysates were collected after 24 h. Cell lysis was performed by adding RIPA buffer and protease inhibitor. Cell lysates in 5  $\times$  sample buffer were boiled at 100 °C for 10 min and then subjected to 4–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The gel was transferred onto a polyvinylidene difluoride membrane and blocked for 1 h with 5% skim milk. Blots were incubated with SARS-CoV-2 Spike antibody (Sino Biological, Beijing, China) at 4 °C overnight and then with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Invitrogen™) for 3 h at 20–25 °C. Visualization was achieved on the 4CN plus chromogenic substrate (PerkinElmer, Waltham, MA, USA).

### 2.3. Mouse immunization

Mouse studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Centers for Disease Control and Prevention (KCDC-061-20-2A). C57BL/6 mice were immunized three times at 3-week intervals with 50, 20, and 5  $\mu$ g of DNA vaccine candidates. The tibialis anterior muscle of anesthetized mice was shaved, and a single dose of plasmid DNA was injected. For electroporation *in vivo*, a two-needle array electrode pair was inserted into the muscle immediately after DNA delivery. The distance between electrodes was 5 mm, and the array was inserted parallel to the muscle fibers. Three pulses of 100 V (50 ms duration) were delivered twice using an ECM 830 square wave electrophoresis system (BTX, Holliston, MA, USA). Blood samples were collected at 4 and 7 weeks after the first vaccination to analyze humoral immune responses.

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well plates were coated with 50 ng/well of SARS-CoV-2

S1+S2 ECD protein (Sino Biological) and incubated overnight at 4 °C. The protein was removed, and the wells blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 2 h at 37 °C. After washing with PBS containing 0.02% Tween-20 (0.02% PBST), 2-fold serial dilutions of sera (initial dilution 1:100) from immunized mice were added, and the plates were incubated at 37 °C for 1 h. After washing three times with 0.02% PBST, HRP-conjugated anti-mouse IgG was added, and the plates incubated at 37 °C for another hour. Plates were washed five times and developed for 10 min at RT on tetramethyl benzidine substrate. After adding the stop solution for TMB substrate (Thermo Fisher Scientific, Waltham, MA, USA), absorbance was measured at 450 nm on a microplate reader device (Spectra Max i3X, Molecular Devices., San Jose, CA, USA).

### 2.5. Viral neutralization assay

Vero cells were seeded in 12-well plates (2.5  $\times$  10<sup>5</sup> cells/well) and incubated at 37 °C and 5% CO<sub>2</sub> overnight. SARS-CoV-2 at 60 PFU was mixed with an equal volume of 2-fold serial dilutions of heat-inactivated mice sera and incubated at 37 °C for 1 h. The mixture was used to infect Vero cells, and after 1 h, the monolayer was overlaid with 0.75% agar in 4% FBS 2  $\times$  minimum essential medium. Following incubation at 37 °C and 5% CO<sub>2</sub> for 3 days, the cells were fixed with formaldehyde and stained with crystal violet. The neutralizing antibody (NAb) titer was defined as the dilution factor corresponding to 50% plaque reduction compared to the positive control (virus only).

### 2.6. Cytokine expression analysis

Enzyme-linked immunosorbent spot (ELISPOT) assay for interferon gamma (IFN- $\gamma$ )-secreting splenocytes was performed using the Mouse IFN- $\gamma$  ELISpot commercial kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol. Splenocytes (5  $\times$  10<sup>5</sup> or 1  $\times$  10<sup>6</sup> cells) from vaccinated mice were added to each 96-well and stimulated with 1  $\mu$ g SARS-CoV-2 spike glycoprotein peptide pool (GenScript Biotech). This pool included 315 peptides derived from a peptide scan (15 mers with 11 amino acid overlap) through the entire S protein of SARS-CoV-2. The splenocytes were stimulated and cultured at 37 °C for 18 h. Plates were washed with wash buffer, and biotinylated anti-IFN- $\gamma$  antibody was added. After incubation at RT for 2 h, alkaline phosphatase-conjugated streptavidin was added, and plates were kept at RT for another 2 h. Finally, the 3-amino-9-ethylcarbazole chromogen (ACE) solution included in the kit was added for 20 min, and colored spots were monitored and counted using CTL's Immunospot reader (Cleveland, OH, USA). The expression of cytokines was examined using the MILLIPLEX Mouse High Sensitivity T Cell Magnetic Bead Panel kit (Millipore, Burlington, MA, USA) according to the manufacturer's instructions and analyzed using the MAGPIX system (Luminex Corp., Austin, TX, USA).

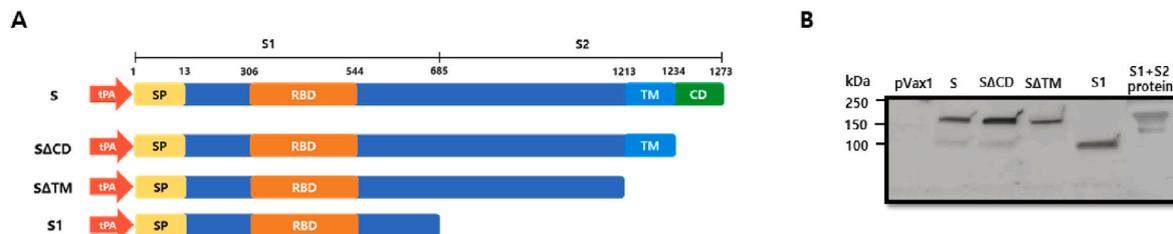
### 2.7. Statistical analyses

Statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. The post-hoc test was used to adjust for multiple comparisons between the different groups. All statistical analyses were performed on GraphPad Prism software (San Diego, CA, USA). Statistical significance was considered at  $P < 0.05$ .

## 3. Results

### 3.1. Construction of DNA vaccine candidates against SARS-CoV-2

We constructed four DNA plasmid vaccine candidates expressing the full-length S protein and other constructs expressing a truncated S protein (Fig. 1A). The optimized DNA sequence of SARS-CoV-2 S protein included a tPA leader sequence to enhance expression and



**Fig. 1.** Design of SARS-CoV-2 DNA vaccines. (A) Schematic representation of the DNA vaccine constructs. Four constructs were generated. One construct expressed the full-length S protein, whereas the other three expressed a truncated S protein by deleting the cytoplasmic domain (SΔCD), transmembrane domain (SΔTM), or S2 subunit (S1). (B) *In vitro* expression of the DNA vaccines in cell cultures as confirmed using western blot with S1+S2 protein (positive control) and anti-S1 polyclonal antibody. The protein marker molecular weight (kDa) is indicated on the left.

immunogenicity. The optimized DNA sequence was digested with BamH1 and Xho1 and then cloned into the expression vector pVax1.

The DNA vaccine candidates were transfected into 293A cells and measured by western blot analysis using an antibody against the SARS-CoV-2 S protein on cell lysates. The molecular weight of the obtained blots was similar to that of the predicted S (140 kDa), SΔCD (135 kDa), SΔTM (133 kDa), and S1 (78 kDa) proteins, with slight shifts likely due to potential N-linked glycans (Fig. 1B).

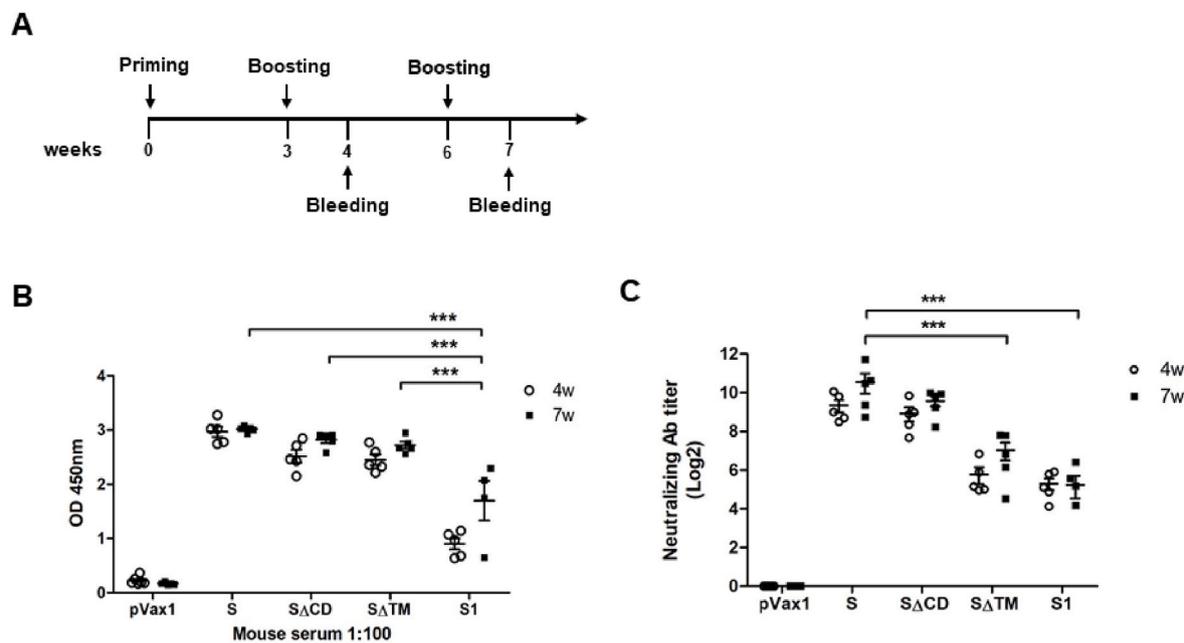
### 3.2. Immune response of the different SARS-CoV-2 spike DNA vaccines in mice

To confirm humoral immune response, we performed ELISA on the sera of mice immunized three times at 3-week intervals with the S, SΔCD, SΔTM, and S1 vaccine candidates delivered by electroporation. The levels of SARS-CoV-2 S1+S2-specific total IgG antibodies in the sera of mice immunized with S, SΔCD, and SΔTM were high after two vaccine doses and increased with additional immunizations (Fig. 2B). To determine if the antibodies in sera could neutralize SARS-CoV-2 infection *in vitro*, a plaque reduction neutralizing test (PRNT) was performed using serially diluted serum samples. The full-length S DNA vaccine

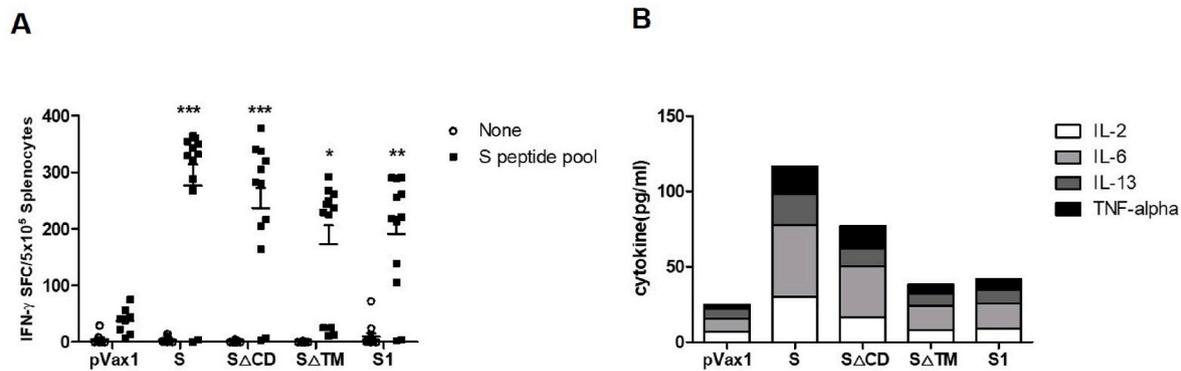
induced a considerably higher NAb titer than the truncated SΔTM or S1 DNA vaccines (Fig. 2C). Antigen-specific T-cell responses in the vaccinated mice were examined using IFN-γ ELISPOT and cytokine multiplex assay. The mice immunized with the full-length S DNA vaccine candidate showed the highest levels of cytokines (Fig. 3). Our results indicated that the deletion of the cytoplasmic and transmembrane domains or S2 ECD of the S protein elicited a lower humoral immune response than the full-length S sequence.

### 3.3. Dose-dependent effects of the SARS-CoV-2 full-length S DNA vaccine

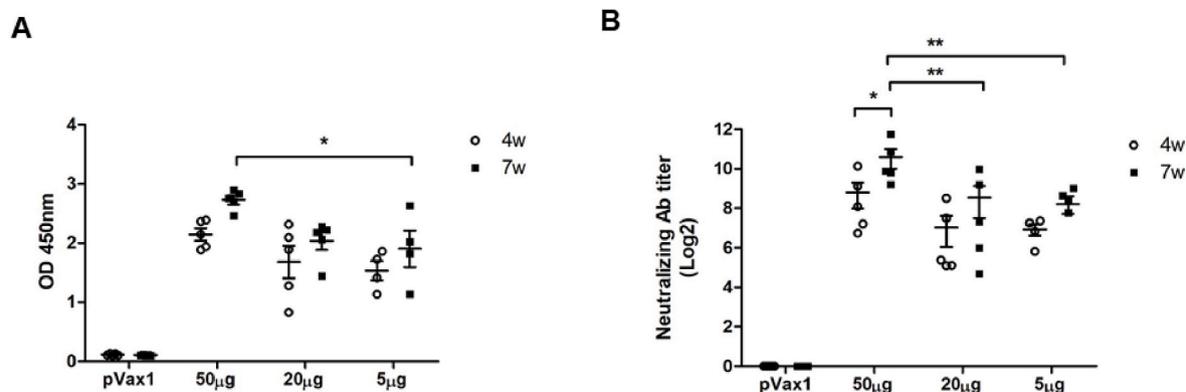
To determine immunogenicity, different groups of mice were administered different doses of the full-length S DNA vaccine. Regardless of the antigen dose, all groups of mice exhibited significantly increased antibody titers compared with control mice (Fig. 4A). The group immunized with 50 μg of the full-length S DNA vaccine showed the highest antibody titer, and both the second and third immunizations confirmed the dose-dependent results. We performed the PRNT using sera obtained at 4 and 7 weeks after immunization with the DNA vaccine candidate. As observed for the IgG titer, the group immunized with 50 μg of the full-length S DNA vaccine showed levels of NAb significantly



**Fig. 2.** Humoral immune response induced by the different SARS-CoV-2 S protein DNA vaccines. (A) Time course of vaccine immunizations and immunity analysis performed in this study. C57BL/6 mice ( $n = 4-5$ ) were immunized with 50 μg DNA vaccine at weeks 0, 3, and 6, and blood samples were collected at weeks 4 and 7 (1 week after the first and second boosts, respectively). (B) Sera collected from the individual mice were serially diluted, and anti-SARS-CoV-2 S1+S2 ECD protein specific total IgG levels were measured using ELISA. (C) NAb responses detected in the viral neutralization assay. Titers are presented as the serum dilution that mediates PRNT<sub>50</sub> of virus infection of the target cells. Data are the mean ± SD of individual mice within each group. *P*-values were determined using one-way ANOVA followed by Tukey’s post-hoc test. \*\*\**P* < 0.001.



**Fig. 3.** Induction of cell-mediated immune responses by the different SARS-CoV-2 S protein DNA vaccines. C57BL/6 mice ( $n = 6$ ) were immunized with 50  $\mu$ g DNA vaccine at weeks 0, 3, and 6. Immunized mice were sacrificed 1 week after the second boost, and splenocytes were isolated for measuring cell-mediated immune response. (A) splenocytes ( $5 \times 10^5$ ) from mice were stimulated for 18 h with 1  $\mu$ g SARS-CoV-2 S protein peptide pool. IFN- $\gamma$  ELISPOT was then performed, and the spots were counted using the CTL Immunospot reader. Data are the mean  $\pm$  SD of individual mice within each group.  $P$ -values were determined using one-way ANOVA followed by Tukey’s post-hoc test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (B) Interleukin (IL)-2, IL-6, IL-13, and tumor necrosis factor (TNF)-alpha expression levels were measured in a mouse cytokine multiplex assay using the culture supernatants of mice splenocytes. Bars present the mean of each group.



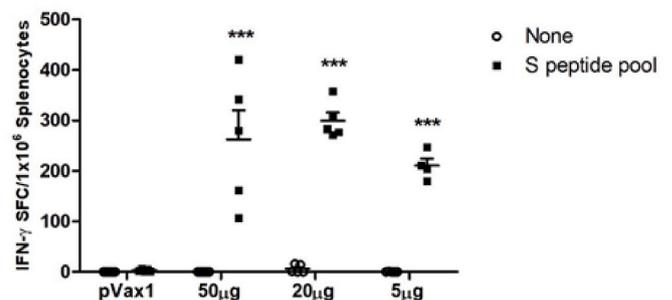
**Fig. 4.** Humoral immune response after SARS-CoV-2 full-length S protein DNA vaccination at different doses. C57BL/6 mice ( $n = 4-5$ ) were immunized with different doses of full-length S DNA vaccine at weeks 0, 3, and 6, and blood samples were collected at weeks 4 and 7 (1 week after the first and second boosts, respectively). (A) Sera collected from individual mice were serially diluted, and anti-SARS-CoV-2 S1+S2 ECD protein specific total IgG values were measured using ELISA. (B) NAB responses were detected in a viral neutralization assay. Titers are presented as the serum dilution that mediates the PRNT<sub>50</sub> of virus infection of the target cells. Data are the mean  $\pm$  SD of individual mice within each group.  $P$ -values were determined using one-way ANOVA followed by Tukey’s post-hoc test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

higher than that observed in the other groups (Fig. 4B). The level of NAB titer was also significantly increased at the third immunization with 50  $\mu$ g of the full-length S DNA vaccine compared to that obtained after the second immunization. All groups had significantly more IFN- $\gamma$  secreting cells than the control group (Fig. 5). In particular, splenocytes from mice immunized with 50 and 20  $\mu$ g of the full-length S DNA vaccine candidate showed higher levels of IFN- $\gamma$  than mice immunized at the lowest dose (5  $\mu$ g). These results suggested that three immunizations with 50  $\mu$ g of antigen was the most effective protocol for the rapid induction of humoral and cell-mediated immune responses against SARS-CoV-2 in mice.

#### 4. Discussion

COVID-19, formerly known as 2019-nCoV pneumonia, has emerged as a global public health crisis in 2020, joining SARS, emerged in 2003, and MERS, emerged in 2012, in the pool of diseases transmitted from animals to human (i.e., zoonosis). SARS-CoV-2 was first isolated from airway epithelial cells of infected patients and then sequenced. In Korea, the first COVID-19 case was reported on January 20, 2020, when SARS-CoV-2 was detected in a traveler entering Korea from China.

In the present study, four DNA vaccines were designed and optimized based on the S protein of the first SARS-CoV-2 isolate from a Korean



**Fig. 5.** Induction of T cell responses in mice SARS-CoV-2 full-length S protein DNA vaccination at different doses. Immunized mice were sacrificed 1 week after the second boost, and splenocytes were isolated for measuring T cell responses using ELISPOT. Splenocytes ( $5 \times 10^5$ ) were stimulated for 18 h with 1  $\mu$ g SARS-CoV-2 S protein peptide pool. IFN- $\gamma$  ELISPOT was then performed, and the spots were counted using the CTL Immunospot reader. Data are the mean  $\pm$  SD of individual mice ( $n = 4-5$ ) within each group.  $P$ -values were determined using one-way ANOVA followed by Tukey’s post-hoc test. \*\*\* $P < 0.001$ .

patient (BetaCoV/Korea/KCDC03/2020) (Kim et al., 2020) and used for immunoassays. To increase translation efficiency, the tPA signal sequence was attached to the N-terminal sequence of the S protein. In the intracellular synthesis of plasmid DNA antigens, these are mostly presented after digestion by the proteasomes and peptidases present in the endoplasmic reticulum (ER) involved in the major histocompatibility complex class I pathway. As proteasomes and ER are present in the cytoplasm, ensuring the presence of many antigens in the cytoplasm may be highly effective for inducing immunogenicity. The tPA is a specific signal targeting the expressed antigen directly to the ER, avoiding its processing and transfer to the cytoplasm (Luo et al., 2008). Based on the results of previous studies that developed vaccines against the SARS and MERS coronaviruses, the S protein was selected as the target antigen in the present study. The S protein is a class I membrane fusion protein and the major envelope protein on the coronavirus surface. Previous studies have shown that the interaction of the virus with the angiotensin-converting enzyme 2 host receptor can be blocked using antibodies (Benton et al., 2020; Glowacka et al., 2011).

Results of the present study revealed that the SARS-CoV-2 DNA vaccine based on the pVax1 vector induced antigen-specific immune responses in mice. The total IgG titers in the groups immunized with S,  $\Delta$ CD, and  $\Delta$ TM vaccines were higher than that of the group immunized with the S1 vaccine. Moreover, the group immunized with the S vaccine showed significantly higher NAb titers than the other groups, except that immunized with the  $\Delta$ CD vaccine (Fig. 2). These results are similar to the PRNT results obtained for the positive sera of challenged mice (unpublished data). The cell-mediated immune responses in the group immunized with the S vaccine was also higher than that in the other groups (Fig. 3). In a previous study of SARS-CoV-2 DNA vaccine on Rhesus macaque, the group immunized with the DNA vaccine encoding the full-length S protein presented a reduced viral load in the bronchoalveolar lavage and nasal mucosa compared to sham controls after challenge with SARS-CoV-2 (Yu et al., 2020). Similar to the MERS-CoV DNA vaccine, the S1 antigen showed higher NAb titers and effective T cell responses than S,  $\Delta$ CD, and  $\Delta$ TM antigens (Al-Amri et al., 2017). Similar to our results, the antisera of mice immunized with the  $\Delta$ CD vaccine elicited the strongest NAb response, and SARS-CoV replication was not observed in the challenged mouse model (Yang et al., 2004). These results suggest that coronaviruses within the same family may have different antigen domains that induce an effective immune response according to their characteristics.

We selected the full-length S DNA vaccine as the best candidate and performed additional experiments to determine its appropriate dose. This vaccine induced sufficient levels of IgG antibody and NAb titers in all groups of mice administered 5, 20, or 50  $\mu$ g antigen (Fig. 4). The groups inoculated with 5 or 20  $\mu$ g antigen each showed similar antibody titers, whereas that inoculated with 50  $\mu$ g antigen showed significantly higher titers. For the group inoculated with 50  $\mu$ g antigen, antibody titers were also significantly higher after the third inoculation (7 weeks) than after the second inoculation (4 weeks), revealing the boosting effect of up to three times vaccination, which might affect long-lasting immunogenicity. In the case of the cell-mediated immune response analysis using IFN- $\gamma$  ELISPOT, there was no significant difference between groups, but all showed higher antigen specific spot forming unit values compared to unstimulated experimental groups (Fig. 5).

Among the various vaccination methods, electroporation was used in our study. This method can introduce macromolecules, such as nucleic acids, into cells *in vivo* or *in vitro* by applying short electric pulses to induce transient and reversible permeation of cell membranes (Sardesai and Weiner, 2011). Electroporation *in vivo* was shown to promote the transfer of DNA to the nucleus of muscle cells, and protein expression was also increased by about 18-folds compared to intramuscular injection (Dupuis et al., 2000). In addition, the level of expression varied according to differences in muscle mass compared to the inoculation volume, explaining the relevance of decreased immunogenicity in animals larger than mice. Further research is needed to determine a way to

effectively apply electroporation inoculation in small animals including mice to human.

In summary, the results of the present study showed that the DNA vaccine candidates induced humoral and cell-mediated immune responses against SARS-CoV-2. Nevertheless, further studies are needed to confirm its ability to protect against virus infection *in vivo* by selecting an appropriate animal model. These findings are useful for understanding the role of S glycoproteins in SARS-CoV-2 infection and vaccine development and as a reference for the design and development of vaccines against related emerging pathogens.

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## CRedit authorship contribution statement

**Heeji Lim:** Conceptualization, Methodology, Investigation, Writing – original draft. **Se Eun Kim:** Methodology, Investigation. **Yun Ha Lee:** Methodology, Investigation. **Yun-Ho Hwang:** Investigation. **Su Hwan Kim:** Investigation. **Mi Young Kim:** Project administration. **Gyung Tae Chung:** Project administration. **You-Jin Kim:** Writing – review & editing, Project administration. **Dokeun Kim:** Writing – review & editing, Project administration. **Jung-Ah Lee:** Conceptualization, Methodology, Investigation, Writing – review & editing, Supervision, Project administration.

## Declaration of interests

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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