Article

Cellular and humoral immunogenicity of a candidate DNA vaccine expressing SARS-CoV-2 spike subunit 1

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Abstract: The urgent need for effective, safe and equitably accessible vaccines to tackle the ongoing spread of COVID-19 led researchers to generate vaccine candidates targeting varieties of immunogens of SARS-CoV-2. Because of its crucial role in mediating binding and entry to host cell and its proven safety profile, the subunit 1 (S1) of the spike protein represents an attractive immunogen for vaccine development. Here, we developed and assessed the immunogenicity of a DNA vaccine encoding the SARS-CoV-2 S1. Following in vitro confirmation and characterization, the humoral and cellular immune responses of our vaccine candidate (pVAX-S1) was evaluated in BALB/c mice using two different doses, 25 μg and 50 μg. Our data showed high levels of SARS-CoV-2 specific IgG and neutralizing antibodies in mice immunized with three doses of pVAX-S1. Analysis of the induced IgG subclasses showed a Th1-polarized immune response as demonstrated by the significant elevation of spike-specific IgG2a and IgG2b compared to IgG1. Furthermore, we found that immunization of mice with three doses of 50 μg of pVAX-S1 could elicit significant memory $CD4^+$ and $CD8^+$ T cell responses. Taken together, our data indicates that pVAX-S1 is immunogenic and safe in mice and is worthy of further preclinical and clinical evaluation.

Keywords: SARS-CoV-2 vaccine; cellular and humoral immunogenicity; DNA vaccine

1. Introduction

The emergence and rapid spread of the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), the causative agent of the coronavirus disease 2019 (COVID-19) pandemic, represent a serious public health and economic burden to humanity [1-4]. While the majority of COVID-19 patients are either asymptomatic carriers or have mild symptoms such as fever, myalgia and cough, millions have suffered from life-threatening

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acute respiratory infections and deaths. As of June 2021, around 178 million confirmed cases have been reported with at least 3.8 million deaths [5]. Furthermore, the high transmissibility rate of SARS-CoV-2 among humans as well as the emergence of new variants of concern (VOC) of the virus pose significant obstacles toward controlling its spread [6-8], highlighting the urgent need for the development of safe, effective and equitably accessible vaccines.

Hundreds of SARS-CoV-2 vaccines have been or being developed using traditional and innovative technology platforms [9]. Several of these vaccines were approved for emergency use by multiple regulatory agencies across the globe. Examples of traditional vaccines include killed/inactivated vaccines which although demonstrated safety and efficacy in humans, several potential risks may still exist [10]. Other developers have adopted innovative technologies and/or novel approaches for antigen design, gene expression and vector optimization including adenovirus-based vaccines [11-14] as well as mRNA vaccines [15]. Additional platforms being used include novel viral vectors, recombinant subunit proteins, nanoparticles and plasmid DNA [16-18].

The spike (S) protein of SARS-CoV-2 is composed of a globular head S1 subunit containing the receptor-binding domain (RBD), and a membrane-proximal S2 subunit containing the fusion machinery of the virus [19]. Most of these aforementioned vaccines rely on using either the full-length S protein or the RBD as the immunogen because of their critical roles in viral entry and host tropism [19,20], and ability to elicit protective immunity in animals and humans after vaccination or infection [9, 10, 21]. Use of full-length S protein as immunogen, however, could be associated with undesired responses by inducing non-neutralizing antibodies which may contribute to disease enhancement, immunopathological inflammation and fatality [22-28]. As such, targeting the S1 subunit could help minimize potentially undesirable effect. Here, we evaluated the humoral and cellular immunogenicity of a plasmid DNA vaccine candidate expressing the S1 subunit of the S protein in an attempt to focus the immune response towards the neutralizing-epitope rich domains.

2. Materials and Methods

2.1 Generation of DNA construct

SARS-CoV-2 S1 coding sequence was PCR amplified from codon-optimized full-length S gene (GenBank accession number: MN908947) synthesized by GenScript USA Inc. (Piscataway, NJ). The coding sequence of S1 (1-681 aa) was subcloned into the mammalian expression vector pVAX1 under the control of the cytomegalovirus immediate-early promoter, denoted as pVAX-S1. The construct was cloned between *NheI* and *KpnI* restriction sites. The construct was then confirmed by restriction digestion and sequencing. Bulk endotoxin-free preparations of pVAX-S1 and empty vector (pVAX) were prepared using the GenElute™ HP Select Plasmid Gigaprep Kit (Sigma, Germany) for animal studies.

2.2 Detection of SARS-CoV-2 S1 protein expression by Western blot

HEK-293A cells (70-90% confluent) in 6-well plates were transfected with 2 μg of pVAX-S1, pVAX1, or pcDNA3.1 expressing full-length S protein (pcDNA-S) using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, followed by incubation at 37°C in a 7% CO_2 incubator for 48 h. After that, media were removed, and transfected cells were then washed with phosphate-buffered saline (PBS) and lysed with radioimmunoprecipitation assay buffer (RIPA buffer) (Sigma, Germany). The lysates were subjected to western blot analysis to test protein expression using in-house anti-S1 (SARS-CoV-2) mouse polyclonal antibodies at a 1:1000

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dilution. Also, β-actin was detected with anti β-actin antibodies at a 1:3000 dilution (Ori-Gene Technologies, Inc., Rockville, MD) as a loading control.

2.3 Detection of SARS-CoV-2 S1 protein expression by immunofluorescence

HEK-293A cells were seeded on cell culture slide and incubate at 37°C in a 7% CO_2 incubator to be 70% confluent by the next day. Cells were then transfected with 1 μg of either pVAX-S1 or pVAX control plasmid using Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions and incubated at 37°C in a 7% CO_2 incubator for 36 h. The media was removed, cells were washed with PBS, fixed with 4% formaldehyde at 4°C for 10 min and permeabilized with 0.2% PBS-Triton 100 (PBS-T) at 4°C for 20 min. Cells were washed twice with PBS-T and blocked with 2% goat serum/PBS-T at room temperature for 30 min. Cells were then stained with in-house mouse anti-S1 primary polyclonal antibodies at a 1:1000 dilution in 2% goat serum/PBS-T at 4°C for 1 h. This was followed by three washes and staining with Alexa Fluor-488 labeled goat anti-mouse IgG H&L secondary antibody (Abcam, UK) at 1:500 dilution in blocking buffer in the dark at room temperature for 1 h. Cells were finally washed three times with PBS-T and mounted with VECTASHIELD with DAPI counterstain antifade mounting medium (Invitrogen, Carlsbad, CA). Images were captured using Olympus BX51 Fluorescence Microscope and analyzed using ImageJ 1.53e Software.

2.4 Immunization and samples collection

6- to 8-week-old female BALB/c mice were provided from King Fahd Medical Research Center (KFMRC) core animal facility, King Abdulaziz University (KAU). All animal experiments were done according to guidelines and the approval of the Animal Care and Use Committee (ACUC) at KFMRC and ethical approval from the bioethical committee at KAU (approval number 04-CEGMR-Bioeth-2020). Mice were randomly divided into three experimental groups (8 mice/group) and immunized with three doses of 25 μg or 50 μg of pVAX-S1, or 50 μg of pVAX on days 0, 21 and 42. Mice were immunized intramuscularly using customized needle-free Tropis system (PharmaJet, Golden, CO). Serum samples were collected every three weeks and mice were euthanized on day 63 to collect final bleed and spleens for immune response analysis.

2.5 Binding antibodies measurement by indirect ELISA.

End-point titers of anti-S1 total IgG or its isotypes (IgG1, IgG2a and IgG2b) from immunized mice were determined by ELISA as previously described [29]. Briefly, 96-well plates were coated with SARS-CoV-2 S1 protein (Sino Biological, China) at 1 μg/ml in at 4°C overnight. Plates were washed three times with PBS containing 0.1% Tween-20 before blocking with 5% skim milk in PBS-T for 1 h at room temperature. After washing, 2-fold serial dilution of mouse sera starting from 1:100 were added to wells and incubated for 1 h at 37°C. Then, peroxidase-conjugated rabbit anti-mouse IgG secondary antibodies (Sigma, Germany) were added at the recommended concentrations and incubated for 1 h at 37°C. After extensive washing, 3,3',5,5'-tetramethylbenzidine (TMB) substrate (KPL, Gaithersburg, MD) was added for 30 min to develop a colorimetric reaction. Finally, reaction was stopped with 0.16 M sulfuric acid, and absorbance was read spectrophotometrically at 450 nm on ELx808™ Microplate Reader (BioTek, Winooski, VT). End-point titers were determined as the reciprocals of the highest dilution with an OD above the cut-off value which was defined as the OD mean from the control group plus three standard deviations (SDs).

2.6 Neutralizing antibodies measurement by pseudovirus neutralization assay

To assess the ability of induced antibodies to inhibit virus entry, pseudovirus neutralization assay was performed as previously described [30]. Briefly, recombinant

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vesicular stomatitis virus expressing codon-optimized full-length SARS-CoV-2 S protein (GenBank accession number: MN908947) (rVSV-ΔG/SARS-2-S*-luciferase pseudovirus) was generated in BHK21/WI-2 cells. Pseudovirus was collected and titrated on Vero E6 cells as previously described [30]. Then, neutralization assay was conducted by co-incubating two-fold serial dilutions of heat-inactivated mouse sera from vaccinated and control groups started from 1:20 dilution (in duplicate) with media containing $5x10^4$ relative luciferase unit (RLU) of rVSV-ΔG/SARS- 2-S*-luciferase pseudovirus for 1 h at 37°C in a 7% CO_2 incubator. The mixtures were then transferred onto confluent Vero E6 cell monolayers in white 96-well plates and incubated for 24 h at 37°C in a 7% CO_2 incubator. Following that, cells were lysed, luciferase activity was measured using Luciferase Assay System (Promega) according to the manufacturer's instructions, and luminescence activity was measured using BioTek Synergy 2 microplate reader (BioTek, Winooski, VT). Cell only control (CC) and virus control (VC) were included with each assay run. The median inhibitory concentration (IC_{50}) of neutralizing antibodies (nAbs) was determined using GraphPad Prism version 9.0.2 software.

2.7 Determination of T cell response by flow cytometry

Single cell suspensions of splenocytes were prepared from each mouse in immunized and control groups. One million splenocytes/well were re-stimulated with 5 μg/ml of a pool of 15-mer peptides overlapping with 11 amino acid and covering the SARS-CoV-2 S1 protein (GenScript USA Inc, Piscataway, NJ) for 6 h at 37°C in a 7% CO_2 incubator in the presence of brefeldin A (BD Biosciences, San Jose, CA) at a final concentration of 1:1000. Phorbol myristate acetate/ionomycin was used as a positive control and RPMI 1640 medium as a negative unstimulated control. Cells were then washed in FACS buffer (PBS with 2% heat inactivated FBS) and stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen, Carlsbad, CA) for 30 min at room temperature. After washing with FACS buffer, cells were stained for surface markers with PB-conjugated anti-mouse CD8, PB-conjugated anti-mouse CD4, APC-conjugated anti-mouse CD44 antibody and Pe-Cy7-conjugated anti-mouse CD62L antibodies (BioLegend, UK). The cells were then washed with FACS buffer and fixed and permeabilized using Cytofix/Cytoperm Solution (BD Biosciences, San Jose, CA) according to the manufacturer's protocol. For intracellular staining, cells were labeled with FITC-conjugated anti–mouse IFN-γ (clone XMG1.2), PE-conjugated anti-mouse TNF-α (clone MP6-XT22) and PerCP/Cy5.5–conjugated anti-mouse IL-2 (clone JES6-5H4) antibodies (BioLegend, UK) for 20 min at 4°C. Cells were then washed twice with permeabilization buffer and once with FACS buffer. All data were collected using BD FACSAria™ III flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo v10 software (Tree Star).

2.8 Statistical analysis

Statistical analysis and graphical presentations were generated using GraphPad Prism version 9.0.2 software (Graph-Pad Software, Inc., CA, USA). Statistical analysis was conducted using the Mann–Whitney test or one-way analysis of variance with Bonferroni post-hoc test to adjust for multiple comparisons between groups. All values are represented as mean ± SD and statistical significance is reported as *, P≤0.05, **, P≤0.01, ***, $p \leq 0.001$, and ****, $p \leq 0.0001$.

3. Results

3.1 In vitro confirmation of protein expression from the candidate vaccine

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The generated DNA vaccine candidate (Figure 1a) was evaluated for protein expression in vitro in HEK293A cells prior to animal experiments. As shown in Figure 1b, western blot analysis confirmed that the recombinant construct was able to express S1 subunit protein at the expected molecular weight. A plasmid expressing full-length S protein (pcDNA3.1-Full S) was used as a positive control. Similarly, immunofluorescence analysis showed expression of SARS-CoV-2 S1 protein in transfected cells (Figure 1c), suggesting that the expressed protein maintained structural confirmation to be detected by polyclonal anti-S antibodies. As expected, no protein was detected from cells transfected with the empty control plasmid pVAX (Figures 1b and 1c).

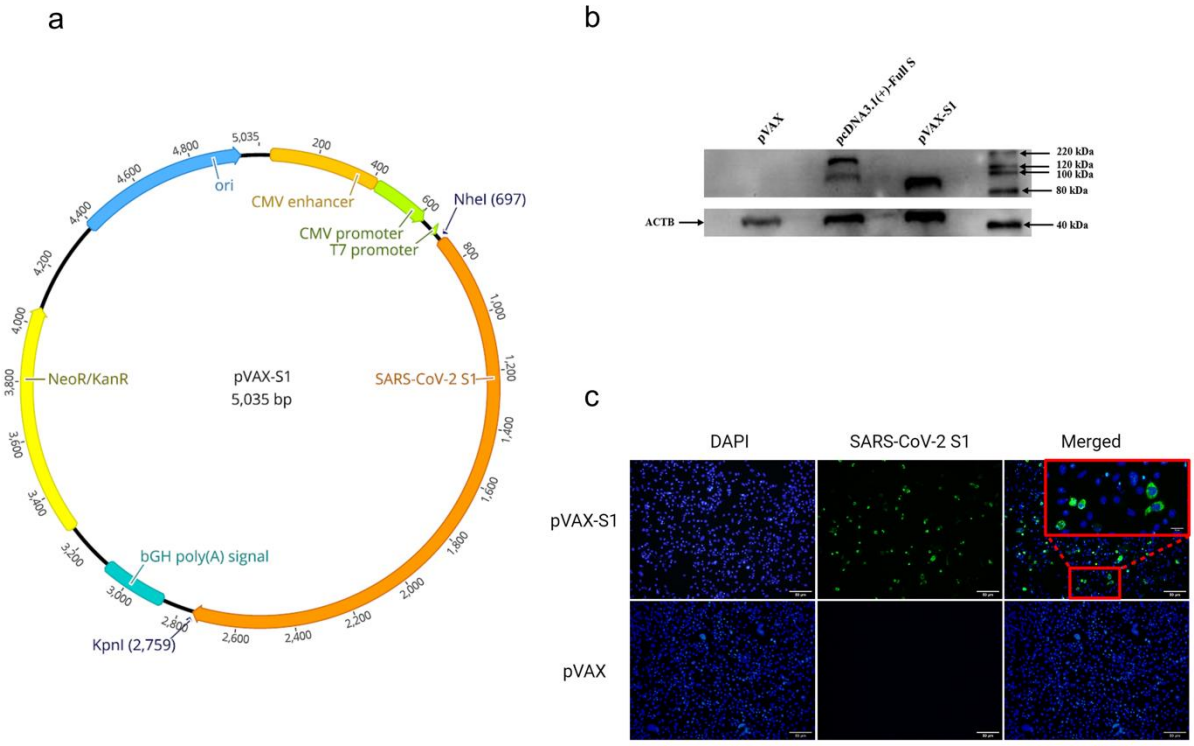

Figure 1. **Vaccine design and characterization.** a) DNA vaccine (pVAX-S1) map. The inserted gene (SARS-CoV-2 S subunit 1) is indicated by orange color in the pVAX1 plasmid. b) Western blot analysis. Figure shows bands of expressed full-length S from cells transfected with pcDNA3.1-Full S (positive control) and S1 subunit protein expressed from pVAX-S1. Empty pVAX was used as negative control. c) Immunofluorescence analysis. Cells transfected with pVAX-S1 or empty control pVAX were stained with anti-SARS-CoV-2 S1 mouse polyclonal antibodies. Scale bars are 50 μm. Red square is magnified to scale bar of 10 μm. Merging and magnification were processed by ImageJ 1.53e.

3.2 Evaluation of binding and neutralizing antibodies in immunized mice

Mice were intramuscularly immunized with 3 doses of 25 μg or 50 μg of pVAX-S1 in a three-week interval regimen (Figure 2a). As a control, a group of mice was immunized with 50 μg of empty control vector (pVAX). Vaccine-induced binding antibodies were assessed by indirect ELISA from serum samples collected on weeks 3, 6 and 9. As shown in Figure 2b, analysis of S1-specific total IgG showed significant levels only after 3 doses with both doses of pVAX-S1 vaccine compared to control group (pVAX). Although no significant difference was found between 25 μg and 50 μg of pVAX-S1, the 50 μg dose of pVAX-S1 showed significantly higher level of antibodies than the 25 μg dose when both were compared to the control group. Testing levels of IgG subclasses (IgG1, IgG2a and IgG2b) in samples collected on week 9 from mice immunized with 50 μg suggested a Th1-skewed immune response as shown by the high IgG2a:IgG1 and IgG2b:IgG1 ratios (Figure

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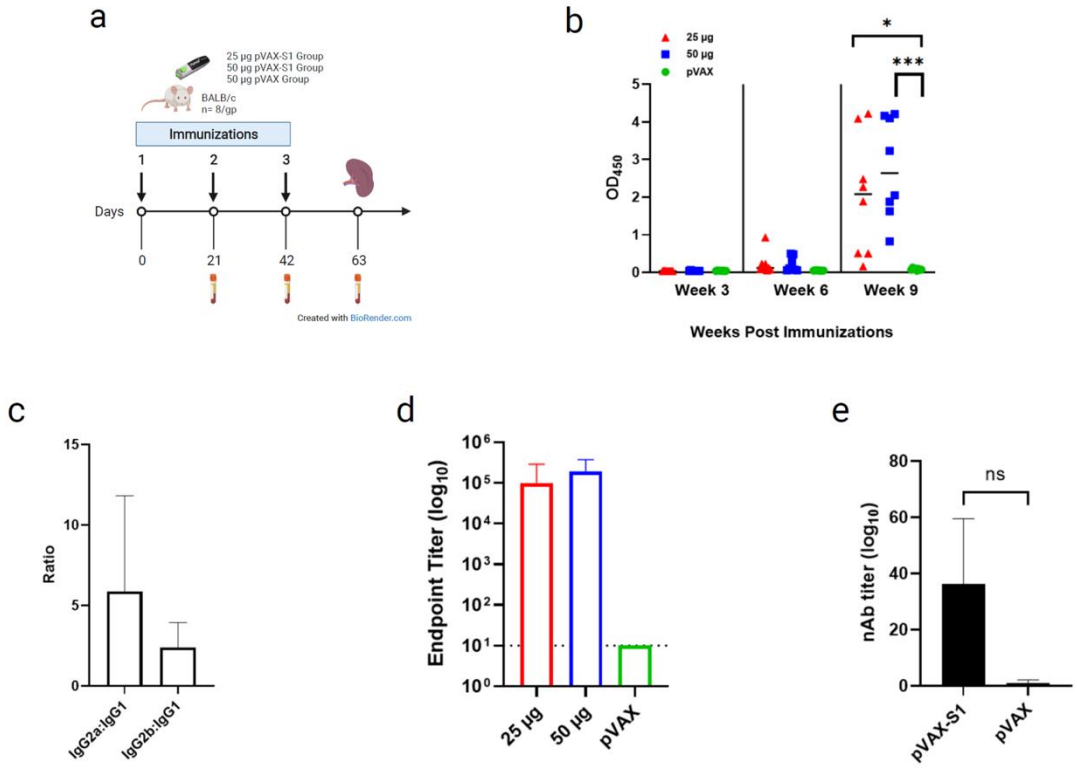
2c). Determination of end-point titers of S1-specific total IgG also confirmed the induction of significant levels of binding antibodies in samples collected on week 9 from each immunized mouse (Figure 2d). To investigate whether the vaccine-induced antibodies were able to inhibit viral entry in cells, levels of nAbs were determined using pseudovirus neutralization assay from samples collected on week 9 in the 50 µg group. As shown in Figure 2e, immunized mice were only able to induce low levels of nAbs against SARS-CoV-2 pseudovirus in Vero cells. Collectively, these results confirm the ability of the vaccine to elicit significant Th1-skwed humoral immunity against SARS-CoV-2.

Figure 2. Humoral immunity in pVAX-S1 immunized mice. a) Animal experimental plan. Mice were divided into three experimental groups (n=8) and immunized intramuscularly with three doses on days 0, 21 and 42 using 25 µg or 50 µg of pVAX-S1, or 50 µg of pVAX1 using a customized needle-free Tropis system (PharmaJet, Golden, CO). b) Values of optic density (OD) of S1-specific



binding total IgG at 1:100 dilution from every single mouse were determined by ELISA at 3, 6 and 9 weeks after immunizations. c) IgG2a:IgG1 and IgG2b:IgG1 ratios were calculated in samples collected on week 9 from immunized mice in the group that received the 50 µg dose of pVAX-S1. d) End-point titers of S1-specific total IgG were determined by ELISA in samples collected on week 9 from each mouse in all groups. e) The median inhibitory concentration (IC_{50}) of neutralizing antibodies (nAbs) was determined against rVSV-ΔG/SARS-2-S*-luciferase pseudovirus as described in the materials and methods.

3.3 Evaluation of cellular immune response in immunized mice

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Next, we investigated the memory $CD8^+$ and $CD4^+$ T cell response from 9-week samples (3 weeks post last immunization) using peptide pool covering the entire S1 protein. As shown in Figure 3a, memory $CD4^+$ T cells ($CD4^+CD62L^-CD44^+$ T cells) from mice immunized with 50 µg dose of pVAX-S1 produced significant levels of IFN-γ and TNF-α but not IL-2 compered to control group. Similarly, antigen-specific CD8+CD44+CD62L+ central memory T cells showed significant levels of IFN-γ and TNF-α but not IL-2 compared to pVAX control group (Figure 3b). On the other hand, effector $CD8^+CD62L^-CD44^+$ memory T cells only produced IFN-γ at significant level compared to control group (Figure 3c). These data show that pVAX-S1 could elicit significant memory $CD4^+$ and $CD8^+$ T cell responses in mice.

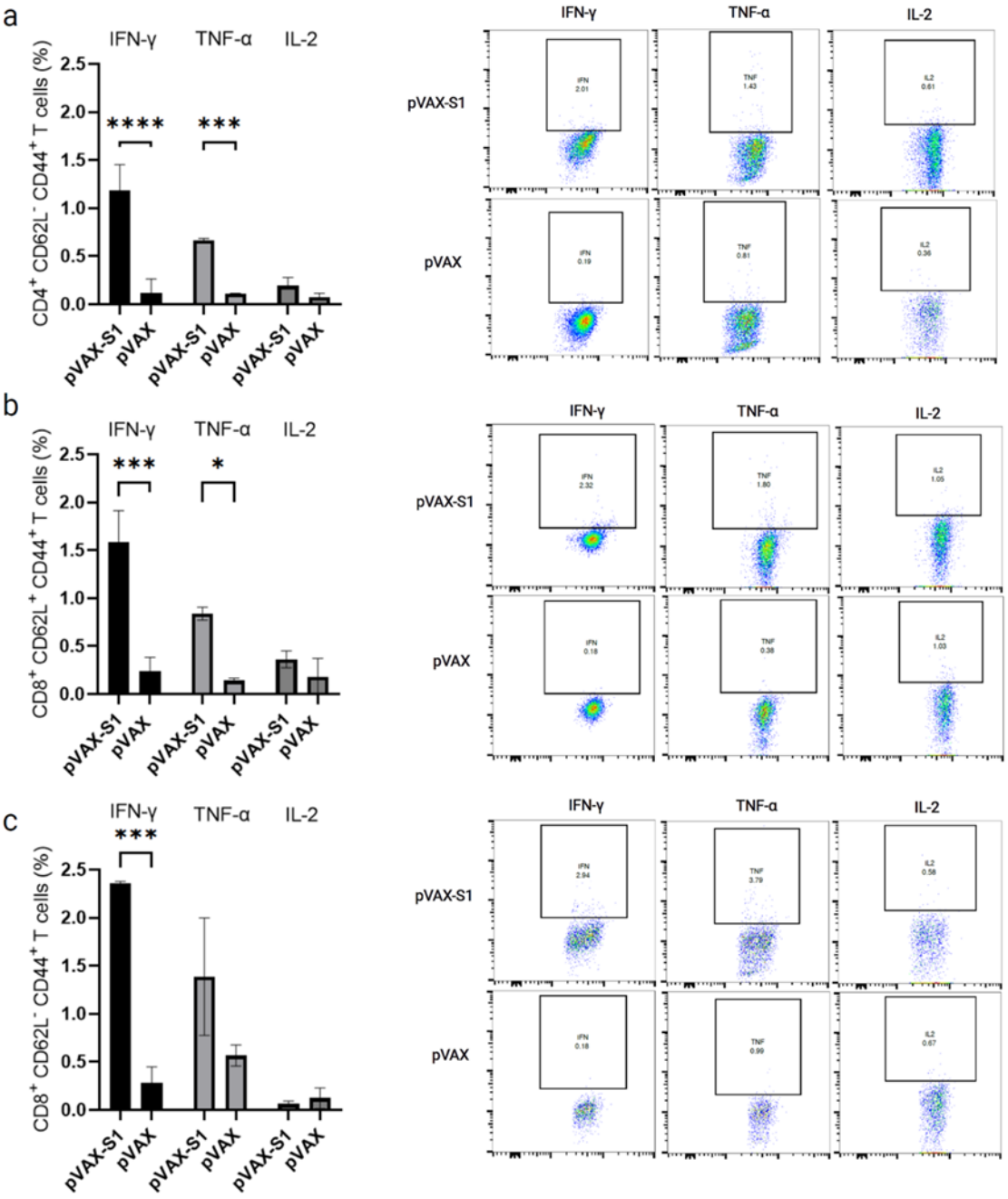

Figure 3. Memory T cells response in immunized mice. Histograms and FACS plots display IFN-γ, TNF-α and IL-2 expression from ex vivo re-stimulated a) memory CD4 ($CD4^+CD62L^-CD44^+$ T cells), b) central memory CD8 ($CD8^+CD62L^+CD44^+$ T cells) and c) effector memory CD8 ($CD8^+CD62L^-CD44^+$ T cells). Data in histograms are shown as percentages of induced cytokines from peptides-stimulated cells after subtracting levels produced by unstimulated splenocytes from each

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mouse. Representative FACS plots are shown. Data are shown as mean ± SD for each group from one experiment.

4. Discussion

There is still an urgent need for multiple safe and protective vaccines against SARS-CoV-2 to combat the ongoing COVID-19 pandemic [9]. DNA-based vaccines represent a fast and safe approach to develop vaccines for such unprecedented situations [31]. Numerous studies on SARS-CoV-2 and other pathogenic human CoVs such as MERS-CoV and SARS-CoV have dementated that most of the neutralizing antibodies that are generated to either natural infection or full-length S based vaccines candidates target the S1 subunit, making S1 an attractive and probably safer immunogen for vaccine development [32-36]. This is due to the fact S1 contains the RBD and the N-terminal motif (NTD) which are critical for mediating binding to the host receptor. In this work, we successfully developed and evaluated the immunogenicity of a new DNA vaccine candidate against SARS-CoV-2 encoding the S1 subunit of the S protein. After *in vitro* confirmation and characterization of the S1 expression, we evaluated the immunogenicity and safety of the vaccine in mice. Overall, our data showed that pVAX-S1 was able to induce strong antibody responses in mice after three doses regimen of intramuscular immunization in a dose dependent manner. Furthermore, we showed that pVAX-S1 induced a Th1-biased protective immune response, characterized by antibody production predominantly of IgG2a and IgG2b subclasses and secretion of significantly elevated levels of Th-1 cytokines (IFN-γ and TNF-α) by memory $CD4^+$ and $CD8^+$ T cells. Interestingly, while the vaccine candidate induced high levels of S1 specific Abs titers, we noticed that the level of the produced nAbs was relatively low which should be investigated for further improvement may be through novel antigen design or use of molecular adjuvants. Nonetheless, similar immune response induced by a similar DNA vaccine provided protection in non-human primates [37], suggesting protection through multiple mechanisms.

It is of note that several vaccine candidates have been developed using S1 subunit as immunogen such as DNA vaccine expressing S1 domain with a foldon trimerization motif [37], live-attenuated YF17D expressing S1 (YF-S1) [38], S1-Fc fusion subunit protein [39], and S1 subunit protein alone (S1) or fused to the norovirus shell domain (S1-S) [33]. Those candidates used different technologies to test S1 immunogenicity in numbers of animal models. Similar to our work, single-dose of YF-S1 in hamsters or two doses of DNA vaccine expressing S1-foldon in rhesus macaques induced significant levels of binding antibodies and low-to-medium levels of nAbs compared to other tested vaccines expressing full-length cleavable S protein, prefusion-stabilized S or other truncated versions such as those lacking the transmembrane or the cytoplasmic domains [37, 38]. Also consistent with our data, both of these S1-based vaccines induced highly elevated levels of Th1-skewed T cell responses compared to other vaccines [37, 38]. Interestingly, while YF-S1 failed to protect hamsters from viral replication [38], S1-foldon DNA vaccine led to reduction of viral RNA after SARS-CoV-2 challenge in rhesus macaques [37]. On the other hand, other developed subunit vaccines such as S1-Fc and S1-S fusion proteins elicited significantly high levels of nAbs in multiple animal models that exceeded the levels observed in acutely infected individuals [33, 39]. These previous reports as well as our current data clearly show the potential of SARS-CoV-2 S1 as a promising immunogen [33, 37-39].

The use of S1 as an immunogen has been proposed for other highly pathogenic coronaviruses such as MERS-CoV and SARS-CoV because its potential high safety profile compared to the use of full-length S. Although full-length S protein can induce the highest immune response, some reports suggest its association with possible side effects in the currently used COVID-19 vaccines [22, 23]. Additionally, previous reports on MERS-CoV, SARS-CoV and other coronaviruses have suggested that the use of full-length S based vaccine could lead to undesired immune response upon infection [24-28]. Although, exact mechanism of this vaccination-induced immunopathology and/or disease enhancement

has not been fully elucidated, it has been postulated that the non-neutralizing epitopes within the S protein may be responsible for the harmful immune response in vaccinated hosts [40, 41]. These data suggested that using the neutralizing-epitope rich S1 subunit of the S protein could be a better approach to avoid any potential safety concerns.

Within the past year and half, several COVID-19 vaccines have been approved for emergency use with hundreds of others being currently in different stages of clinical development [9, 42, 43]. Among the ones that have been approved and in late stages of clinical development are the nucleic acid-based vaccines. DNA and mRNA vaccines have several advantages over the platforms. For example, these vaccines can be developed rapidly without the need for the cultivation of the target pathogen and can be easily produced in a large industrial scale. Furthermore, nucleic acid-based vaccines can be rapidly and easily adapted to respond to potential mutations/variants. Compared to mRNA vaccines which requires a cold-chain system, DNA vaccine are more thermo-stable with less stringent storage condition and easier formulation, enabling it as a promising platform for wide distribution across the globe. Our approach using plasmid DNA (pVAX) to encode the SARS-CoV-2 S1 as a proof of principle have demonstrated that S1 can lead to high humoral and cellular immunity in mice with a predominant Th1-biased response. Such approach can be further enhanced by the use of efficient adjuvant and improved method of delivery.

Author Contributions: K.A.A., R.Y.A., M.H., A.A., X.L. and A.M.H. designed and conceptualized the work. K.A.A., R.H.A., R.Y.A., N.D.A., S.S.A., M.B., R.H., M.A.A., L.T., and W.Z. performed and optimized experiments and analyzed data. W.A., M.H., A.A., X.L., and A.M.A. provided resources and materials. A.M.A. and A.M.H. acquired the funding. K.A.A., A.A., X.L. and A.M.H. drafted the manuscript. All authors have reviewed and edited the manuscript and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and all animal experiments were done according to guidelines and the approval of the Animal Care and Use Committee (ACUC) at KFMRC and ethical approval from the bioethical committee at KAU (approval number 04-CEGMR-Bioeth-2020).

Data Availability Statement: The raw data collected in this study are available on request from the corresponding author.

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Conflicts of Interest: A.M.H., M.A.A., K.A.A., S.S.A. and A.A. are named as inventors on a US patent application related to this work. A.M.H. and A.A. work as scientific consultants in SaudiVax ltd and receive fees for consulting. M.H. is an employee of SaudiVax ltd and receives salary and benefits. All other authors report no competing interests.

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