



Successive Site Translocating Inoculation Improved T Cell Responses Elicited by a DNA Vaccine Encoding SARS-CoV-2 S Protein

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A variety of methods have been explored to increase delivery efficiencies for DNA vaccine. However, the immunogenicity of DNA vaccines has not been satisfactorily improved. Unlike most of the previous attempts, we provided evidence suggesting that changing the injection site successively (successively site-translocated inoculation, SSTI) could significantly enhance the immunogenicity of DNA vaccines in a previous study. To simplify the strategy and to evaluate its impact on candidate SARS-CoV-2 vaccines, we immunized mice with either a SARS-CoV-2 spike-based DNA vaccine or a spike protein subunit vaccine *via* three different inoculation strategies. Our data demonstrated that S protein specific antibody responses elicited by the DNA vaccine or the protein subunit vaccine showed no significant difference among different inoculation strategies. Of interest, compared with the conventional site fixed inoculation (SFI), both successive site-translocating inoculation (SSTI) and the simplified translocating inoculation (STI) strategy improved specific T cell responses elicited by the DNA vaccine. More specifically, the SSTI strategy significantly improved both the monofunctional (IFN- γ ⁺IL-2⁻TNF- α ⁻CD8⁺) and the multifunctional (IFN- γ ⁺IL-2⁻TNF- α ⁺CD8⁺, IFN- γ ⁺IL-2⁻TNF- α ⁺CD4⁺, IFN- γ ⁺IL-2⁺TNF- α ⁺CD4⁺) T cell responses, while the simplified translocating inoculation (STI) strategy significantly improved the multifunctional CD8⁺ (IFN- γ ⁺IL-2⁻TNF- α ⁺CD8⁺, IFN- γ ⁺IL-2⁺TNF- α ⁺CD8⁺) and CD4⁺ (IFN- γ ⁺IL-2⁻TNF- α ⁺CD4⁺, IFN- γ ⁺IL-2⁺TNF- α ⁺CD4⁺) T cell responses. The current study confirmed that changing the site of intra muscular injection can significantly improve the immunogenicity of DNA vaccines.

Keywords: successive site translocating inoculation, DNA vaccine, protein vaccine, T cell response, antibody response

INTRODUCTION

According to the data released by WHO, there have been more than 360 million confirmed cases of COVID-19, including over 5.6 million deaths by the end of January 2022. Multiple vaccines have been developed and deployed to reduce severe disease and death, as well as protecting health systems (Interim Statement on COVID-19 vaccines in the context of the circulation of the Omicron SARS-CoV-2 Variant from the WHO TAG-CO-VAC). Both the classical and next-generation platforms have been employed in developing COVID-19 vaccines (1), including whole inactivated virus, live attenuated virus, protein subunit, viral vector, RNA, and DNA vaccines (2, 3). The advantages and disadvantages of these platforms have been described and compared elsewhere (4, 5). Among different types of COVID-19 vaccines, the mRNA vaccines displayed extraordinary performance in preventing COVID-19 according to clinical trials (6, 7). Although there is no parallel comparison of the immunogenicities and protective efficacies, a retrospective review of data generated on rhesus macaque models suggested that mRNA vaccine showed better protection efficacy against SARS-CoV-2 than DNA vaccine (8). Compared with mRNA vaccines, it usually required to use higher dosages of DNA vaccines to stimulate desired immunities (9, 10). A major hurdle that restricts the application of DNA vaccine is the lack of efficient delivery approach (11). A variety of physical methods (gene gun, electroporation, jet injection, etc.), chemical methods (liposome, synthetic polymer, etc.) and adjuvants have been explored to improve the immunogenicity of DNA vaccine (12). However, the extracellular and intracellular barriers that restrain the transportation of DNA into the nucleus have not been satisfactorily resolved (11).

Unlike most of the preceding efforts, in a previous study, we provided the first evidence suggesting that changing the injection site successively (successively site-translocated inoculation, SSTI) could significantly enhance the immunogenicity of a DNA vaccine encoding OVA protein (13). In the current study, we further demonstrate that the SSTI strategy can be leveraged to improve both the quantity and the quality of T cell responses elicited by a candidate DNA vaccine expressing the full length of SARS-CoV-2 S protein. In comparison, the strategy is not conducive to augment the immunogenicity of an alum adjuvanted S protein subunit vaccine.

MATERIALS AND METHODS

Ethics Statement

All experiments and methods were performed in accordance with relevant guidelines and regulations. Experiments using mice were approved by the Research Ethics Review Committee of the Shanghai Public Health Clinical Center Affiliated to Fudan University.

Construction and Preparation of a Candidate DNA Vaccine Encoding SARS-CoV-2 Full Length S Protein

The full-length s gene sequence of the reference SARS-CoV-2 strain was optimized according to the preference of human codon usage and synthesized by Genewiz (Genewiz Biotech Co., Ltd., Suchow, China).

The codon optimized spike gene was subcloned into a eukaryotic expression vector (pJW4303, kindly gifted by Dr. Shan Lu at the University of Massachusetts). And the sequence of inserted gene was verified by Sanger sequencing (Sangon Biotech Co., Ltd., Shanghai, China). An EndoFree plasmid extraction kit (Cat# 12391, Qiagen, Hilden, USA) was used to prepare the recombinant plasmid for mouse vaccination.

The Preparation of an Alum Adjuvanted Protein Vaccine

Purified SARS-CoV-2 S protein (Cat# 40591-V08H, Sino Biological, China) was reconstituted with PBS at a concentration of 80 μ g/ml. Then, it was mixed with a commercialized alum adjuvant (Cat# 77161, ThermoFisher Scientific Co., Ltd., USA) at a ratio of 1:1 (v/v). The final concentration of the purified S protein was 40 μ g/ml.

Mouse Vaccination

Female C57BL/6 mice, 6 to 8 week-old, were housed under a specific-pathogen free (SPF) environment. As shown in **Figure 1**, the mice were immunized intramuscularly with the pJW4303-CMV-S DNA vaccine (50 μ g/mouse) or the alum adjuvanted S protein subunit vaccine (2 μ g/mouse) for three times at an interval of 2 weeks. The control group was inoculated with PBS. Peripheral blood was collected at 2 weeks post each vaccination. Five weeks post the third vaccination, the mice were euthanized. Peripheral blood, bronchial alveolar lavage fluid (BALF) and splenocytes were collected for assays of antigen-specific immune responses.

Detection of SARS-CoV-2 RBD Binding Antibodies

An in-house enzyme-linked immunosorbent assay (ELISA) was developed to measure SARS-CoV-2 RBD specific binding antibody responses. High-binding 96-well EIA plates (Cat# 9018, Corning, USA) were coated with purified SARS-CoV-2 RBD protein (Cat# 40592-V08B, Sino Biological, China) at a final concentration of 1 μ g/ml in carbonate/bicarbonate coating buffer (30mM NaHCO₃, 10mM Na₂CO₃, pH 9.6). Subsequently, the plates were blocked with 1 \times PBS containing 5% skimmed milk for 1 hour at 37°C. Next, 100 μ l of serially diluted mouse serum or plasma was added to each well. After 1-hour incubation at 37°C, the plates were washed with 1 \times PBS containing 0.05% Tween20 for 5 times. Then, 100 μ l of an HRP labeled goat anti-mouse IgG antibody (Cat# 115-035-003, Jackson Immuno Research, USA) diluted in 1 \times PBS containing 5% skimmed milk were added to each well and incubated for 1 hour at 37°C. After a second round of wash, 100 μ l of TMB substrate reagent (Cat# MG882, MESSGEN, China) was added to each well. 15 minutes later, the color development was stopped by adding 100 μ l of 1M H₂SO₄ to each well and the values of optical density at OD₄₅₀_{nm} and OD₆₃₀_{nm} were measured using 800 TS microplate reader (Cat# 800TS, Biotek, USA). The cut-off value was defined as 2-fold of the average OD₄₅₀-630 of PBS group at 1:100 dilution.

Competitive ELISA

The binding antibody titers against the full-length S protein were measured using a method of competitive ELISA (14), which can

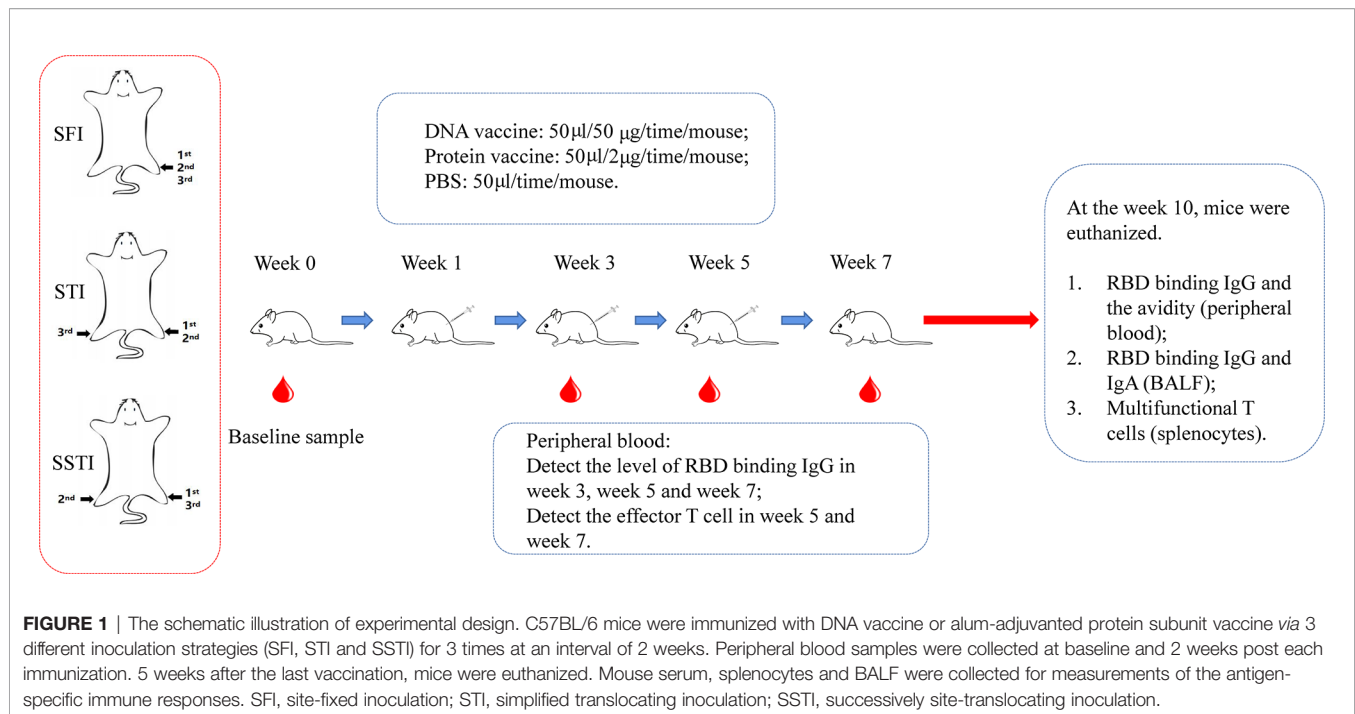


FIGURE 1 | The schematic illustration of experimental design. C57BL/6 mice were immunized with DNA vaccine or alum-adsorbed protein subunit vaccine via 3 different inoculation strategies (SFI, STI and SSTI) for 3 times at an interval of 2 weeks. Peripheral blood samples were collected at baseline and 2 weeks post each immunization. 5 weeks after the last vaccination, mice were euthanized. Mouse serum, splenocytes and BALF were collected for measurements of the antigen-specific immune responses. SFI, site-fixed inoculation; STI, simplified translocating inoculation; SSTI, successively site-translocating inoculation.

help to avoid the interference of pre-existing cross-reactive antibody responses against S2. Briefly, high-binding 96-well EIA plates were coated with purified SARS-CoV-2 S protein (Cat# VISC2-S002, East Mab, China) at a final concentration of 1µg/ml in carbonate/bicarbonate coating buffer. The experiment procedure was generally similar with the aforementioned in-house ELISA assays, except that the diluted mouse serum were incubated with a synthesized peptide (P144, SFKEELDKYFKNHT) (10µg/ml) for 1 hour at 37°C before adding into the coated EIA plates.

Antibody Avidity Assay

Avidity of Ag-specific Ab was determined by avidity ELISA as reported (15–17) with minor modifications. Briefly, plates were coated as the regular ELISA assay described above. Diluted mouse sera were added into each well. After 1-hour incubation, ELISA plates were washed with washing buffer and incubated with 1.5M NaSCN or PBS for 15 minutes at room temperature and then immediately washed with washing buffer. Ab avidity index was defined as the ratio of the OD value of a sample with 1.5M NaSCN treatment versus the OD value of the same sample with PBS treatment.

Flowcytometry Assays

Freshly isolated splenocytes or peripheral blood mononuclear cells were plated into round-bottom 96-well plates (2×10^6 cells per well) and incubated with either R10 (RPMI1640 with 10% FBS) or R10 containing synthesized peptides encompassing the full length of S protein (0.66µg/ml for each peptide) (Synthesized by Gill Biochemistry Co., Ltd., Shanghai, China). Two hours later, brefeldin A and monensin were added to each well at final concentrations of 1µg/ml and 1µM, respectively. Another 12 hours later, the cells were washed and stained sequentially with

Live/Dead dye (Fixable Viability Stain 510, cat# 564406, BD Pharmingen), surface markers (PE/Cyanine7-labeled anti-mouse CD3, cat# 100220, BioLegend; APC-labeled anti-mouse CD4, cat# 100412, BioLegend; PE-labeled anti-mouse CD8, cat# 100708, BioLegend) and intracellular markers (BV421-labeled anti-mouse IFN-γ, cat# 505830, BioLegend; FITC-labeled anti-mouse IL-2, cat# 503806, BioLegend; BV711-labeled anti-mouse TNF-α, cat# 506349, BioLegend). Stained samples were analyzed using a BD Fortessa flow cytometer and data were analyzed with the FlowJo software version 10 (TreeStar Inc., Ashland, OR, USA). The gating strategy was shown in **Supplementary Figure 1**.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between two groups were conducted by the method of t-test. $P < 0.05$ was considered as statistically significant.

RESULTS

The S Protein Specific Binding Antibody Responses Were Not Improved by SSTI for Mice Immunized With Both the DNA and Protein Subunit Vaccines

C57BL/6 mice were immunized via three different inoculation strategies (**Figure 1**): Mice in the group of site-fixed inoculation (SFI) were immunized by injection into the tibialis anterior of the same limb. Mice in the group of successively site-translocated inoculation (SSTI) were immunized by injecting into the left hind limb, the right hind limb and the left hind limb,

sequentially. The mice in the group of simplified translocating inoculation (STI) were injected into the left hind limb for the 1st and 2nd shots, and into the right hind limb for the 3rd shot. Peripheral blood samples were collected at 2 weeks post each immunization. Five weeks post the last vaccination, the mice were euthanized. Serum, BALF and splenocytes were collected for detection of antigen-specific immune responses (Figure 1).

Our data showed that the serum levels of RBD binding IgG titers induced by the DNA and the protein subunit vaccines were not improved by either the SSTI or the STI strategies (Figures 2A, B). Of note, the average binding antibody responses elicited by the protein subunit vaccine for the STI and SSTI groups tended to be lower than that of the SFI group (Figure 2B). Besides, we found that the binding antibody titers reached the plateau after two shots of DNA vaccine, while it required at least 3 shots for the protein subunit vaccine to boost the binding antibody responses. Similarly, binding antibody titers against the full-length S protein also showed that there was no significant difference among different inoculation strategies (Supplementary Figure 2). The avidities of RBD binding antibodies determined at 5 weeks post the 3rd immunization were similar among different strategies (Figure 2C). The levels of RBD binding IgG in BALF were different between mice immunized with the DNA vaccine and the protein vaccine. However, no significant difference was observed among different inoculation strategies for either the DNA or the protein subunit vaccine (Figure 2D).

The levels of RBD binding IgA in BALF were very low in mice inoculated with either the DNA or the protein vaccine (Supplementary Figure 3).

Changing Inoculation Site for the 2nd Shot of DNA Vaccine Was Essential for Eliciting Optimal Specific T Cell Responses

To characterize the influences of different inoculation strategies on cellular response kinetics during the vaccination process, we collected mouse peripheral blood lymphocytes (PBL) through mandibular vein puncture at 2 weeks post the 2nd immunization and 2 weeks post the 3rd immunization, respectively. S protein specific T cell responses were measured using flowcytometry. Our data showed that the cellular immune responses elicited by the alum adjuvanted S protein were obviously weaker than those induced by the S DNA vaccine (Figures 3 and 4). Both the CD8⁺ and CD4⁺ T cell responses at the two time points showed no significant differences among groups of mice immunized with the protein vaccine (Figure 4). For mice immunized with the SARS-CoV-2 S DNA vaccine, the average frequency of circulating specific IFN- γ secreting CD8⁺ T cells in the SSTI group ($1.310 \pm 0.393, 6$) was significantly higher than that of the SFI group ($0.621 \pm 0.177, 7$) ($P=0.0015$) at 2 weeks post the 3rd immunization (Figure 3B), whereas the STI strategy did not significantly improve the frequency of circulating IFN- γ ⁺CD8⁺ T cells at this time point, suggesting that

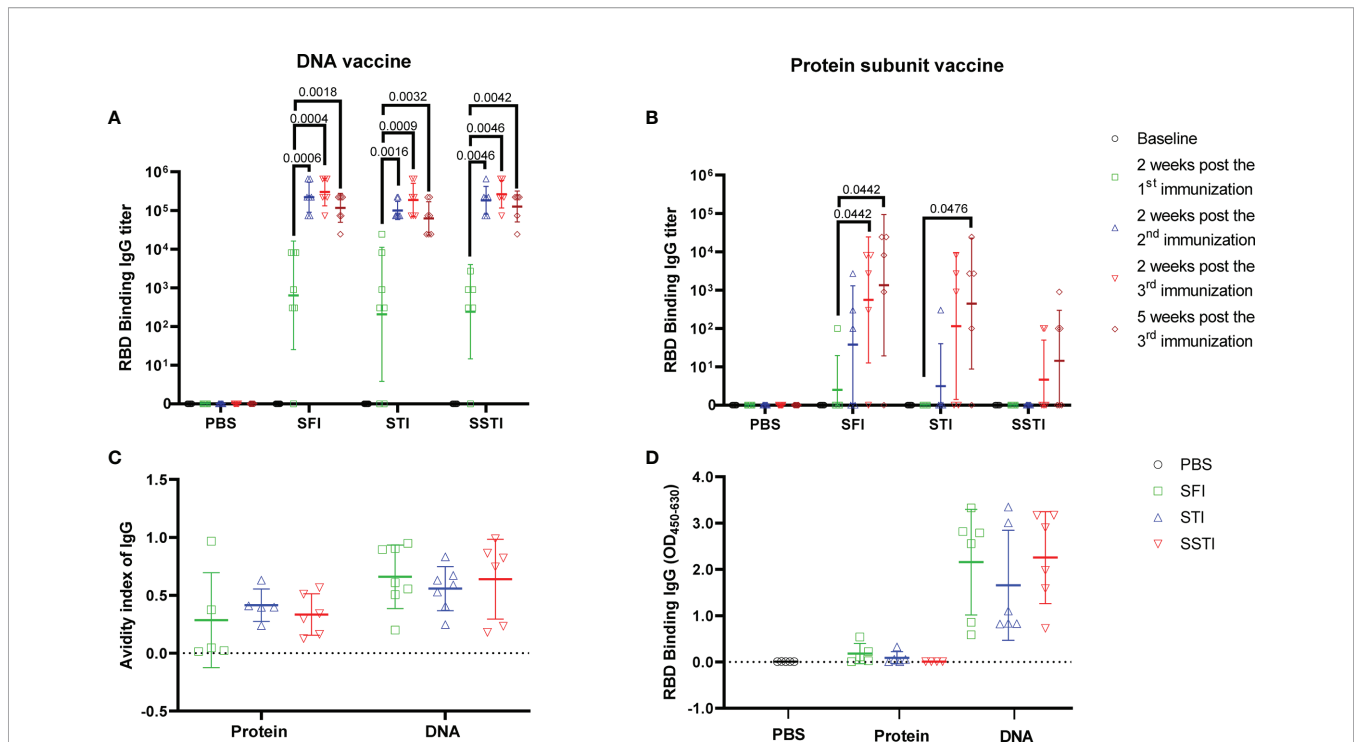


FIGURE 2 | SSTI did not improve the specific antibody responses elicited by the DNA or the protein subunit vaccine. RBD binding antibody titers elicited by the DNA (A) or the protein subunit vaccine (B) were determined at baseline, 2 weeks post the 1st immunization, 2 weeks post the 2nd immunization, 2 weeks post the 3rd immunization and 5 weeks post the 3rd immunization, respectively. (C) The avidity of RBD-specific antibodies was determined at 5 weeks post the 3rd immunization. (D) RBD specific IgG in BALF was detected using an ELISA method after adjusting the total protein for each BALF sample to the same concentration (250 μ g/ml). Data are shown as mean \pm SD. Statistical analyses were performed using the method of t-test.

changing the inoculation site for the second shot of DNA vaccine was essential to induce optimal T cell responses. Interestingly, our data showed that specific CD8⁺ T cell responses elicited by the DNA vaccine were not significantly improved by the SSTI strategy at 2 weeks post the 2nd shot (Figure 3A), implying that the SSTI improved DNA induced T cell responses in an incremental mode. Compared with the CD8⁺ T cell responses, the frequencies of S protein specific IFN- γ secreting CD4⁺ T cells in PBL were weak at these time points and showed no significant difference among different inoculation strategies (Figures 3C, D).

The incremental improvement of T cell response mediated by the SSTI strategy was verified by an independent experiment using a DNA vaccine encoding a CD8⁺ T cell epitope derived from HIV (18). We used the DNA vaccine immunizing mice *via* two different inoculation strategies: SFI and SSTI (Supplementary Figure 4A). Our data showed that antigen-specific CD8⁺ T cell responses in SSTI group were significantly higher than that of the SFI group ($P=0.0484$) at 2 weeks post the 3rd immunization while not at 2 weeks post the 2nd vaccination (Supplementary Figure 4B).

SSTI Improved the Multifunctional T Cell Responses Induced by the SARS-CoV-2 S DNA Vaccine

To further investigate the influences of different inoculations on the cellular immune responses elicited by the S DNA vaccine, we euthanized the mice at 5 weeks post the 3rd immunization and measured the multifunctionality of S protein specific T cells in

spleens using flow cytometry. Compared with the SFI group, the average S protein specific T cell responses (IL-2, TNF- α and IFN- γ) of both the SSTI and the STI groups tended to be higher (Figures 5A, B), of which the frequencies of IFN- γ ⁺CD8⁺ T cells (Figure 5A) and IFN- γ ⁺CD4⁺ T cells (Figure 5B) were found to be significantly improved in the SSTI group. To evaluate the magnitudes of S protein specific T cells more accurately, we calculated the integrated median fluorescence intensity (iMFI) according to previous reports (18, 19). Our data showed that the iMFI of S protein specific IFN- γ ⁺CD8⁺ T cells were significantly improved only by the SSTI strategy (Figure 5C). While, the iMFI of TNF- α ⁺CD4⁺ cells and IFN- γ ⁺CD4⁺ T cells were significantly improved by both the SSTI and STI strategies (Figure 5D). Next, the functional features of S protein specific CD8⁺ and CD4⁺ T cells were further delineated *via* Boolean gating. Our data showed that the mean frequency of IFN- γ ⁺IL-2⁺TNF- α ⁺ CD8⁺ T cells in the SSTI group was significantly higher than those in the STI and the SFI groups (Figure 6A). The mean frequencies of IFN- γ ⁺IL-2⁺TNF- α ⁺CD8⁺ T cells in both the SSTI and the STI groups were significantly higher than that in the SFI group (Figure 6A). Similarly, the mean frequencies of IFN- γ ⁺IL-2⁺TNF- α ⁺ and IFN- γ ⁺IL-2⁺TNF- α ⁺CD4⁺ T cells were significantly higher in the SSTI and the STI groups than those in the SFI group (Figure 6B).

DISCUSSION

Compared with conventional vaccine formalities, such as whole inactivated virus vaccine and protein subunit vaccine, an

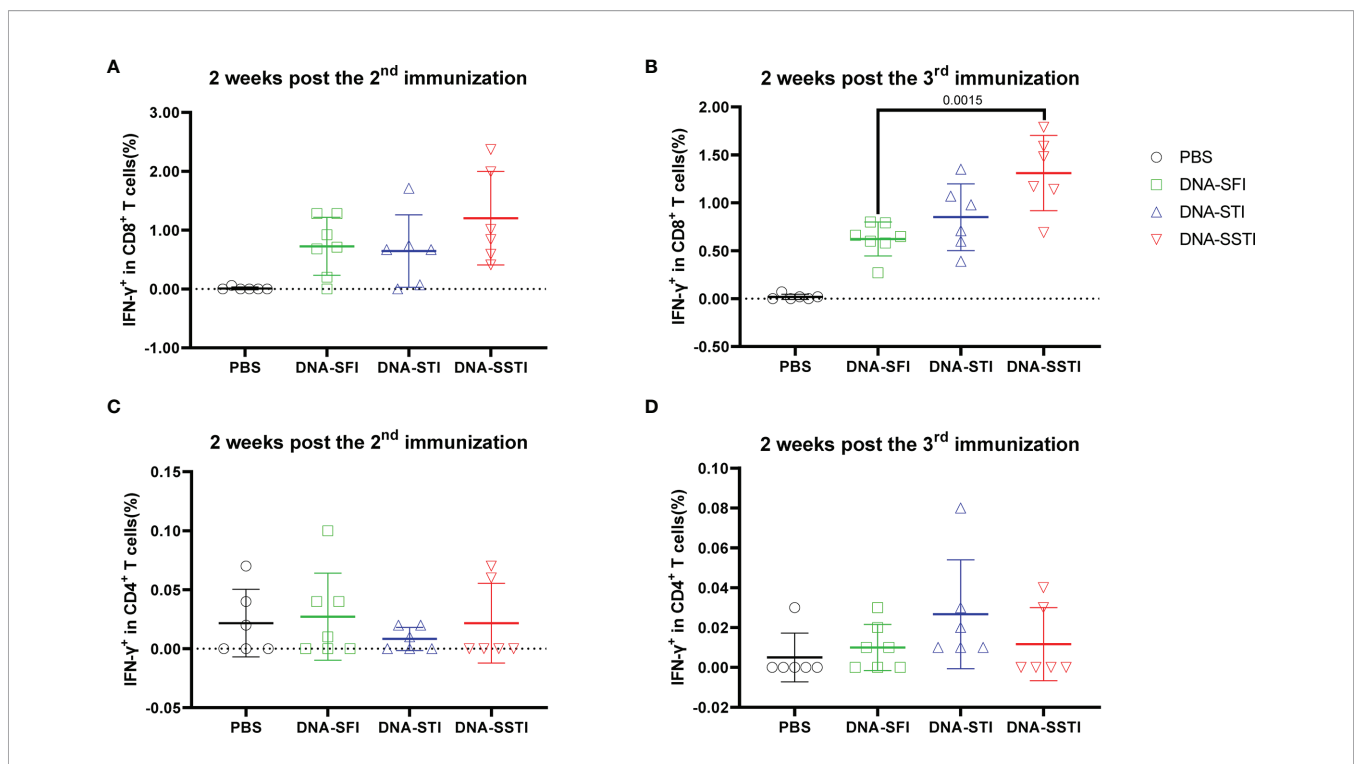


FIGURE 3 | Frequencies of circulating S protein specific IFN- γ secreting CD8⁺ T cells elicited by the DNA vaccine at 2 weeks post the 2nd and 3rd immunization. Peripheral blood were collected from mice immunized with the DNA vaccine at 2 weeks post the 2nd and 3rd immunization. S protein specific IFN- γ secreting CD8⁺ T cells (A, B) and CD4⁺ T cells (C, D) in peripheral blood were detected by intracellular cytokine staining (ICS). Data are shown as mean \pm SD. Statistical analyses were performed using the method of t-test.

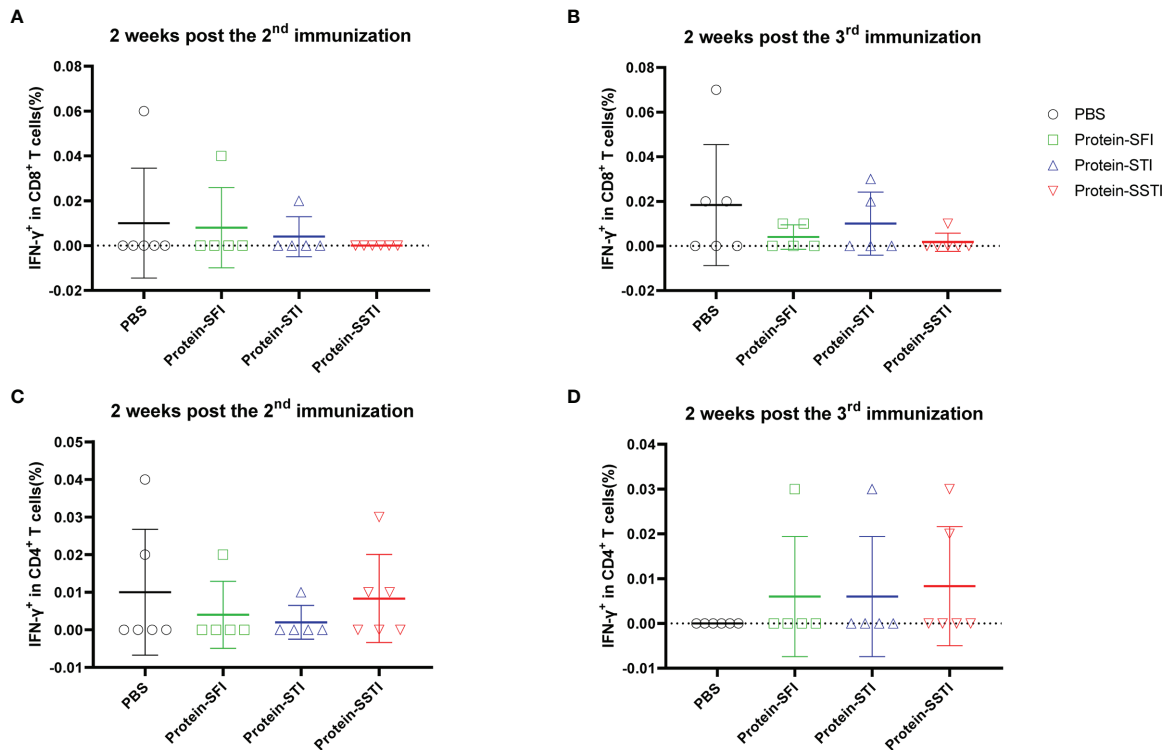


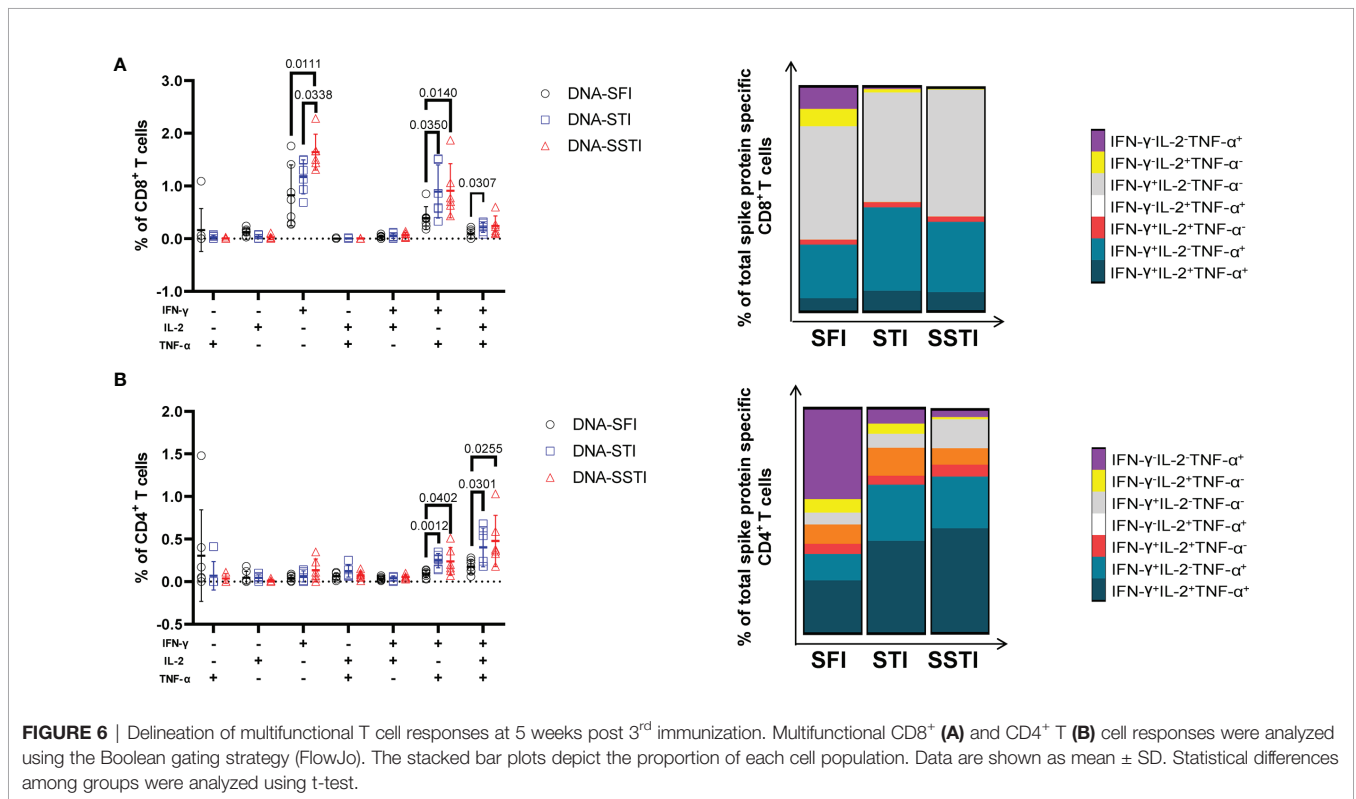
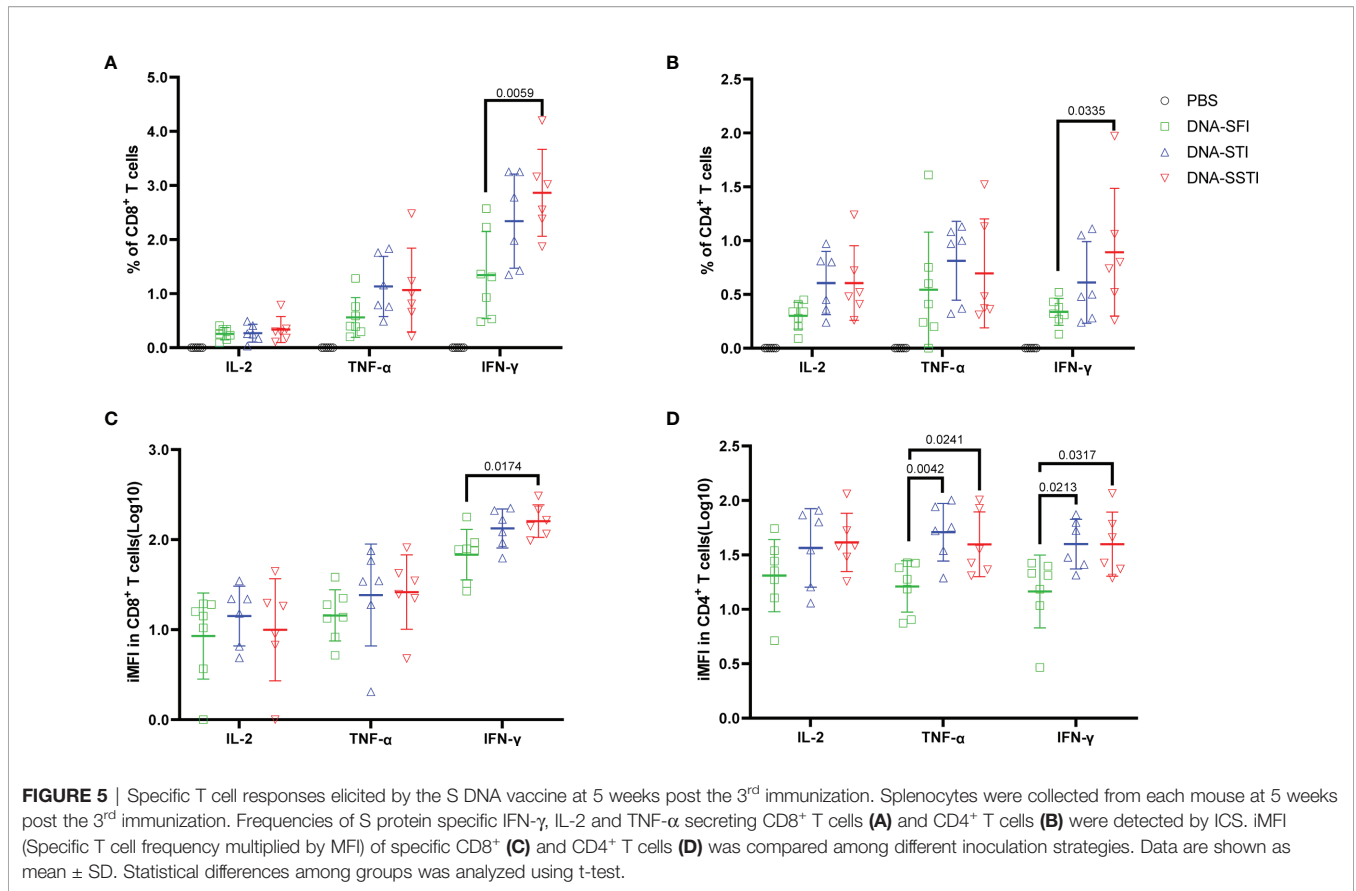
FIGURE 4 | Frequencies of circulating S protein specific IFN- γ secreting CD8⁺ T cells elicited by the protein subunit vaccine at 2 weeks post the 2nd and 3rd immunization. Peripheral blood were collected from mice immunized with the DNA vaccine at 2 weeks post the 2nd and 3rd immunization. S protein specific IFN- γ secreting CD8⁺ T cells (**A, B**) and CD4⁺ T cells (**C, D**) in peripheral blood were detected by intracellular cytokine staining (ICS). Data are shown as mean \pm SD. Statistical analyses were performed using the method of t-test.

advantage of nucleic acid vaccine is the capability of stimulating stronger antigen specific T cell response (20), which is thought to be important for vaccine mediated protection (21, 22). The contribution of cellular immune response to vaccine-induced protection against COVID-19 has been intensively discussed (23, 24). Its potential to confer cross-protection against different SARS-CoV-2 variants is demonstrated by a few most recent studies (25–27). As a promising nucleic vaccine platform, DNA vaccine has unique advantages such as the low cost and extraordinary stability (28). The major limitation of DNA vaccine is the relatively low immunogenicity compared with mRNA vaccine. Most previous studies seek to improve the immunogenicity of DNA vaccine through optimizing the delivery techniques and/or developing new adjuvants. In a previous study, we showed for the first time that changing the injection site for each shot could significantly improve specific T cell responses elicited by DNA vaccines (13). In this study, we tested whether the SSTI strategy could be leveraged to enhance the cellular immune responses induced by a candidate DNA vaccine encoding SARS-CoV-2 S protein. Meanwhile, we also tried to simplify the SSTI strategy by changing the injection site only for the third (last) shot, which was designated as STI (simplified translocating inoculation).

In consistent with our previous finding (13), we found that neither the SSTI nor the STI strategy significantly improved the

SARS-CoV-2 RBD binding antibody responses in mice immunized with 3 shots of a SARS-CoV-2 S DNA vaccine at an interval of 2 weeks. While, the S protein specific CD8⁺ and CD4⁺ T cell responses were significantly improved by the SSTI and the STI strategies at 5 weeks after DNA vaccination. A major difference between SSTI and STI is that STI did not significantly improve circulating CD8⁺ T cell responses at 2 weeks post the 3rd shot, suggesting that the change of inoculation site each time is critical in achieving optimal T cell responses. However, we found that SSTI of two doses didn't benefit the T cell responses compared with the SFI strategy, which might be because that the local antibody titers induced by the SFI strategy were not high enough to inhibit the expression of DNA vaccine. Indeed, in an on-going mechanism study, we observed that the numbers of residential antibody secreting cell in muscle tissues after 3 times of inoculation were much higher than in those being inoculated only once (Data not shown). Moreover, our data showed that the CD4⁺ and CD8⁺ T cell responses were not equally improved by the SSTI strategy, which might be due to the inequivalent capabilities of the DNA vaccine to induce CD4⁺ and CD8⁺ T cell responses (29). However, the exact mechanism is not clear, which requires further investigation.

In sharp contrast, neither the antibody responses nor the T cell responses elicited by protein vaccine were benefited from the SSTI strategy, which also consisted with our previous finding (13).



We have demonstrated in a previous work that ensuring the efficient *in vivo* antigen expression is a major mechanism underlying the SSTI strategy (13). While, the protein-based vaccine does not need to be expressed *in vivo*, therefore, the SSTI strategy doesn't benefit its immunogenicity. In addition, our data showed that the mucosal IgG tended to be higher in mice inoculated with the DNA vaccine. We speculate that this might be due to the relatively high binding antibody titers in the sera of mice inoculated with the DNA vaccine. It should be noted that the difference observed here may not reflect the real difference between DNA and protein-based vaccines, because the vaccine formulation and dosage were not comparable.

As being discussed elsewhere (30–32), the influence of inoculation route on vaccine immunogenicity has been intensively investigated. In addition to characterizing and comparing the immune responses elicited by different inoculation routes, previous studies also explored to improve topical immune responses *via* combination of different administrating routes (Prime and pull) (33–36). Our current study together with a previous work (13) further show that simply changing the inoculation site for the same route (intra muscular injection) can significantly improve the immunogenicity of DNA vaccines. Since the mechanism of SSTI strategy is different from other immunogenicity enhanced approaches, it is very likely that the strategy can be applied in combination with other techniques, such as gene gun (37) and electroporation (38), which is worth of further investigation. Moreover, as anatomical site-fixed inoculation of any types of vaccine may induce local antibody responses in the injection site, we speculate that the SSTI strategy may also help to improve the immunogenicity of a DNA vaccine even if it is administered as a booster dose.

Several limitations should be noted. First, as aforementioned, our previous study proved that SFI inhibited the *in vivo* antigen expression by local antibodies against the antigen encoded by the DNA vaccine. However, due to technical limitation, we were not able to reliably track and quantify the *in vivo* uptake of plasmid DNA which is a key factor that may impact the *in vivo* expression of DNA vaccine. Second, a challenging experiment with live virus can help to delineate how the enhanced T cell immunities may impact the pathogenicity of SARS-CoV-2. We plan to collaborate with other teams to do the experiment in an ABSL-3 lab in near future. Third, our results were generated using a mouse model, which might not completely mimic the characteristics of human immune responses. We are going to test this strategy through a phase I clinical trial designed to test a DNA based therapeutic TB vaccine in the near future.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Research Ethics Review Committee of the Shanghai Public Health Clinical Center Affiliated to Fudan University.

AUTHOR CONTRIBUTIONS

YW and WW designed the study and revised the manuscript. XT, YZ, SL and ZH conducted the experiments and analyzed the data. XT drafted the manuscript. DY and ZZ curated the data and edited the manuscript. All authors listed have made a direct contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.875236/full#supplementary-material>

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