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Unmethylated CpG motif-containing genomic DNA fragments of bacillus calmette-guerin improves immune response towards a DNA vaccine for COVID-19

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ABSTRACT

The development of an effective vaccine to control the global coronavirus disease-2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus- 2 (SARS-CoV-2) is of utmost importance. In this study, a synthetic DNA-based vaccine candidate, known as pSV10-SARS-CoV-2, expressing the SARS-CoV-2 spike protein was designed and tested in 39 BALB/c mice with BC01, an adjuvant derived from unmethylated CpG motif-containing DNA fragments from the Bacillus Calmette-Guerin genome. Mice vaccinated with pSV10-SARS-CoV-2 with BC01 produced early neutralizing antibodies and developed stronger humoral and cellular immune responses compared to mice that received the DNA vaccine only. Moreover, sera from mice vaccinated with pSV10-SARS-CoV-2 with BC01 can neutralize certain variants, including 614G, 614G + 472 V, 452R, 483A, 501Y.V2, and B.1.1.7. The results of this study demonstrate that the addition of BC01 to a DNA-vaccine for COVID-19 could elicit more effective neutralizing antibody titers for disease prevention.

1. Introduction

Coronavirus disease 2019 (COVID-19) has emerged as a global health crisis. As of November 17, 2020, >55 million people were infected with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which has caused over one million deaths [1]. SARS-CoV-2 is a single, positive-strand ribonucleic acid (RNA) virus with a spike protein that determines infectivity of the virus and transmissibility in the host [2]. The spike (S) protein of the virus is also quite unstable, with mutations potentially resulting in increased infectivity of the virus [3–7]. With no effective treatment currently available to the public, there is an urgent need for effective prevention methods, particularly a vaccine. Currently, 48 candidate vaccines are in clinical evaluation, and at least four of these are deoxyribonucleic acid (DNA)-based vaccines (https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines).

DNA vaccines have previously been successful in preventing various infectious diseases and are advantageous compared to conventional vaccines, as their design is straightforward and only requires one-step cloning into a plasmid vector [8]. Moreover, the expression of an antigen gene *in vivo* can maintain native protein structures, ensuring appropriate processing and immune presentation. Unfortunately, eliciting adequate immunogenicity is still the biggest challenge for practical DNA vaccine use. Many strategies have been applied in preclinical models to solve this problem, including formulation of DNA vaccines with molecular adjuvants [9]. Adjuvants are immunomodulators, which have been used in conjunction with vaccines to treat various clinical diseases for decades. The purpose of adding adjuvants to vaccines is to enhance, accelerate and prolong antigen-specific immune responses. For example, CpG-oligodeoxynucleotides (CpG-ODN) have been recognized as an immune adjuvant for various vaccines, because it can promote the activation of innate and adaptive immune responses

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in animals and humans [9]. CpG-ODN can also directly stimulate monocytes, macrophages, and dendritic cells to secrete various cytokines, such as TNF-α and GM-CSF, which in turn stimulate helper T cells to elicit immune responses [10,11]. BC01 was derived from the unmethylated CpG motif-containing DNA fragment from the BCG genome, as it displays strong adjuvant properties [11].

In the present study, we constructed a DNA vaccine expressing the SARS-Cov-2 S protein using BC01 as an adjuvant. The DNA vaccine with BC01 elicits effective neutralizing antibodies and cell-mediated responses to protect against SARS-CoV-2 virus infection.

2. Materials and methods

2.1. DNA vaccine production

DNA vaccines were designed based on the SARS-CoV-2 S protein sequence Wuhan-1 (GenBank: MN_908947). The full-length sequence of the S protein was synthesized. Synthetic genes were cloned into the mammalian expression plasmid pSV10, and the DNA vaccine sequence was confirmed using Sanger sequencing.

2.2. Western blotting

The 6-well plates seeded with 293T cells at 70% confluency were transfected with pSV10-SARS-CoV-2 plasmids (4 μg) using Lipofectamine 3000 (Invitrogen). Cell lysates were harvested 48 h post transfection, heated for five minutes at 95 °C, and run on a precast 10% SDS-PAGE gel (Bio-Rad). Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and membrane blocking was performed overnight at 4 °C in a phosphate-buffered saline T (PBST) containing 0.2% Tween 20 (Sigma) (V/V) and 5% (W/V) non-fat milk powder. Following overnight blocking, the PVDF membrane was incubated for 1 h in 5% milk PBST containing a 1:1000 dilution of monoclonal mouse anti-SARS-CoV-2 S1 antibody. Then, the PVDF membrane was washed five times with 5% milk PBST and subsequently incubated with 1:10,000 goat anti-mouse secondary antibodies in 5% milk PBST. Thereafter, the PVDF membrane was washed again five times with 5% milk PBST, and protein expression was detected using a Touch Imager XLI system (e-BLOT).

2.3. Animal specimens

Thirty-nine female mice, four to six weeks old, were classified into four groups: (1) pSV10-SARS-CoV-2 with BC01 (N = 10), (2) pSV10-SARS-CoV-2 only (N = 10), (3) BC01 only (N = 10), (4) PBS only (N = 9). Each mouse was intramuscularly injected with 50 μg DNA vaccine and/or 10 μg BC01, followed by CELLECTRA® *in vivo* electroporation (EP) at week 0, week 2, and week 4. The CELLECTRA® EP delivery consisted of two sets of pulses with 0.2 Amp constant current. Second pulse sets were delayed by 3 s. Within each set there were two 52 ms pulses with a 198 ms delay between the pulses. The serum was collected at four and six weeks after immunization.

2.4. Production and titration of pseudotyped viruses

The production of SARS-CoV-2 pseudovirus was performed as described previously [12]. Briefly, the plasmid expressing the SARS-CoV-2 S protein was inserted into 293T cells, and the cells were subsequently infected with G*ΔG-VSV (VSV G pseudotyped virus) at a concentration of 7.0×10^4 TCID 50/ml. G*ΔG-VSV packages expression cassettes for firefly luciferase instead of VSV-G in the VSV genome. Luciferin and luciferase, which are bioluminescent systems, can detect gene expression very sensitively and efficiently. Thereafter, the supernatant was discarded after cells were incubated for six to eight hours at 37 °C, and 15 mL fresh cell medium was added to the flask, followed by further culture for 24 h. Finally, culture supernatants containing SARS-CoV-2 pseudotyped viruses were harvested, filtered (0.45 mm pore size, Millipore, Cat#SLHP033RB), and stored at −80 °C. SARS-CoV-2 pseudotyped viruses were measured in titers and yielded more than ten times the relative luminescence units (RLU) compared to negative controls (cells only) after 24 h of infection.

2.5. Antigen binding enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed to detect sera antibody binding titers. ELISA plates were coated with 1 μg/ml SARS-CoV-2 S protein in 1 × DPBS overnight at 4 °C. Plates were washed thrice with wash buffer and then blocked with 3% BSA in DPBS with 0.05% Tween for two to three hours at room temperature. The blocking solution was discarded, plates were washed and incubated with serial dilutions of heat-inactivated mice serum for one hour at room temperature. Plates were washed and incubated with a 1:4000 dilution of anti-mouse IgG HRP-conjugated antibody in the dark for one hour at room temperature. Plates were then washed five times with wash buffer, and 100 μL TMB solution was added to each well. The reaction was halted by the addition of 100 μL TMB Stop solution per well. The absorbance was recorded at 450 nm and 630 nm.

2.6. Pseudovirus neutralization assay

In addition, the neutralization level of the vaccine against the currently prevalent pseudoviruses D614G, D614G + I472V, L452R, 501Y.V2, B.1.1.7, and V483A were evaluated. The sera of five vaccinated mice from each group at week 6 were used for the neutralization test. Mouse sera from different vaccinated groups were heat inactivated for 30 min at 56 °C and serially diluted threefold, starting at a 1:30 dilution for each assay. Sera were mixed with 50 μL of pseudovirus for 60 min. Huh-7 cells stably expressing angiotensin-converting enzyme 2 (ACE2) were added after 60 min, followed by incubation at 37 °C for 24 h. Next, cells were lysed using Britelite plus luminescence reporter gene assay system (Perkin Elmer Catalog no. 6066769) and RLU values were measured. The Reed-Muench method was used to calculate the virus neutralization titer (ID_{50}).

2.7. Enzyme-linked immune absorbent spot (ELISPOT) assay

Mice spleens were collected and ground into single cell suspensions in RPMI1640 media supplemented with one percent penicillin/streptomycin (R0). Cell pellets were resuspended in 5 mL ACK lysis buffer for 5 min, thereafter 8 mL PBS was added to stop the reaction. The samples were centrifuged at 1500*g* for 5 min, and then cells were resuspended in RPMI1640 media containing 10% FBS (R10). Mouse IFN-γ ELISpotPLUS plates and Mouse IL-2 ELISpotPLUS plates (MABTECH) were activated using 200 μL R10/well for 30 min. The 5×10^5 mouse splenocytes were seeded into each well and stimulated with pools of 18-mer peptides overlapping by nine amino acids from the SARS-CoV-2 Spike proteins. Additionally, matrix mapping was performed using peptide pools in a matrix designed to identify immunodominant responses. Mouse splenocytes were stimulated with a final concentration of 5 μg/ml of each peptide per well in R10. R10 and cell stimulation PAM + ION (Invitrogen) were used for negative and positive controls, respectively. Spots were counted using the ImmunoSpot CTL reader.

2.8. Cellular immune response

We used IFN-γ ELISPOT to detect the cellular immune response. We performed epitope mapping on splenocytes of BALB/c mice receiving 50 μg of the pSV-10-SARS-CoV-2 vaccine. The S protein was covered with 20 peptide libraries. Each peptide library contained seven peptides: each peptide had 18 amino acids, and the adjacent peptide overlapped nine amino acids. Cellular immune response was detected in multiple peptide pools, but the strongest response was in peptide pool 5, located at 234–297 regions. Peptide 5 was used as the peptide stimulant in subsequent cellular immune experiments. BALB/c mice were sacrificed at week 4 and week 6, and spleen cells were harvested. The single cell suspension was stimulated by peptide 5 for 20 h. The IFN-γ ELISPOT kit was used for detection.

2.9. Statistical analyses

Analysis was performed using GraphPad Prism 8.4.2 (GraphPad Software). Comparison of data between groups was performed using One-way ANOVA and Holm-Sidak's multiple comparisons test. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Construction of the DNA vaccine

We produced a prototype DNA vaccine expressing the SARS-CoV-2 S protein (Fig. 1A). pSV10-SARS-CoV-2 recombinant plasmid constructed by inserted the SARS-CoV-2 Spike protein (GenBank: MN_908947) into pSV10. The molecular weight of the S protein was 140–142 kDa, and its slight shift was due to 22 potential N-linked glycans in the S protein (Fig. 1B). The spike protein could be expressed after transfection of the vaccine vector.

3.2. Humoral immune responses in mice

The 39 mice (aged 4–6 weeks) were immunized with the DNA vaccine: pSV-10-SARS-CoV-2 vaccine (n = 10), pSV-10-SARS-CoV-2 with BC01 (n = 10), BC01 (n = 10), and PBS (n = 9). A neutralization ID_{50} average titer of 87 was observed in pSV-10-SARS-CoV-2 vaccinated mice, an ID_{50} average titer of 141 was detected in pSV-10-SARS-CoV-2 with BC01 vaccinated mice. No neutralizing antibodies were detected in the BC01 only and PBS only groups. At week six, the average titer of neutralizing ID_{50} was 262 in the pSV-10-SARS-CoV-2 group, 309 in the pSV-10-SARS-CoV-2 with BC01 group, and no neutralizing antibodies were detected in either the BC01 or PBS control groups (Fig. 2A). The pSV-10-SARS-CoV-2 with BC01 vaccine induced the development of protective neutralizing antibodies in 80% of mice, which was significantly higher than that of the pSV-10-SARS-CoV-2 group (50%). These results indicate that BC01 promotes the early production of neutralizing antibodies in mice (Table 1).

At week 4, the binding antibody titer of the pSV-10-SARS-CoV-2 group was not significantly different from that of pSV-10-SARS-CoV-2 with BC01 group ($p > 0.05$). No binding antibody was detected in the BC01 and PBS only groups. At week 6, the binding antibody titer of the DNA vaccine group was not significantly different from that of pSV-10-SARS-CoV-2 with BC01 group ($p > 0.05$). No binding antibody was detected in the BC01 and PBS only groups (Fig. 2B).

At week 6, most animals vaccinated with pSV-10-SARS-CoV-2 with BC01 exhibited neutralization of D614G, D614G + I472V, L452R, V483A, 501Y.V2, and B.1.1.7 variants with average ID_{50} titers of 74, 123, 96, 110, 125, and 203, respectively. The pSV-10-SARS-CoV-2 group exhibited neutralization of D614G, D614G + I472V, V483A, and B.1.1.7 variants, but not of L452R and 501Y.V2 variants. Those variant pseudoviruses neutralizing activity with average ID_{50} titers of 112, 71, 86, 0, 0, and 68 were detected in pSV-10-SARS-CoV-2 immunized mice, respectively. The BC01 and PBS only groups could not neutralize these variants (Fig. 3A–F).

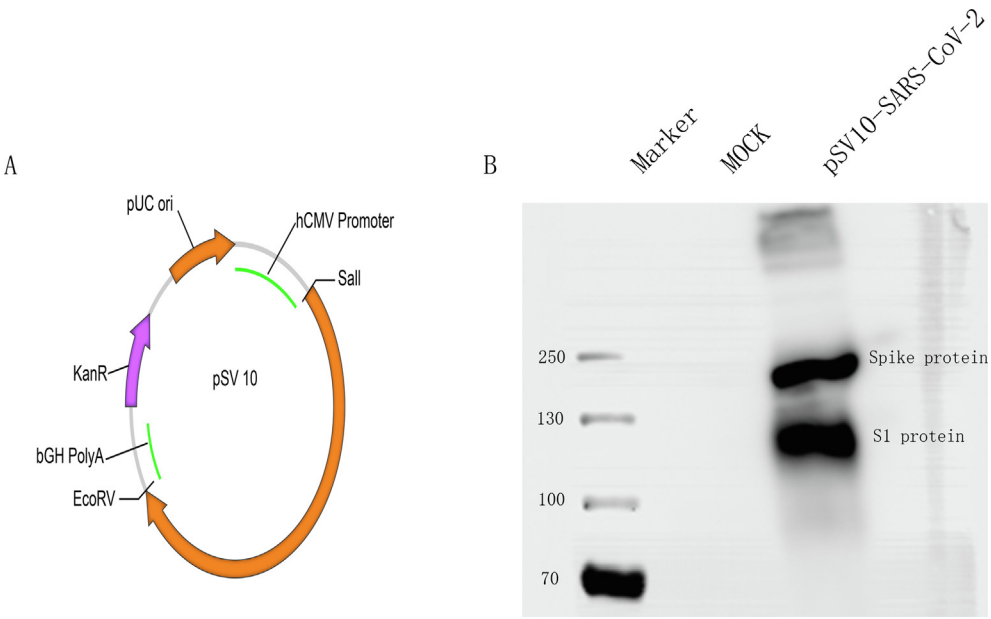

Fig. 1. Design and expression of the synthetic SARS-Cov-2 DNA vaccine constructs. (A) Schematic diagram of the synthetic DNA vaccine, pSV10-SARS-CoV-2 containing SARS-CoV-2 Spike protein insert. (B) Western blot analyses indicating S protein expression *in vitro* after transfection of 293T cells with pSV10-SARS-CoV-2 or MOCK plasmid. The 293T cell lysates were collected and analyzed using an anti-SARS-CoV-2 Spike protein polyclonal antibody.

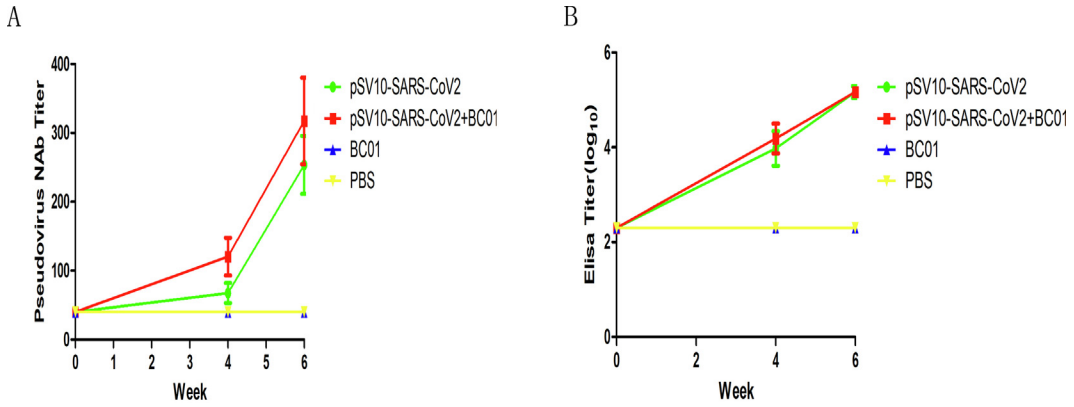

Fig. 2. Humoral immune responses in vaccinated mice receiving different types of vaccines. (A) Neutralization 50 in different groups. (B) SARS-CoV-2 S1 + 2 protein antigen binding of IgG in serial serum dilutions from mice at week 4 and week 6.

Table 1
Neutralizing activity of sera after pSV10-SARS-CoV-2 with BC01 administration to mice.

Model	Vaccine	N	Sample timepoint	Serum ID50 (reciprocal dilution)	Positive rate (ID50 > 40)
BALB/c	pSV10-SARS-CoV-2	10	week 4	183,104,78,57,54,<40,<40,<40,<40,<40	50%
	pSV10-SARS-CoV-2 + BC01	10		302,66,81,147,215,65,165,84,<40,<40	80%
	BC01	10		<40,<40,<40,<40,<40,<40,<40,<40,<40,<40	0
	NC	9		<40,<40,<40,<40,<40,<40,<40,<40,<40	0
	pSV10-SARS-CoV-2	10	week 6	173,364,251,421,153,130,197,516,151,180	100%
	pSV10-SARS-CoV-2 + BC01	10		424,332,150,728,161,140,212,431,114,488	100%
	BC01	10		<40,<40,<40,<40,<40,<40,<40,<40,<40,<40	0
	NC	9		<40,<40,<40,<40,<40,<40,<40,<40,<40	0

3.3. Cellular immune response induced by vaccine

We used IFN-γ ELISPOT to detect the cellular immune response. We performed epitope mapping on spleen cells of BALB/c mice receiving 50 μg pSV-10-SARS-CoV-2 dose. The S protein was covered with 20 peptide libraries. Each peptide library contained 7 peptides, each peptide had 18 amino acids, and the adjacent peptide overlapped 9 amino acids. Cellular immune response was detected in multiple peptide pools, but the strongest response was in peptide pool 5, located at the 234–297 region (Fig. 4).

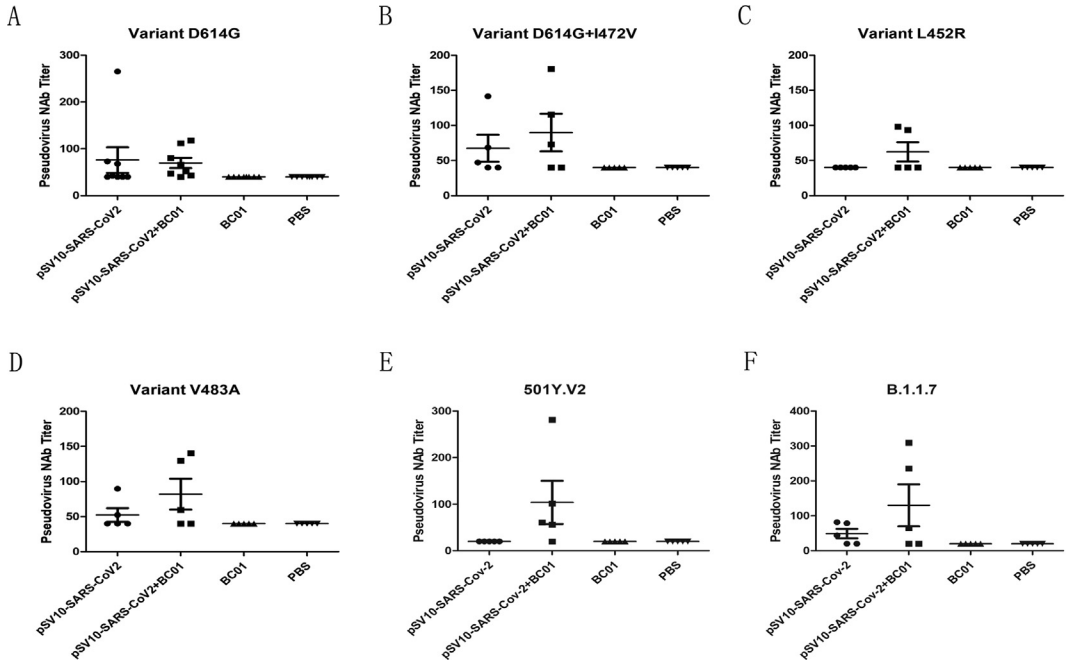

Fig. 3. Sera from mice vaccinated with pSV10-SARS-CoV-2 and BC01 can neutralize variants. (A) Neutralization of D614G variant, (B) Neutralization of D614G + I472V variant, (C) Neutralization of L452R variant, (D) Neutralization of V483A variant, (E) Neutralization of 501Y.V2, and (F) Neutralization of B.1.1.7.

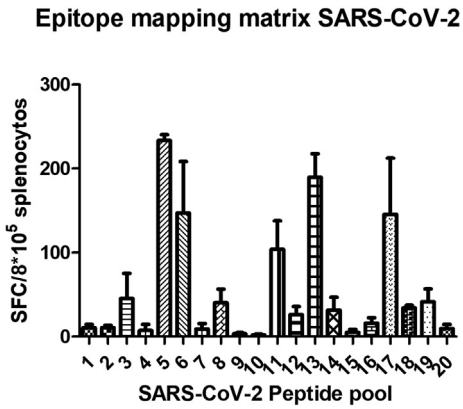

Fig. 4. T cell epitope mapping after administering the pSV10-SARS-CoV-2 vaccine to BALB/c mice.

Cellular immune response results at week 4 showed that IFN -γ levels per 5 × 10^5 splenocytes in the pSV-10-SARS-CoV-2 group averaged 154 SFU, while the pSV-10-SARS-CoV-2 with BC01 group averaged 179.1 SFU. At week 6, IFN -γ levels in the pSV-10-SARS-CoV-2 group averaged 253 SFU per 5 × 10^5 splenocytes, and 389.9 SFU per 5 × 10^5 splenocytes in the pSV-10-SARS-CoV-2 with BC01 group (Fig. 5A). The pSV-10-SARS-CoV-2 with BC01 enhanced the cellular immune response and increased the number of IFN-γ spots.

The cellular immune response was also detected using an IL-2 ELISPOT. The average IL-2 spots were 29.3 SFU per 5 × 10^5 splenocytes in the pSV-10-SARS-CoV-2 group and 37.7 in the pSV-10-SARS-CoV-2 with BC01 group. At week 6, the average IL-2 spots were 39.4 SFU per 5 × 10^5 splenocytes in the pSV-10-SARS-CoV-2 group and 60.4 SFU in the pSV-10-SARS-CoV-2 with BC01 group (Fig. 5B). There was no statistical difference in the number of IL-2 secreting cells between groups ($p > 0.05$) (Fig. 5C, D).

4. Discussion

COVID-19 has become a global pandemic with high morbidity and mortality. At present, many vaccines are undergoing phase III clinical trials, including four DNA vaccines. Additionally, studies have shown that DNA vaccines for COVID-19 can produce antigen-specific T cell responses and neutralizing antibodies [13,14]. Thus, we synthesized a DNA vaccine based on the SARS-CoV-2 S protein with the addition of BC01 to induce a protective immune response. The results of this study show that the DNA vaccine with BC01 elicits a stronger specific T cell response and a higher titer of neutralizing antibodies than a vaccine without a BC01 adjuvant. Moreover, the vaccine with BC01 can neutralize several popular SARS-CoV-2 mutant strains.

In this study, the DNA vaccine with BC01 increased the number of neutralizing antibodies earlier compared to the DNA vaccine without adjuvant. Unmethylated CpG-ODN induces an innate immune response by triggering cells expressing Toll-like receptor 9, which is characterized by the production of Th1 and pro-inflammatory cytokines [15]. In mice, TLR-9 is expressed in multiple cells of the myeloid lineage, including monocytes, macrophages, and dendritic cells [16–18]. B cells and plasmacytoid dendritic cells are the main cell types that express TLR-9 in humans [19–22]. Therefore, the BC01 binds to these immune cells

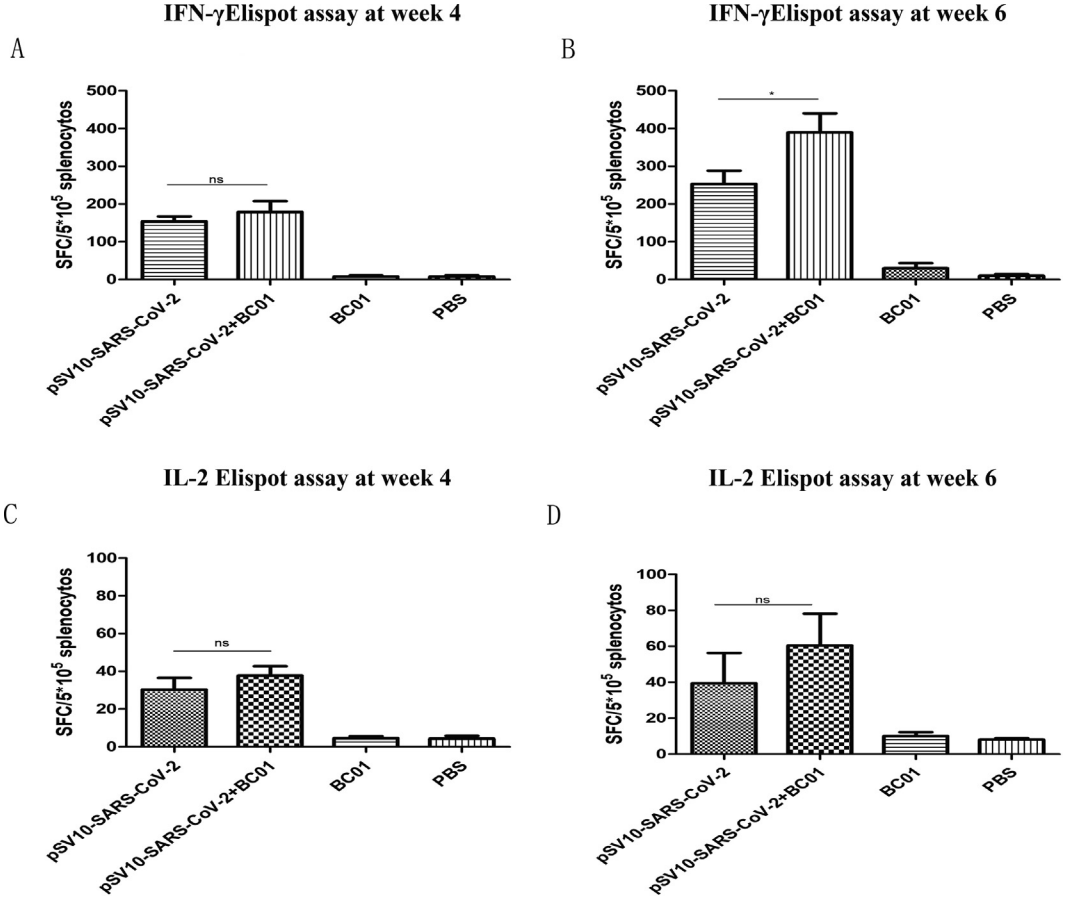

Fig. 5. Induction of T cell responses in mice after receiving the pSV10-SARS-CoV-2 with BC01 vaccine. (A) IFN-γ ELISPOT assay at week 4, (B) IFN-γ ELISPOT assay at week 6, (C) IL-2 ELISPOT assay at week 4, and (D) IL-2 ELISPOT assay at week 6.

expressing TLR-9 and triggers a protective immune response, enhancing the specific cellular and humoral immunity caused by the DNA vaccine. The BC01 used in this study also enhanced the neutralizing antibody titer but did not increase the binding antibody. Therefore, we hypothesized that the neutralizing antibodies and binding antibodies are evaluation indicators for the functional activity and quality of vaccine-induced humoral immune responses. Neutralizing antibodies can directly neutralize the virus, causing the virus to lose its infectious activity, and then be phagocytosed and cleared by macrophages. After the binding antibody binds to the virus, it mediates the virus to macrophages, which in turn kills the virus, and may also cause immune damage. Neutralizing antibody and binding antibody are induced by different antigens. The results of this study revealed that the BC01 adjuvant promoted the increase of neutralizing antibody titer, but did not increase the binding antibody titer. The reason towards the expressed neutralizing antibody is higher than the immune response towards the induced binding antibody, and the adjuvant effect of BC01 is mainly reflected in the improvement of the neutralizing antibody titer. At the same time, it may also be the immune stimulating effect of BC01 itself, such as promoting the release of antigen-specific Th1 cytokines (IFN-γ, IL-2), and biasing the adaptive immune response to promote the production of neutralizing antibodies. Moreover, it has been reported that neutralizing antibodies account for <1% of total antibodies, so an increase in neutralizing antibodies may have no effect on the number of total antibodies [23,24]. Otherwise, it may be the combination of the two or other unknown reasons, but the current *in vivo* test results exhibit that BC01 promotes the vaccine to produce a protective immune response. Several studies suggest that the affinity of antibody to antigen determines the protective effect of antibody, and neutralization reaction is related to affinity. In this study, BC01 can improve protective immune responses induced by DNA vaccine, so further investigations on whether BC01 play a role in vaccines that cause higher affinity antibodies are imperative.

In order to evaluate the vaccine in this study, only detecting the binding antibody may not accurately reflect the immunogenicity induced by the vaccine. The pseudovirus-based neutralization antibody assay used in this study has been validated by previous studies [25–28], which correlates well with the live virus-based neutralization assay, and can be used for neutralization antibody evaluation.

SARS-CoV-2 is a single-stranded positive RNA virus, which has a higher mutation rate than DNA viruses [29–31]. At present, studies have found many mutations in the S protein gene of SARS-CoV-2, of which D614G is the most important mutation along with combined mutations, such as D614G + I472V [32–34]. Other studies have found that certain mutation sites can reduce the infectivity of the virus, such as L452R and V483A, but these sites can tolerate some neutralizing antibodies [34]. Furthermore, the B.1.1.7 variant includes many genetic changes, and has been increasing rapidly in the United Kingdom. This variant may be increasing human ACE2 binding due to an N501Y mutation [35]. The emergence of unique South African lineages (501Y.V2) also include multiple non-synonymous S protein mutations that may have functional significance, such as K417N, E484K, and N501Y in the S-receptor-binding domain [36]. The pSV10-SARS-CoV2 vaccine used in this study was found to neutralize some mutations, but not the L452R and 501Y.V2 variants. However, the pSV10-SARS-CoV2 with BC01 vaccine can neutralize these mutant strains, as it induces higher neutralizing antibody titers. Thus, DNA vaccines with BC01 can provide a more effective neutralizing activity to defend against SARS-CoV-2 infection. However, further research is warranted to determine whether the DNA vaccine can neutralize all mutant strains.

5. Conclusions

A DNA pSV10-SARS-CoV2 vaccine with the addition of a BC01 adjuvant improves efficacy and increases the cellular and humoral immune response in mice. Further research is needed to determine how this adjuvant may impact human trials and if it can be incorporated into ongoing vaccine trials for the prevention of COVID −19.

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 material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2021.08.103.

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