ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
In press.

Humoral Immunogenicity of Homologous and Heterologous Prime–boost Regimens Using DNA, Inactivated, and Recombinant Protein SARS-CoV-2 Vaccines in Mice

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Received: 28 April 2026; Received in revised form: 21 July 2026; Accepted: 26 August 2026

ABSTRACT

Vaccination remains a key strategy for controlling SARS-CoV-2 transmission, especially in the post-pandemic era, this study evaluated the humoral immune responses elicited by homologous and heterologous prime–boost vaccination strategies using established DNA, inactivated virus, and recombinant protein vaccine formulations in a BALB/c mouse model. The focus was on specific IgG and neutralizing antibodies as complementary indicators of vaccine-induced humoral immunity.

BALB/c mice were immunized using homologous (DNA/DNA, Inactivated/Inactivated, Protein/Protein) or heterologous (DNA-Inactivated, DNA-Protein) prime-boost regimens. Antibody titers were measured via ELISA using purified SARS-CoV-2, and neutralization capacity was assessed using the virus neutralization test (VNT). Antibody persistence was evaluated 120 days after the initial immunization, followed by viral exposure to assess recall antibody responses.

Both the inactivated virus vaccine and the four-dose DNA vaccination regimen induced high levels of SARS-CoV-2-specific antibodies, with significantly higher titers than those observed in the control and recombinant protein groups. However, the recombinant protein group exhibited the highest levels of neutralizing antibodies in the VNT assay. Antibody responses remained detectable through day 120 in all vaccinated groups. Following viral exposure, animals receiving inactivated virus-containing regimens exhibited enhanced recall antibody responses, suggesting the capacity for effective secondary humoral responses.

These findings indicate that homologous and heterologous prime–boost vaccination regimens generate distinct humoral immune response profiles. The observed differences should be interpreted within the context of the established vaccine formulations and immunization regimens evaluated in this study and may contribute to the optimization of future vaccination strategies.

Keywords: Antibody persistence; DNA vaccine; Humoral; Inactivated vaccine; Neutralizing antibodies; Prime-boost; Recombinant vaccine; SARS-CoV-2

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INTRODUCTION

The global outbreak of COVID-19, caused by the novel coronavirus SARS-CoV-2, has profoundly impacted health, social systems, and economies worldwide.[1] Although the initial pandemic phase has subsided, SARS-CoV-2 continues to circulate in communities worldwide, causing recurrent outbreaks and posing a persistent public health threat.[2] This has shifted vaccine development priorities from emergency-use prevention to long-term protection, necessitating platforms capable of inducing durable and effective immunity.[3]

Among the structural proteins, the spike (S) glycoprotein is the primary target of neutralizing antibodies and is essential for viral attachment to host cells via the angiotensin-converting enzyme 2 (ACE2) receptor.[4] Consequently, the S protein is the cornerstone of most vaccine platforms and serological assays.[5]

The induction of neutralizing antibodies against the S protein remains the key correlate of protection, [6] and their magnitude, quality, and persistence directly influence vaccine efficacy and long-term immunity. Moreover, humoral immunity is especially important in populations where cellular immune responses may be compromised, such as the older adults or immunosuppressed individuals.[7] In people infected with the virus, the production of antibodies, especially neutralizing antibodies, plays a key role in generating an immune response and preventing reinfection.[8] These antibodies are usually produced a few weeks after the initial infection, and their levels change over time. Conducting a study comparing the immunogenicity and efficacy of different vaccine platforms against SARS-CoV-2 is essential due to the pivotal role of antibodies in vaccine-induced protection.[9]

Different vaccine platforms have been employed to present viral antigens and stimulate protective immunity. DNA vaccines offer several advantages including thermal stability, rapid design, and the ability to elicit both B cell and T cell responses.[10] Inactivated whole-virus vaccines, while traditional, present multiple structural and non-structural viral antigens to the immune system. This broader exposure may confer cross-protection against viral variants but may also dilute the immune focus on critical neutralizing epitopes, such as the receptor-binding domain (RBD) of the S protein.[11] Recombinant subunit vaccines, particularly those based on the S protein, focus immune responses with reduced risk of off-target effects, although they often require adjuvants to boost immunogenicity.[12]

An emerging strategy in vaccinology is the heterologous prime-boost approach, which combines different vaccine platforms in sequential administration to enhance both the breadth and the durability of the immune response.[13] However, the optimal combinations and schedules remain an area of active investigation.[14]

Despite the successful deployment of multiple vaccine types, parallel comparisons of their ability to induce neutralizing antibodies and maintain antibody levels over time, especially in experimental animal models, are still limited. Most comparative studies have focused on acute immune responses without long-term follow-up or have failed to include heterologous regimens.[15]

The present study was therefore designed to evaluate humoral immune responses elicited by homologous and heterologous prime–boost vaccination regimens using established DNA, recombinant protein, and inactivated SARS-CoV-2 vaccine formulations in a BALB/c mouse model. The primary objective was to evaluate homologous and heterologous prime–boost vaccination strategies using established vaccine formulations rather than to establish the intrinsic superiority of individual vaccine platforms. Accordingly, comparisons among the vaccination regimens were interpreted within the context of the experimental conditions employed.

MATERIALS AND METHODS

Virus Characterization

The virus used for inactivation and virus neutralization test (VNT) was isolated from a confirmed COVID-19 patient and validated by specific real-time PCR and electron microscopy. The virus was propagated in Vero E6 cells, and its titer was determined using the Reed-Muench method. Inactivation was confirmed by three blinded passages without cytopathic effect (CPE).

Vaccines Preparation

Inactivated virus was formulated with aluminum hydroxide[16] and the recombinant spike protein was prepared and formulated in Freund adjuvant as described elsewhere.[17] The recombinant protein vaccine was based on the SARS-CoV-2 spike ectodomain (S1+S2) expressed in CHO-K1 cells and purified by affinity

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chromatography and gel filtration. The inactivated vaccine was generated from SARS-CoV-2 isolate, propagated in Vero E6 cells, inactivated with 37% formaldehyde and formulated with aluminum hydroxide, followed by ultrafiltration and sterile filtration. The plasmid used as a DNA vaccine is a commercial plasmid, pCMV32019nCoVSpike (S1+S2)-long from Sino Biological, which contains an optimized spike sequence. pCMV3 expresses the SARS-CoV-2 spike genes under a strong CMV promoter to enhance transcription. To confirm the spike sequence, the gene encoding the spike protein was verified using specific primers. For expression confirmation, the plasmid was transfected into HEK293T cells. Samples were collected from the transfected wells at specific intervals (12, 24, 48, and 72 hours) and subjected to Western blot analysis using an anti-spike primary antibody.

Animals and Immunization Program

Female BALB/c mice (6–8 weeks old) were purchased from Baqiyatallah University of Medical Sciences, Iran and maintained under standard conditions. All animal protocols were approved by the Ethicc Committee of Tarbiat Modares University. The plasmid was prepared at a concentration of 80 µg in 100 µl of PBS. For the injection of recombinant protein and inactivated virus, 10 µg and 3 µg of the formulated solution were injected into the back of the neck, respectively (n=6/group). Dosage levels and booster-dose intervals for each vaccine were selected based on commonly used ranges in murine preclinical studies as reported in previous publications.[16-18] According to Table 1, the vaccination schedule was implemented as follows: DNA vaccines were administered in three doses (on days 1, 14, and 28), while the killed virus and recombinant protein were given in two doses (on days 1 and 14). Additionally, prime-boost groups were established to enhance immunogenicity, receiving three doses (DNA vaccines on days 1 and 14, and either the killed virus or recombinant protein on day 28). In a separate group, four doses of the DNA vaccine were administered (on days 1, 14, 21, and 28). Four DNA doses were used to assess whether an additional booster could enhance antibody persistence and neutralization compared with three-dose schedules. For each vaccine platform, the optimal dose, injection protocol, and delivery route reported in previous studies were used, to allow a controlled comparison between platforms. Blood samples were collected on days 42, 120, and 127 for immunological analysis. Day 42 was selected to represent the post-immunization response after completion of the vaccination schedules, day 120 was used to assess antibody persistence at a late time point, and day 127 was used to evaluate recall responses following viral exposure.

ELISA

To evaluate SARS-CoV-2–specific IgG antibodies, an indirect ELISA was performed using whole inactivated SARS-CoV-2 virus as the coating antigen. As described by El-Duah et al (2019),[19] the purified virus was coated onto the wells of a 96-well high-binding ELISA plate at a concentration of 1 µg/mL in carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were then washed with PBS-T (phosphate-buffered saline containing 0.05% Tween-20) and blocked with 5% skim milk in PBS-T for 1 h at 37°C to prevent nonspecific binding. Mouse sera were serially diluted starting from 1:100 in blocking buffer and added to the wells, followed by incubation for 1–2 h at room temperature. After washing, HRP-conjugated goat anti-mouse was added and incubated for 1 h at 37°C. Subsequently, TMB substrate (3,3′,5,5′-Tetramethylbenzidine) was added to the wells, the reaction was stopped with 2N H_2SO_4, and absorbance was measured at 450 nm using a microplate reader. Negative mouse sera were included as controls, typically yielding absorbance values in the range of 0.1–0.2.

Virus Neutralization Test (VNT)

Neutralizing antibodies were evaluated using a conventional live-virus virus neutralization test (VNT), a well-established method for assessing the functional neutralizing activity of serum antibodies against SARS-CoV-2. Serum samples were heat-inactivated prior to testing and serial 2-fold dilutions were prepared. Equal volumes of each serum dilution were mixed with a standardized inoculum containing 200 $TCID_{50}$ (Tissue Culture Infectious Dose 50%) of live SARS-CoV-2 and incubated to allow antibody–virus interaction. The mixtures were subsequently transferred onto confluent Vero cell monolayers and incubated for 72 h under standard cell culture conditions.

Virus-only and cell-only controls were included in each assay to verify viral infectivity and cell viability, respectively. Neutralizing antibody titers were defined as the reciprocal of the highest serum dilution that

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prevented virus-induced CPE in 50% of the replicate wells, according to the conventional endpoint neutralization method. CPE was evaluated by microscopic examination based on the presence or absence of the characteristic morphological changes observed in infected Vero E6 cells.

Each assay was performed in three independent experiments under identical experimental conditions to ensure reproducibility.

Evaluation of Antibody Stability After 4 Months

To investigate the stability of antibodies, on day 120 after the first dose, blood samples were collected and all mice were exposed to live virus (10^6 TCID50) as a recall antigen, and one week later, on day 127, blood samples were taken again to assessthe antibody titers.

Statistics

Statistical analysis was performed using SPSS software (version 24) and GraphPad Prism (version 8). Data distribution was assessed prior to statistical testing. For comparisons involving multiple groups, data were initially analyzed using the Kruskal–Wallis test, followed by pairwise Mann–Whitney U tests with Bonferroni correction to account for multiple comparisons. Results are presented as mean±SD. Each experiment was performed in triplicate. A p value<0.05 was considered statistically significant.

Table 1. Grouping of mice, schedule and dose of vaccines

Group	Group description	Immunization regimen
Group 1	Control group (PBS)	injection of 100 µl of PBS serum on days 1, 14 and 28
Group 2	DNA vaccine group (S)	injection of three doses of DNA spike vaccine (80 µg/100 µl) on days 1, 14 and 28
Group 3	Recombinant vaccine group (Pro)	injection of two doses of recombinant vaccine (10 µg) on days 1 and 28.
Group 4	DNA vaccine/recombinant vaccine group (S/Pro)	injection of two doses of DNA spike vaccine (80 µg/100 µl) on days 1 and 14 and a single dose of recombinant vaccine (10 µg) on day 28.
Group 5	Inactivated virus group (Inact)	Two doses of inactivated virus (3 µg) on days 1 and 28.
Group 6	DNA/inactivated virus group (S/Inact)	Two doses of spike DNA vaccine (80 µg/100 µl) on days 1 and 14 and a single dose of inactivated virus (3 µg) on day 28.
Group 7	4-dose DNA vaccine group (S4)	Four doses of spike DNA vaccine (80 µg/100 µl) on days 1, 14, 21, and 28.

RESULTS

SARS-CoV-2-specific Antibody Responses (ELISA)

Following immunization, SARS-CoV-2-specific antibody levels were assessed using ELISA. The groups receiving four doses of DNA vaccine (S4) and inactived virus demonstrated the highest antibody titers among all groups, with a statistically significant increase compared to both the 3-dose DNA (S) group and the control (PBS) group (p<0.01). Notably, the S4 group also outperformed all heterologous prime-boost groups. Among the prime-boost strategies, the DNA/Inactivated (S/Inact) group showed moderately lower IgG levels, whereas the DNA/Protein (S/Pro) group elicited a relatively higher IgG response. The recombinant protein-only (Pro) group showed modest IgG levels, significantly lower than those of the S4 group (Figure 1).

Neutralizing Antibody Responses (VNT)

To evaluate the neutralizing ability of antibodies produced against the spike protein of the SARS-CoV-2 virus, a Virus Neutralizing Test was employed. The Pro group achieved the highest neutralizing titers at day 42, significantly greater than those of the S and PBS groups (p<0.001). Among the heterologous groups, the S/Pro group generated partial neutralization but did not match the Pro group in potency. The S4, Inact, S/Pro, and S/Inact groups showed comparable neutralizing activity (Figure 2).

Antibody Stability Over Time

To assess antibody persistence at a late time point, mice were bled on days 42 (post-immunization), 120 (4 months), and 127 (one week after exposure to live-virus as a booster antigen). Longitudinal tracking revealed robust IgG persistence in the Inact and S/Inact groups up

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to day 127. In all groups, no significant decline in IgG titers was observed over time up to day 120, suggesting persistence of the humoral response up to day 120. In contrast, the Pro and S/Pro groups showed slight declines in antibody levels by day 127. The Inact and S/Inact groups showed the most notable increase in IgG titers on day 127, suggestive of an effective recall response (Figure 3).

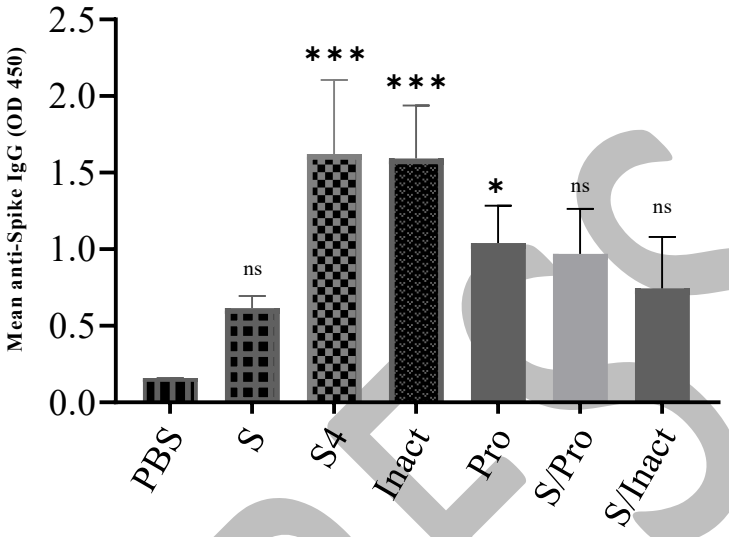

Figure 1. Spike-specific IgG titers (Day 42). Mean anti-Spike IgG antibody titers (OD450) in serum samples collected on day 42 post-primary immunization. Error bars represent SEM. Data were analyzed using the Kruskal–Wallis test followed by Mann–Whitney U tests with Bonferroni correction. *p<0.05, **p<0.01, ns: not significant, using the control as reference. *p<0.05, p<0.01, ns: not significant.

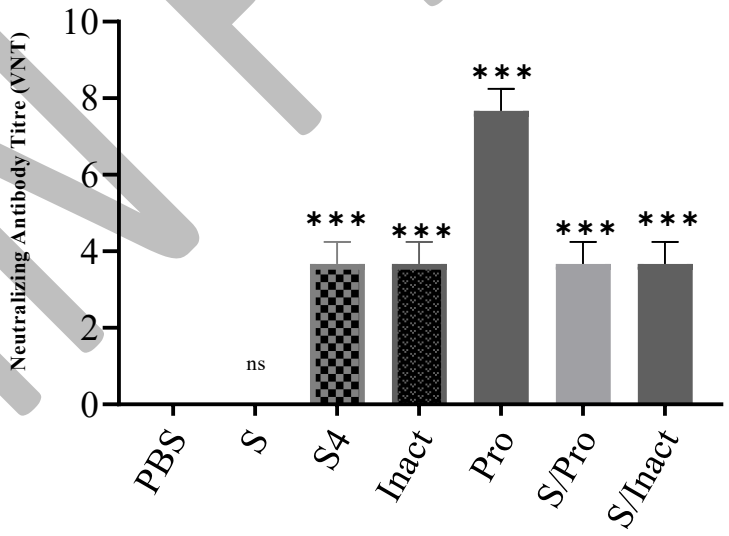

Figure 2. Neutralizing antibody titers measured on day 42 by virus neutralization test (VNT). Mean neutralizing antibody titers are shown for each group. Groups S4, Inact, S/Pro, and S/Inact exhibited equivalent titers, indicating comparable neutralizing capacity. Group Pro showed the highest titer, while S, like the PBS, shows the lowest rate.

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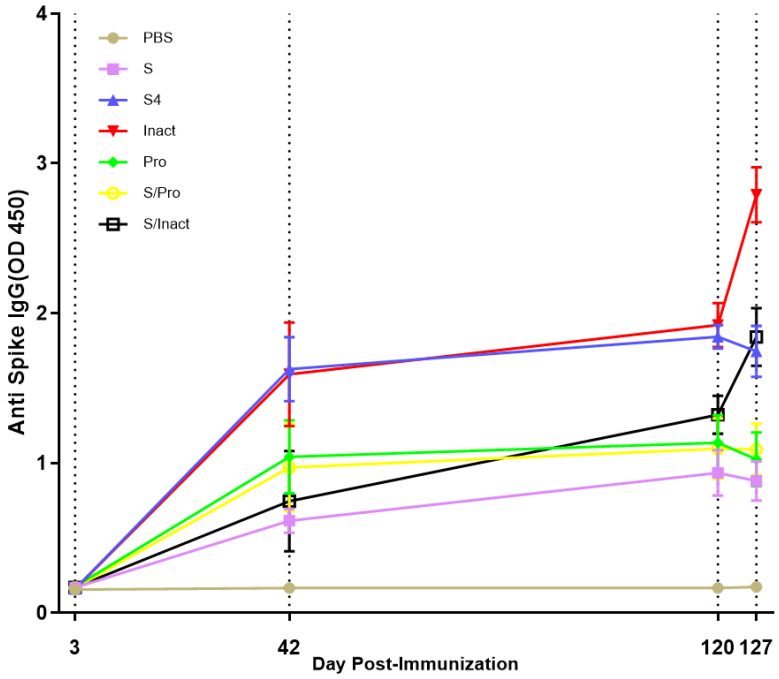

Figure 3. Anti-Spike IgG Persistence Over Time. Longitudinal tracking of spike-specific IgG antibody levels measured at multiple timepoints. Data reflect stability in all groups up to day 120 and increases in Inact and S/Inact at day 127 (after re-exposure to the virus). Values represent OD450 from indirect ELISA.

DISCUSSION

The ongoing circulation of SARS-CoV-2 and the emergence of novel variants underscore the need for vaccines that not only generate strong initial immune responses but also sustain long-term immunity.[20] Our study compared three major vaccine platforms: DNA, inactivated virus, and recombinant protein administered alone or in heterologous prime-boost regimens in a murine model.

Among the DNA groups, the four-dose DNA vaccine (S4) elicited the most robust and stable IgG response. This finding aligns with previous studies showing that repeated DNA immunization enhances antigen expression and B cell activation, which has been associated with enhanced germinal center activity and the generation of long-lived plasma cells in previous studies. Although these mechanisms were not directly evaluated in the present study, they may partially explain the sustained antibody responses observed following repeated DNA immunization.[21]

However, despite high IgG titers, the three-dose DNA group (S3) failed to induce neutralizing antibodies in the VNT, highlighting a functional gap between total antibody production and neutralizing capacity. The S4 group, which received four doses of the DNA vaccine, produced significantly higher levels of neutralizing antibodies than the three-dose S3 group. This marked improvement after that the additional dose may have contributed to enhanced B cell activation and improved antibody quality.

In contrast, the S group may not have provided sufficient stimulation to expand high-affinity B cell clones capable of producing potent neutralizing antibodies. Previous studies have shown that repeated immunization can support affinity maturation through germinal center reactions. Although germinal center responses were not directly assessed in this study, this mechanism may contribute to the observed findings, leading to improved antibody quality.[22] The observed discrepancy between total spike-specific IgG and neutralizing activity likely reflects differences in antibody quality rather than quantity alone, as ELISA detects all binding antibodies, including non-neutralizing populations, whereas VNT specifically measures functional neutralization. Differences in antigen presentation and epitope conformation among the vaccine platforms may also contribute to this pattern.

Although in vitro expression of the DNA-encoded Spike protein was confirmed, in vivo expression level, native trimeric conformation, and glycosylation status were not directly evaluated. Therefore, the lower neutralizing activity despite relatively high IgG levels may reflect the induction of non-neutralizing spike-binding antibodies or differences in antigen conformation and epitope presentation.

These findings are consistent with the possibility that repeated antigen exposure contributes to improved antibody maturation.[23]. This emphasizes the need for vaccine platforms not only to elicit antibodies but to ensure they are of high functional quality.[24]

The four-dose DNA group (S4) elicited anti-spike IgG titers and neutralizing antibodies, comparable to those induced by the inactivated virus platform. This outcome of using more booster doses of DNA than of inactivated virus is particularly relevant as DNA vaccines are often considered weakly immunogenic in the absence of adjuvants or delivery enhancers.[25] Our results suggest that repeated DNA dosing may overcome this limitation through mechanisms such as sustained antigen expression or other mechanisms associated with repeated antigen exposure. This aligns with findings from prior studies where repeated DNA vaccination enhanced germinal center reactions and memory B cell formation.[26]

The recombinant protein (Pro) vaccine group achieved the highest neutralizing titers, outperforming both the inactivated and DNA-only groups in terms of functional antibody generation. This result may be attributed to the high conformational integrity of the recombinant spike protein, which allows optimal epitope exposure for B cell recognition. However, the Pro group also demonstrated a faster decline in IgG levels by day 127. This transient profile suggests strong initial immunogenicity but weaker durability, possibly due to lack of persistent antigen exposure or insufficient induction of long-lived plasma cells in the absence of Toll-like receptor (TLR) stimulation.[27]

Notably, the inactivated and S/inact groups despite inducing slightly lower neutralizing titers (~1:4) showed significantly better long-term IgG stability. One explanation is the presentation of full viral particles in inactivated vaccines, which may activate a broader repertoire of B cells and engage multiple pattern recognition receptors (PRRs), leading to more polyfunctional and long-lasting responses.[28] For the DNA S4 group, the improved persistence might result from continued transcription of the plasmid in muscle cells, creating a pseudo-persistent antigen exposure.

The heterologous groups (S/Pro and S/Inact) demonstrated intermediate profiles, with neutralizing titers similar to those of the Inact and S4 groups. These results support the hypothesis that prime-boost combinations can broaden the immune repertoire and improve antibody quality, especially when priming with DNA and boosting with a protein or viral-particle format.[29] However, the absence of clear superiority of heterologous regimens in this study suggests that dose, timing, and immunogen quality may be more critical than platform variety alone.

The time-course results obtained on days 120 and 127 showed two distinct trends. In the S and S4 groups, antibodies remained stable without further increase upon viral antigen exposure, suggesting that the antibody response had reached a plateau with limited additional recall following re-exposure. In contrast, the Inact and S/Inact groups showed a delayed boost in antibody titers, suggesting a recall antibody response that may be consistent with functional immunological memory.

One notable observation was the modest increase in neutralizing antibody titers in some groups between days 120 and 127. This may reflect recall responses consistent with memory B cell activation or the delayed activity of long-lived plasma cells.[30] We anticipated a rise in antibody titers following the booster dose on day 120. Although this response was observed in several groups, others particularly the Pro and S/Pro groups showed only minimal changes or even a slight decline in titers. This suggests that the priming platform plays a critical role in shaping the breadth, quality, and magnitude of the subsequent recall antibody response[31], thereby potentially influencing responsiveness to subsequent boosters.[32] These findings are consistent with previous reports indicating that protein-based vaccines, especially in the absence of potent adjuvants, may fail to induce long-lived humoral responses.[33] Such findings highlight the importance of evaluating not just early responses but also the recall capacity of each vaccine regimen over time.[34]

Notably, mice immunized with the inactivated virus vaccine exhibited a marked increase in antibody titers after viral exposure, despite having lower antibody levels at day 120 compared with the DNA vaccine group. This observation suggests a stronger recall antibody response that may be consistent with enhanced

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immunological memory**.** One possible explanation is that the inactivated vaccine presents the immune system with the full repertoire of viral antigens, thereby stimulating a broader range of B cell clones than vaccines based solely on the spike protein[35]. Consequently, this broader antigenic exposure may contribute to more diverse recall responses following re-exposure to the virus. Similar findings have been reported for whole-virus vaccines, which can induce broader epitope recognition.[36]

The significant rise in antibody levels in the S/Inact group post-booster also suggests that DNA priming followed by inactivated virus exposure may promote broader recall antibody responses, possibly through epitope spreading or enhanced antigen presentation.

Collectively, our findings highlight the importance of prime-boost vaccine design in shaping humoral immune responses. Heterologous combinations involving DNA priming followed by recombinant protein or inactivated virus boosting showed promising humoral responses under the experimental conditions used in this study. However, these findings should be interpreted within the context of the platform-specific formulations, adjuvant systems, and antigen doses employed, which were selected to represent established vaccine formulations rather than to enable direct comparisons of the intrinsic immunogenicity of different vaccine platforms. These observations support the potential utility of heterologous vaccination strategies.[37]

The comparative immunogenicity of the various vaccine regimens including their ability to induce initial IgG and neutralizing titers, sustain humoral responses, and recall memory after booster injection is summarized in Table 2.

Several limitations should be considered when interpreting the findings of this study. First, the vaccine formulations differed with respect to adjuvant composition and antigen dose. These formulations were selected to represent established vaccine platforms rather than to standardize individual formulation components across groups. Therefore, the observed differences in humoral responses should be interpreted within the context of the combined effects of vaccine platform, formulation, adjuvant system, and antigen dose, rather than as direct comparisons of the intrinsic immunogenicity of different vaccine platforms.

Table 2. Comparative Immunogenicity and Memory Response Across Vaccine Regimens

Vaccine Group	Prime/Boost Type	Initial IgG (Day 42)	Neutralizing Ab	IgG Durability (to Day 120)	Booster Response (Day 127)	Memory B Cell Strength	Immunological Interpretation
PBS	Not observed	Not observed	Not observed	Not observed	Not observed	Not observed	Negative control
S	DNA/DNA	Weak	Not observed	Moderate	Weak	Weak	Moderate memory; limited boost
S4	DNA/DNA	Strong	High	Weak	Weak	High	Strong early response; booster plateau
Inact	Inact/Inact	Strong	High	Strong	Strong	Strong	Robust memory; strong recall
Pro	Protein/ Protein	High	Strong	Weak	Weak	Not observed	Weak priming; poor memory
S/Pro	DNA/Protein	Moderate	High	Weak	Weak	Weak	Effective priming; suboptimal memory
S/Inact	DNA/Inact	Moderate	High	High	High	High	Durable combo; effective booster

This study provides insight into the antibody responses of three major SARS-CoV-2 vaccine platforms—DNA, inactivated virus, and recombinant protein—in homologous and heterologous prime-boost regimens in a murine model, with a specific focus on the induction and persistence of virus-specific and neutralizing antibodies. The DNA vaccine, especially when administered in four doses, induced the highest and most persistent antibody responses through day 120. However, these responses lacked strong neutralizing

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activity, emphasizing the need for improved functional quality of the elicited antibodies. In contrast, recombinant protein vaccines effectively induced potent neutralizing antibodies but exhibited a decline in total IgG titers over time. Inactivated vaccines demonstrated a moderate profile in both IgG levels and neutralization potential but uniquely showed a strong recall response upon re-exposure, as indicated by a significant increase in SARS-CoV-2-specific antibody levels at day 127. These findings highlight the distinct humoral immune response profiles generated by the homologous and heterologous prime–boost regimens evaluated in this study and underscore the importance of matching vaccination strategies with desired immunological outcomes. DNA vaccines may serve as a durable foundation, whereas protein subunit and inactivated platforms can complement through boosting or enhancing functional immunity. These findings underscore the pivotal role of the priming platform in determining not only short-term antibody generation but also the magnitude and persistence of recall antibody responses, which are essential for long-term protection and responsiveness to booster immunizations. Strategic pairing of vaccine platforms—particularly those capable of robust memory imprinting during priming—may enhance the efficacy and adaptability of next-generation COVID-19 vaccines and other future viral immunizations.

STATEMENT OF ETHICS

All animal experiments and procedures performed in this study were reviewed and approved by the Institutional Ethics Committee of Tarbiat Modares University, under the ethical approval code IR.MODARES.AEC.1405.027. All efforts were made to minimize animal suffering, and the experiments were conducted in strict accordance with the institutional guidelines for the care and use of laboratory animals and the ARRIVE guidelines.

FUNDING

The authors received no financial support for the research, authorship, and/or publication of this article.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

We wish to thank the deputy of research, Tarbiat Modares University for the financial supports (Med IR.MODARES.REC.7510) and assistances.

DATA AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

ChatGPT (OpenAI) was used solely to assist with English-language editing, including grammar correction and improving clarity and readability. The authors reviewed the AI-assisted text and take full responsibility for the final manuscript.

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