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Evaluation of Heterologous Prime-boost Vaccine Strategy Using Full-length Cytomegalovirus Glycoprotein B to Trigger BALB/c Mice Immunity

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ABSTRACT

Human cytomegalovirus glycoprotein B (gB) emerges as a viable candidate for eliciting neutralizing antibodies. This research specifically focused on exploring the immune reaction prompted by the nonglycosylated variant of the gB, with a comprehensive assessment of humoral immunity in mice.

The gB coding sequence was optimized and expressed in pET-15b. Additionally, pcDNA3.1(+) vectors were also used for cloning the same gB sequence as the DNA vaccine. The gB was purified using a Ni-NTA chromatographic column. SDS-PAGE and Western blotting were used to confirm protein expression and purification. Using the prime-boost strategy, 8 different BALB/c mice were injected with DNA vaccine plus gB heterologous vaccine at 3 intervals. We evaluated the interferon (IFN-γ), interleukin (IL-4), immunoglobulin (Ig) G1, IgG2a, and IgG2b using enzyme-linked immunosorbent assay.

It was shown that the mice administered with DNA vaccine plus gB had higher IFN- γ and IL-4 levels compared to controls. On the other hand, the mice that received 3 doses of gB showed the highest levels of IgG1 and IgG2a. However, IgG2b was at its highest in mice administrated with DNA vaccine plus gB. The total IgG was higher in mice that received gB than in other interventions.

According to the findings, the DNA vaccine enhanced total IgG in immunized mice more effectively than the gB. This could be attributed to conformational changes owing to a lack of glycan moiety. Furthermore, combining nonglycosylated gB with DNA as a heterologous vaccine strategy enhances innate immunity by increasing the IFN- γ levels.

Keywords: Cytomegalovirus; Glycoprotein B, Heterologous immunity

INTRODUCTION

Human cytomegalovirus (HCMV) is a prevalent virus worldwide, with over 50% of adults naturally infected.[1] Transplant recipients receiving immunosuppressive therapy, patients with HIV, and

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pregnant women are susceptible to severe HCMV infections.[2–5] Immunity against CMV involves humoral and cell-dependent immune responses. Most primary HCMV infections in normal cases are mild with no certain clinical presentation.[4,6] Natural immunity to HCMV cannot prevent reinfection, although cellular immunity is important in limiting acute disease in healthy people and those with weakened immune systems.[6,7] Immunological determinants predisposing people to disease and infection are not completely characterized. After primary CMV infection, a large CD4+ and CD8+ T-cell response is established by the virus for the host's life.[1,4] Therefore, antiviral antibodies, these responses, and natural cytotoxicity play a role in controlling HCMV pathogenesis.

The high prevalence of HCMV disease, the high expense of current anti-CMV therapies, and significant side effects[8] highlight the need for alternative therapeutic interventions. Also, the limitation of the HCMV infection severity that happens in the nonimmune host following prenatal infection or under immunosuppression conditions needs an effective vaccine.[9] Identification and characterization of HCMV proteins that elicit a protective response pave the way to controlling the infection and limiting disease in the infected host.[10] The anti-CMV vaccination age began in the 1970s with the introduction of the first generation of live attenuated viral strains, which did not match the criteria for clinical trials II and III.[11] Novel HCMV viral strains developed to express the pentameric complex (PC) are under investigation, however, safety concerns are still present. On the contrary, recombinant protein subunit vaccines are highly safe and effective in humans.[12]

The HCMV virus has different types of glycoprotein complexes (gC-I to III) needed for the infection of the host cells. gC-I contains a pan-Herpesviridae conserved glycoprotein, homotrimers of glycoprotein B (gB), a membrane fusion mediator, which can rearrange during the entrance into the cell from a prefusion conformation to a postfusion conformation.[13] Additionally, the gB effect on the entrance into all cells makes it an appropriate target for vaccination. A protective vaccine requires neutralizing antibodies preventing the infection of endothelial and epithelial cells.[14] Most infected individuals have antibodies against gB, which can neutralize viral infectivity.[15] Most (40% to 70%) serum-neutralizing antibodies for HCMV are directed toward gB in vivo. Active or inactive immunity against gB has been shown to protect mice or guinea pigs against CMV-associated diseases. Maintaining a high immunoglobulin titer against HCMV in pregnancy prevents vertical transmission.[6,7] Cui et al have shown that HCMV trimeric gB elicits a high rate of HCMV-neutralizing antibodies in the animal model.[16] Neutralizing and function-blocking antibodies targeting gB could effectively inhibit HCMV infection in vitro. However, gB, the primary target for neutralizing antibodies, is highly glycosylated.[17] The glycosylation of HCMV surface proteins either enhances immune escape or facilitates viral entry.[18] gB contains 18 *N*-linked glycan sites shielding viral epitopes. Furthermore, our knowledge of each epitope's role in eliciting neutralizing antibodies is limited. In this regard, it has been shown that epitope AD-3 plays as a decoy to subvert the neutralization of HCMV.[19]

The rationale for such vaccine developments is their safety and inducing neutralizing antibodies. Although glycosylation plays a crucial role in the biology of HCMV, this study specifically aims to evaluate the immune response triggered by the nonglycosylated full-length CMV gB. The evaluation includes examining the humoral immune response, lymphocyte proliferation, and cytokine production in response to the nonglycosylated gB in mice. By focusing on the nonglycosylated form, we can gain insights into the immunogenic potential of the gB independent of glycosylation effects. This evaluation contributes to the understanding of CMV vaccine strategies and their potential to induce protective immunity.

MATERIALS AND METHODS

Production of Recombinant Glycoprotein B

The recombinant gB was provided as mentioned in our previous study.[1] Briefly, the full-length sequence of the gB gene, which encodes a protein consisting of 906 amino acids, was codon-optimized for expression in the prokaryotic system. The full-length gB sequence, with the accession number ACL51135.1, corresponds to the full-length HCMV sequence with the accession number FJ527563.1. The gB gene was cloned into the pET-15b vector and purified under hybrid conditions using a Ni–NTA (Qiagen, Germany) affinity chromatography column.[1] Purification of the recombinant gB was achieved by dialysis in Phosphate Buffered saline (PBS) with pH 7.4.

Developing gB-based DNA Vaccine Construct

To construct the gB-based DNA vaccine, the full-length sequence of the gB gene (FgB) with the accession number FJ527563.1 was cloned into the pcDNA3.1(+) vector. The cloning process involved double digestion of the vector with Nde1 and XhoI enzymes (Biomatik Co., Canada). A Maxiprep of the pcDNA3.1(+)-FgB construct and control pcDNA3.1(+) vector was prepared using a plasmid extraction kit (Qiagen EndoFree Plasmid Maxi, Germany). The quality and quantity of the extracted DNA were evaluated by assessing its optical density (OD) at 260/230 nm and 260/280 nm. The cloning was further confirmed by double digestion of the purified vector with Nde1 and XhoI enzymes through gel electrophoresis analysis. For the immunization process, the DNA was dissolved in PBS (pH 7.4).

Transfection and Expression of gB in HEK293T Cells

EK293T cells (2×10^6 cells.mL^{-1}.well^{-1}) underwent culturing in complete RPMI medium with 1% penicillin-streptomycin (v/v) and 10% (v/v) fetal bovine serum (FBS) in a 6-well tissue culture plate. After 48 hours, transfection of the cells was done with the pcDNA3.1(+)-FgB (4 μg/well) by the lipofectamine 2000 transfection kit (Invitrogen, USA) as instructed. Cells transfected with equimolar levels of the pcDNA3.1(+) vector were considered the control. HEK-293T cells were used as a negative control. Then, 72 hours after transfection, the collection of culture supernatant was performed by centrifugation. Culture supernatants (50 μL) related to the transfected cells were resolved using reducing SDS-PAGE (10%), followed by transferring into nitrocellulose membrane for Western blotting. Expression of the gB was detected using 1:5000 dilution of goat polyclonal anti-gB-HRP (Abcam, USA). The color was developed using DAB (Sigma Aldrich, USA) to visualize antigen-antibody reaction.[1]

Animal Preparation and Strategy of Vaccination

Eight groups of female BALB/c mice (6–8 weeks) (Pasteur Institute of Iran, Tehran) were used, including 5 vaccination groups and 3 control groups (PBS, pcDNA3.1[+], and sham). Each group was made of 5 mice. The administration of DNA vaccine and gB is shown in Table 1. The final dose was determined based on the vaccination schedule and the subsequent evaluation of lymphocyte proliferation, cytokine production, and humoral immune response.

Accordingly, the mice were injected with 100 μL of either DNA vaccine or gB and PBS on days 0, 14, and 28. The intramuscular injection was performed at a rate of 50 μg in each left and right flank muscle. Ten days following the final injection, blood sampling was done from the heart, and sera were stored at −20°C. Furthermore, 3 mice from each group were randomly selected for sacrifice, and splenocytes underwent harvesting for cytokines and lymphocyte proliferation assays.

Table 1. Dosage of different administrations and grouping of mice

Groups	Compounds	Day 0 (Prime)	Day 14 (Booster)	Day 28 (Booster)
1	DNA vaccine	DNA vaccine (90 μg)	DNA vaccine (90 μg)	DNA vaccine (90 μg)
2	gB	gB (20 μg)	gB (20 μg)	gB (20 μg)
3	DNA vaccine + gB	DNA vaccine (90 μg)	DNA vaccine (90 μg)	gB (20 μg)
4	gB + DNA vaccine	gB (20 μg)	gB (20 μg)	DNA vaccine (90 μg)
5	Simultaneous DNA vaccine + gB	DNA vaccine (90 μg) + gB (10 μg)	DNA vaccine (90μg) + gB (10 μg)	DNA vaccine (90 μg) + gB (10 μg)
6	pcDNA3.1(+)	pcDNA3.1(+) (90 μg)	pcDNA3.1(+) (90 μg)	pcDNA3.1(+) (90 μg)
7	PBS	PBS	PBS	PBS
8	Sham	Sham	Sham	Sham

gB: glycoprotein B; PBS: phosphate-buffered saline.

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Animal Safety Parameters

In this study, several measures of animal general health, including close monitoring of their activity levels, appetite, and weight changes, were recorded to ensure the safety and well-being of the experimental animals for their immunological tolerance. Throughout the vaccination process, any signs of distress or adverse reactions were also recorded. No adverse events or abnormal behaviors were observed. The administration of the DNA vaccine and gB followed established protocols, with doses within the commonly used range. Ethical guidelines were strictly adhered to, and the Ethics Committee of Golestan University of Medical Sciences approved the research (IR.GOUMS.REC.1396.95). While safety parameters were not explicitly measured or reported in this study, we ensured the welfare of the animals and followed established protocols and guidelines.

Lymphocyte Proliferation Assay and Interleukin Test

A lymphocyte proliferation assay was performed using the harvested splenocytes of immunized mice to assess the immune response. Splenocytes were seeded in microplates with 96 wells 2×10^5 cells/well. Subsequently, 100 µL of complete medium was mixed with 10 mg/mL of gB, and Phytohemagglutinin (Gibco, USA) was used as a positive control. After incubation for 48 and 72 hours at 37°C with 5% CO_2, each well was filled with 5 mg/mL of methyl thiazole tetrazolium (MTT, Sigma), and the plates were subjected to incubation for 4 hours. The supernatant was aspirated, and MTT formazan crystals were solubilized in dimethyl sulfoxideDMSO for 30 minutes. The resulting solution's absorbance was measured at 570 nm using an ELISA reader, referencing it against the value at 630 nm.

In addition to the lymphocyte proliferation assessment, the secretion of specific interleukins was also evaluated. Splenocytes from all groups (3 mice per group) were cultured at a density of 2×10^6 cells/well in complete RPMI-1640 supplemented medium. The cell stimulation was done using 10 µg/mL of the gB for 72 hours. Untreated cells were considered negative controls. Culture supernatants were collected and kept at −80°C for net analyses. The IFN-γ and IL-4 levels were measured by commercial ELISA kits following the producer's instructions (Mabtech, Sweden), with absorbance readings taken at 450 nm.

The lymphocyte proliferation assay and the evaluation of specific interleukins were performed 3 times, and the findings were reported as a percentage of the control, which was regarded as 100%. The stimulation index as the proportion of stimulated cells to unstimulated cells, was used to quantify lymphocyte proliferative responses.[1]

Cytokine Assays

To assess the IFN-γ and IL-4 secretion, splenocytes from all groups (3 mice per group) underwent culturing in 96-well at 2×10^6 cells/well in complete RPMI-1640 (Sigma, USA) supplemented medium. Then, cell stimulation was done using 10 µg/mL of the gB (20 µg/mL) for 72 hours. Untreated cells were regarded as negative controls. Supernatants related to unstimulated and stimulated cells were collected and kept at −80°C until usage. The supernatants' cytokine levels were assessed by commercial ELISA kits as instructed (Mabtech, Sweden), and absorbance was measured at 450 nm.

Humoral Immune Response

The humoral immune reactions were assessed using indirect ELISA in immunized mice. In brief, plates (96 wells) were filled with the recombinant protein gB (10 µg/mL) in PBS at 4°C overnight, and then blocked with 5% non-fat dry milk in PBS 0.01% Tween 20 at 37°C for 2 hours. Then, the wells were added with the diluted serum (100 µL; 1:1000) followed by incubation at 37°C for 90 minutes. The plates were then washed 5 times and underwent incubation with 100 µL of 1:7000 dilution of anti-mouse HRP-conjugated IgG total (IgG2a, IgG1, and IgG2b) antibodies (Mabtech, Sweden) at 37°C for 90 minutes. Following extensive washing, the reaction development was done by the addition of 100 µL/well 3,3',5,5'-tetramethylbenzidine (TMB) substrate. Color development was stopped by the addition of 100 µL/well 0.5N H_2SO_4 following incubation for 10 minutes in the dark at ambient temperature. The optional density measurement was performed at 450 nm.

Statistical Analyzes

The samples of this study were collected and analyzed by GraphPad Prism 8.0.1 statistical software. Data were parametric and one-way analysis of variance was used to analyze the findings. The IgG results and its

isotypes represented the mean ± standard deviation in 5 mice.

RESULTS

To evaluate the immune responses, the proliferation of the immune cells can be measured after their proximity to the antigen. Therefore, this study investigated the proliferation of lymphocytes in response to restimulation in vaccinated mice with cell lysates expressing gB. Cell lysates without gB were used as a negative control. The results showed an increased lymphocyte proliferation following the administration of mice with the DNA vaccine (Figure 1). In addition, slight cytotoxicity was observed in the mice groups administrated with gB.

The results showed that the first 5 intervention groups had the highest level of IFN-γ stimulation than the controls (Figure 2A). There was a significant difference in the production of IFN-γ between mice immunized with gB (Group 2) than that observed in the DNA vaccine, but it was not significant. Furthermore, mice administrated with DNA vaccine plus gB (Groups 3 and 4) prime-boost showed higher levels of IFN-γ ($p=0.01$), and it was also higher than simultaneous administration of DNA vaccine and gB ($p>0.05$). The highest IFN-γ production was observed in Group 4 (1295 pg/ml) and Group 3 (1274.8 pg/mL).

Production of IL-4 cytokine was assessed in the lymphocytes of mice administrated with different interventions (Figure 2B). After the last boost, the mice received simultaneous administration of DNA vaccine, and gB had the highest level of IL-4 production (69.51 pg/mL). Group 1 had a significant difference from the control groups of PBS and sham ($p<0.05$). The prime boosts of DNA vaccine plus gB (Groups 3 and 4) induced significant IL-4 production compared to other control groups ($p<0.05$). Furthermore, there was a significant IL-4 production in mice receiving pcDNA3.1(+) (55.35 pg/mL), comparable to Group 1 (53.59 pg/mL).

The levels of gB-specific antibodies were assessed by an ELISA method. As presented in Figure 3A, immunization with the DNA vaccine alone or in combination with gB resulted in a significant increase in gB-specific antibody titers compared to the controls ($p<0.001$). The group receiving the DNA vaccine plus gB exhibited the highest antibody levels among all the immunized groups. However, it is important to note that the ELISA results for IgG1 and IgG2a, b subtypes may be limited due to the low range of OD (0.2 to 0.3) obtained. This low range makes it challenging to accurately check and analyze the subtype-specific antibody responses.

Also, the level of anti-gB IgG1 antibody was at its highest level in the DNA vaccine group, and it was followed by Group 4 in which mice were administrated with prime gB on day 0 and boosted with gB on day 14 and DNA vaccine on day 28, respectively. No significant differences were observed between the groups except for Group 1 with control Group 8 (Figure 3B). Additionally, the IgG2a antibody was at its highest level in the group of mice administrated with DNA vaccine, followed by Group 3 and Group 4 (Figure 3C). Furthermore, the increased production of IgG2b antibody was observed in mice of Group 3 that were administrated with the prime DNA vaccine followed by 2 gB boosters. Mice immunized with the DNA vaccine also had a significantly elevated level of IgG2b than that in the control Sham group ($p<0.035$) (Figure 3D). Among the IgG isotypes, IgG1 and IgG2a had the highest levels in the DNA vaccine-administrated mice. DNA vaccination was led to elicit higher antibody levels than that induced by gB only.

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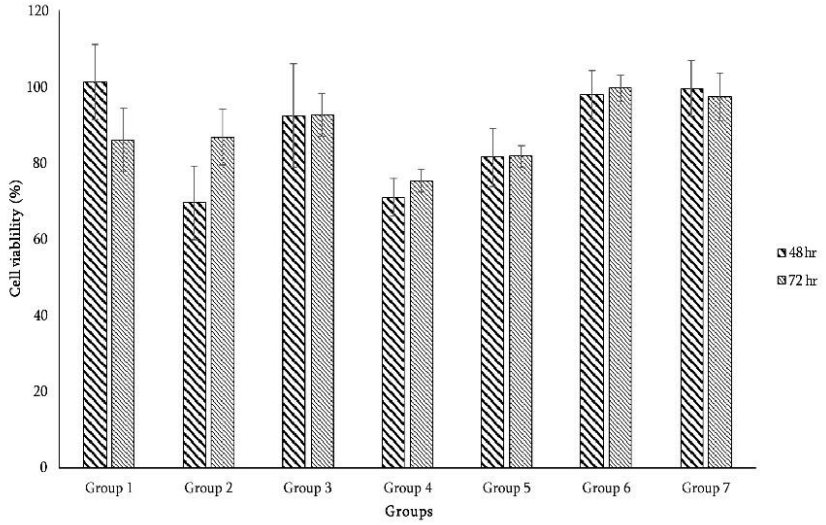

Figure 1. Methyl thiazole tetrazolium (MTT) assay results of cell viability after administration. Lymphocyte stimulation was observed at 10 mg/µL concentrations for 48 and 72 hours. The Sham group (Group 8) was also used as the control group for the MTT analysis.

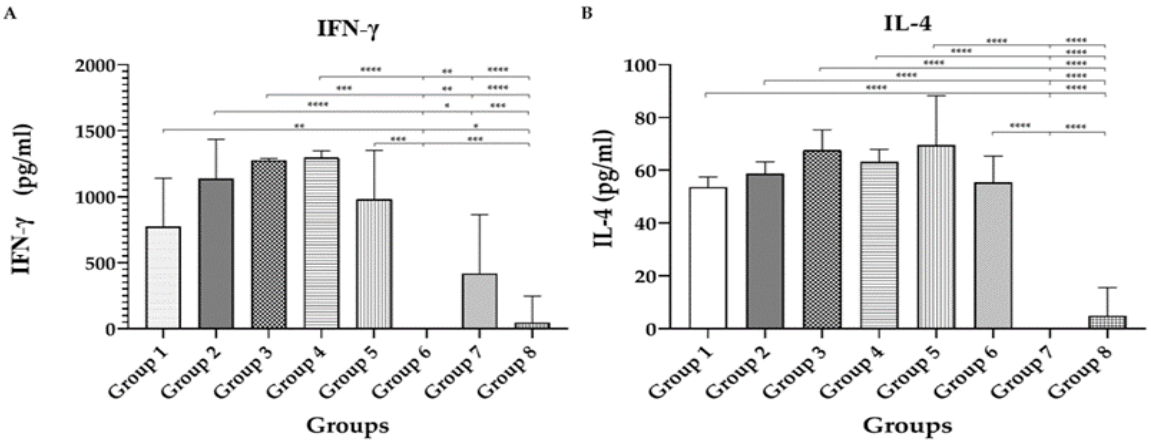

Figure 2. interferon-gamma (IFN-γ) and interleukin-4 (IL-4) levels in immunized groups with various formulations. Pooled splenocyte cultures were prepared from three mice in each group (n=3 per group) and restimulated with glycoprotein B (gB) in vitro. The levels of IFN-γ were determined in the supernatant with enzyme-linked immunosorbent assay. * p<0.5; ** p<0.01; *** p<0.001; **** p<0.0001.

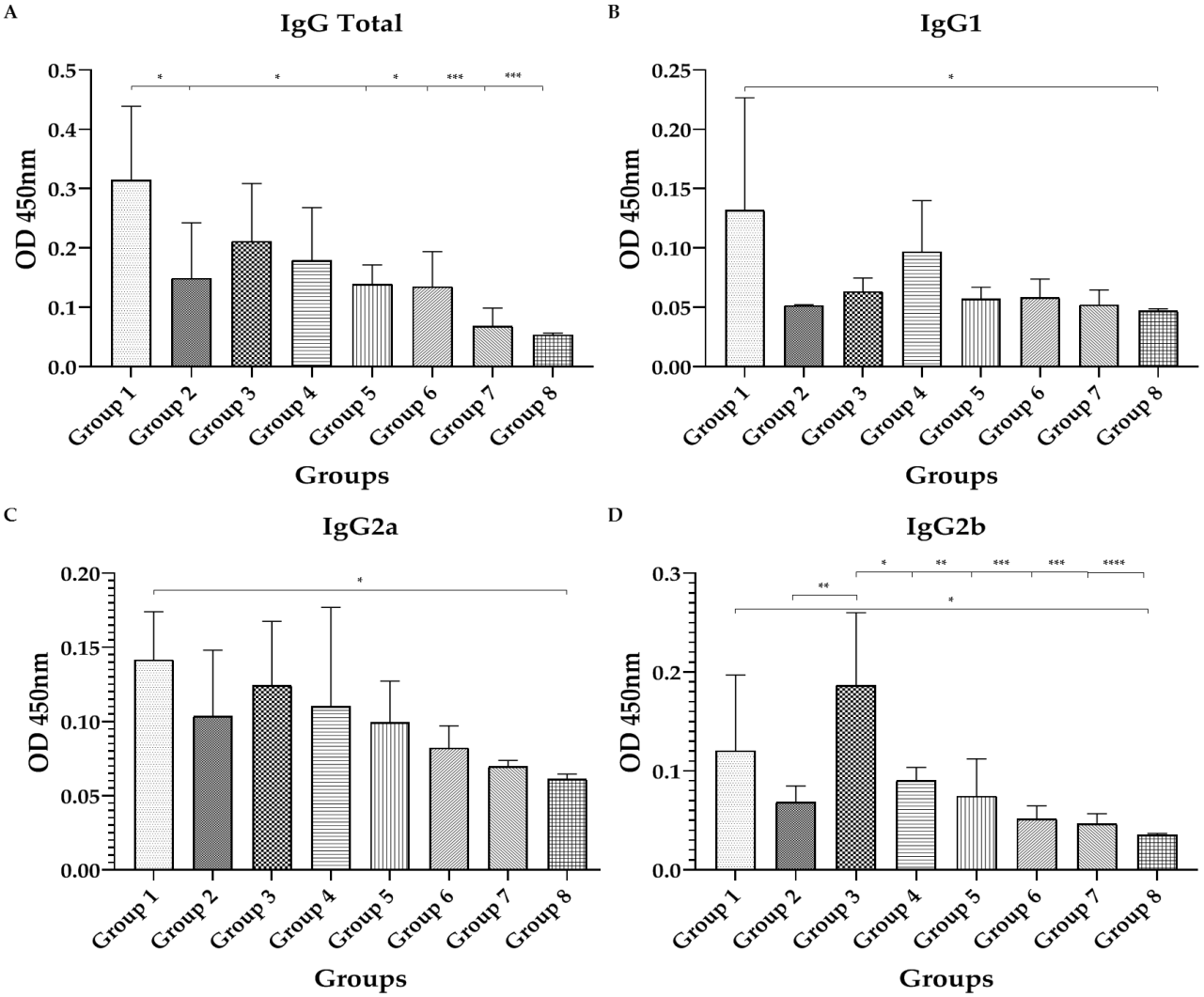

Figure 3. The level of total immunoglobulin (Ig) G and its isotypes among different vaccinated groups. A: total IgG; B: IgG1 isotype; C: IgG2a isotype; D: IgG2b isotype. The whole blood samples were prepared from mice sera of each group (n=5). Serum dilutions are shown as mean absorbance at 450 nm±standard deviation. * $p<0.5$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

DISCUSSION

Developing vaccines to prevent or control HCMV-associated diseases has been hampered by a broad range of viral factors contributing to immune escape,[1] lack of a suitable animal model, and glycosylation of viral glycoproteins that mask the viral epitopes.[17] Various strategies have been utilized to elicit anti-HCMV immune responses. However, there is a lack of studies on antibody responses that overcome the glycosylation state of HCMV gB. Furthermore, heterologous protein-based and DNA vaccines are effective in generating antigen-specific immune responses for viral diseases. Also, prime-boost vaccination is an appropriate alternative to cause effective immune responses to infections.[12,20] We assessed cellular and humoral immune responses caused by 3 modalities (gB, DNA, and prime-boost) in the BALB/c mice model.[20] Besides, the groups injected with DNA vaccine plus gB significantly induced IFN-γ production, which was more pronounced than other groups. This could be attributed to the DNA vaccine, which was more elevated by the combination with heterologous gB. On the other hand, considering that the DNA vaccine's cytotoxicity was

lower than the gB, it can be concluded that the higher dose of the DNA vaccine may enhance lymphocyte proliferation. However, gB showed a cytotoxic effect, and increasing its dosage might show adverse effects.

Further results showed nonspecific enhanced IL-4 levels. The increased amounts of IL-4 were observed in almost all immunized mice. However, an increased IL-4 level was also observed after administering pcDNA3.1(+), indicating nonspecific induction of IL-4. However, a slightly higher IL-4 production was observed in the mice that received the DNA vaccine and gB. These findings indicate that the above vaccine constructs effectively stimulated lymphocytes to produce IL-4. The findings highlight the role of the synthesized protein in inducing IL-4 production. By measuring the cytokine pattern (T_H1/T_H2 balance), the heterologous injection induces T_H1 and T_H2 responses by stimulating IFN-γ and IL-4 production. In the study of Dasari et al, T-cell response in terms of IFN-γ stimulation was higher in the group that received the prime-boost than in other groups, and this difference was significant in comparison to the control group.[21] In a study by Gil et al, a prominent gB-specific T-cell immune response was detected in the mice vaccinated with the gB-DNA vaccine. In addition, a 2.5-fold increase in the T-cell immune response was observed in the mice that received the HCMV booster after being vaccinated with the DNA vaccine.[22]

T_H1 cells, which produce IFN-γ, are responsible for inducing the cellular immune responses characterized by IgG2b and IgG2a secretion. Conversely, T_H2 cells play a role in the humoral immune response and promote IgG1 production by IL-4–mediated signaling pathways.[21] Although humoral immunity, especially in the presence of neutral antibodies alone, cannot prevent the progression of the HCMV disease, prophylactic agents such as passively transferred anti-HCMV antibodies reduce the infection severity. A significant part of neutralizing antibodies is derived from the anti-gB antibody.[23]

Our study focused on evaluating the immune response triggered by the nonglycosylated full-length CMV gB in the context of vaccine development. While N-linked glycosylation plays a crucial role in the neutralization antibody response for HCMV vaccine development,[24,26] our study aimed to investigate the immunogenic potential of the nonglycosylated form of gB independently. N-linked glycosylation in the gB has been reported to contribute to viral immune evasion and modulate the antigenicity of CMV. These glycans can shield key epitopes, preventing their recognition by neutralizing antibodies. Consequently, vaccine strategies targeting glycosylated gB forms have been explored to induce a robust neutralizing antibody response. However, it is important to note that the nonglycosylated form of gB can still elicit immune responses, particularly cellular immune responses, which play a significant role in controlling viral infections. Our findings demonstrate that the nonglycosylated gB elicited significant cellular and humoral immune responses in BALB/c mice, as evidenced by the observed lymphocyte proliferation, cytokine production, and induction of specific antibodies. These results suggest that even in the absence of N-linked glycosylation, the gB retains immunogenic properties that can contribute to vaccine-induced immunity against HCMV.

In this regard, this study investigated total IgG levels and showed that the highest stimulation rate was related to the DNA-vaccinated mice, and the nonglycosylated gB followed in administrated mice. This finding could be attributed to the potential of decoy epitopes within gB. Accordingly, it has been shown that more than 70% of elicited gB-induced antibodies that target the AD-3 epitope are not functional.[18] Therefore, the role of nonglycosylated gB with a deletion at AD-3 should be further studied. Moreover, the conformational changes due to the lack of glycan moieties may involve invoking immune responses, which should be assessed in the future. Additionally, the mice administrated nonglycosylated gB before the DNA vaccine had the highest IgG1 level. Similarly, in a study by Saraji et al, IgG1 was predominant in all studied groups among IgG subclasses, while IgG2a and IgG2b had comparable levels.[27] Consistently, gB-specific IgG2a level was substantially elevated in mice vaccinated with gB. Contrary to IgG1, the level of IgG2a was also increased in DNA vaccine plus nonglycosylated gB-vaccinated mice. This indicates that the IgG1 response is mainly gB-stimulated. However, IgG2b antibodies were higher in DNA vaccinated plus nonglycosylated gB than gB alone.

These findings implicate that whole nonglycosylated gB stimulates IgG isotypes and total IgG antibodies, specifically in immunized animals. It is also worth noting that boosting the prime gB in the 2-week intervals significantly enhanced the total IgG production. In the study of Endrész et al, the administration of a DNA

vaccine (prime) followed by the gB subunit (boost) amplified antibody responses by a mean of 0.28 ± 0.27. Several studies have shown a predominant IgG2a immune response after immunization (intramuscular) with a DNA vaccine conjugated with either a membrane-bound protein or a plasmid expressing an intracellular protein.[28,30] The finding of the present study helps our knowledge on improving HCMV vaccination strategy by enhancing specific humoral immunity by utilizing whole gB. There are a few limitations, including assessing the neutralizing activity of IgG responses in a challenge with a virus strain and evaluating cellular immunity responses toward DNA vaccine and gB.

The present study assessed the effects of the prime-boost strategy using a heterologous vaccination with nonglycosylated gB and a DNA vaccine expressing gB on the immune responses in mice. It was demonstrated that the vaccination could stimulate T_H1 and T_H2 immunities. Accordingly, the prime-boost DNA vaccine plus nonglycosylated gB was an excellent inducer of IFN-γ and IL-4. Vaccination of mice with whole nonglycosylated gB resulted in a substantial induction of gB-specific IgG1 and IgG2a immunity that might confer neutralizing acidities owing to diverting the epitope masking by glycan sites.

STATEMENT OF ETHICS

This study was approved by the Ethics Committee of Golestan University of Medical Sciences, Gorgan, Iran (Ethics Code: IR.GOUMS.REC.1396.95)

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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