Original Article

Avicenna Journal of Medical Biotechnology
Volume 18, Issue 4, 332-340
https://doi.org/10.18502/ajmb.v18i4.22930

Check for updates

MF-59 Adjuvant *vs.* Alum in an Inactivated SARS-CoV-2 Vaccine Model: Focusing on Heterologous Antigen-Binding Response

Tadeh Sepanian [1], Akbar Khorasani [2*], Bizhan Sadighi-Moghaddam [1], Rasoul Baharlou [1,3] and Mehdi Mahdavi [4,5*]

1. Department of Immunology, School of Medicine, Semnan University of Medical Sciences, Semnan, Iran
2. Department of FMD Vaccine Production, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran
3. Cancer Research Center, Semnan University of Medical Sciences, Semnan, Iran
4. Advanced Therapy Medicinal Product (ATMP) Department, Breast Cancer Research Center, National Cancer Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran
5. Biotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

*** Corresponding authors:**
Akbar Khorasani, Ph.D., Department of FMD vaccine production, Razi Vaccine & Serum Research Institute, Agricultural Research, Education & Extension Organization (AREEO), Karaj, Iran

Mehdi Mahdavi, Ph.D., Department of Advanced Therapy Medicinal Product (ATMP), Breast Cancer Research Center, National Cancer Institute, ACECR, Tehran, Iran
Biotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
Tel/Fax: +98 21 88203915
E-mail:
A.khorasani@rvsri.ac.ir,
akbarkhorasani@gmail.com,
Mahdavivac@gmail.com
Received: 30 May 2026
Accepted: 7 Sept 2026

Abstract

Background: Clarifying the role of an adjuvant in vaccine formulation on the induction of a higher cross-reactivity response is very important in the vaccine formulation. In this study, the inactivated Wuhan-Hu-1 strain of *SARS-CoV-2* virus was formulated in MF-59 and Alum adjuvants and the immunogenicity of these vaccines in the induction of cellular and humoral immune responses was assessed. In addition, the cross-binding IgG response Alpha variant (B.1.1.7) "English variant" was assessed by ELISA.

Methods: Experimental BALB/c mice were immunized subcutaneously three times with MF-59- and Alum-based vaccines. Three weeks after the last immunization, lymphocyte proliferation of spleen cells was evaluated by BrdU method, and IL-4 and IFN-γ cytokines were assessed on the spleen cell culture supernatant by quantitative ELISA kits. In addition, specific total IgG and IgG1/IgG2a isotypes were assessed with an optimized indirect ELISA. Furthermore, the cross-binding IgG titer Alpha variant was assessed by ELISA.

Results: The results showed that immunization with MF-59-inactivated SARS-CoV-2 vaccine led to a significant increase in lymphocyte proliferation, IFN-γ-associated responses, specific total IgG, and IgG1, and IgG2a isotypes compared with Alum group. In addition, inactivated SARS-CoV-2-MF59 vaccine revealed a numerically higher (70.83%) increase in the cross-binding IgG titer the inactivated SARS-CoV-2-Alum group.

Conclusion: MF-59 could be used as a suitable adjuvant in SARS-CoV-2 vaccine development due to a better induction of IgG and IFN-γ responses than alum adjuvant and a higher induction of the cross-binding IgG response the Alpha variant. These findings showed that vaccine formulation is important in inducing cross-binding responses.

Keywords: Adjuvant, T cell proliferation, IFN-g, Heterologous IgG response, Immunoglobulin G, SARS-CoV-2
To cite this article: Sepanian T, Khorasani A, Sadighi-Moghaddam B, Baharlou R, Mahdavi M. MF-59 Adjuvant *vs.* Alum in an Inactivated SARS-CoV-2 Vaccine Model: Focusing on Heterologous Antigen-Binding Response. Avicenna J Med Biotech 2026;18(4):332-340.

Introduction

The Wuhan City Health Commission reported an increase in cases of pneumonia on December 31, 2019, which determined and named SARS-CoV-2 [1]. The virus spreads rapidly around the world with a high mutation rate, and emerging new mutants cause great health damage [2]. Several vaccines were approved and commercialized for COVID-19, but some vaccines were not highly effective, and in new waves of mutated ones, the vaccines could not neutralize new mutants, because some mutants of SARS-CoV-2 change amino acids that correlate with the neutralization activity of the humoral immune response [3]. Therefore, develop-

Copyright © 2026, Avicenna Journal of Medical Biotechnology

This work is licensed under a Creative Commons Attribution–NonCommercial 4.0 International License.

ment of new vaccines based on these variants is needed or change in the vaccine formulation may abrogate this weakness. An adjuvant, as a component of vaccine formulation, has a critical role in immune response polarization and formation [4]. Numerous different classes of compounds have been evaluated as vaccine adjuvants, including mineral salts, microbial items, emulsions, saponins, cytokines, polymers, microparticles, and liposomes based on their proposed components of activity and immunostimulatory adjuvants [5-7]. Particulate adjuvants have been designed to form a neighborhood pro-inflammatory environment to trigger expression of cytokines, chemokines, immune receptors, and some other biological molecules, which were commonly balanced by Alum, MF59, and other adjuvants at the location of vaccine injection [8,9]. The MF59 adjuvant up-regulates the expression of CCR2, a receptor for CCL2, which is involved in monocyte and other immune cell migration to the injection site [10]. In addition, Alum, as the first human vaccine adjuvant, was demonstrated to promote Th2-type and humoral immune responses [11]. Several inactivated SARS-CoV-2 vaccines were formulated in Alum and various experiences have suggested that Alum generally favors humoral/Th2-biased responses in many formulations [12,13].

However, immune response analysis in COVID-19 patients demonstrated the need for both Th1 and Th2 responses to efficiently clear viruses from the lung and body [12,14]. Furthermore, several waves of SARS-CoV-2 outbreak due to emerging new mutants of the virus led to the inefficacy of approved vaccines, especially inactivated vaccine platforms, and showed a huge challenge in the protection of societies [15-17].

Therefore, the current vaccines must be optimized by changing either the antigen present in the vaccines, the adjuvant and/or the vaccine formulation to trigger both robust immune responses and cross-reactive immune responses against new variants. Studies show the potency of MF59 adjuvant in the induction of both humoral and cellular immune responses. Some studies have shown the superiority of MF59 adjuvant in the induction of immune responses and even protection in comparison to alum adjuvant [18,19].

In the present study, the inactivated Wuhan-Hu-1 strain of *SARS-CoV-2* virus was formulated in Alum and MF-59 adjuvants and the immune response and cross-binding activity of these vaccines were assessed in the experimental mice. In this study, we compared the immunogenicity and Alpha-binding IgG responses induced by inactivated SARS-CoV-2 vaccine formulations containing MF59 or Alum in mice.

Materials and Methods

Experimental animals

Six- to 8-week-old male inbred Balb/c mice were obtained from the Razi Vaccine and Serum Research Institute of Iran (Karaj, Iran). The mice were housed for 7 days prior to the experiments and given food and water *ad libitum* in standard conditions (light/dark cycle, 12 *hr*/12 *hr*, and 20±2°*C* temperature).

Virus preparation and inactivation

The Wuhan-Hu-1 strain and Alpha variant (B.1.1.7) "English variant" were isolated from patients living in the Alborz province (Karaj, Iran) who had clinical symptoms of COVID-19 infection. The infection was confirmed by Real-time PCR and the isolated viruses were cultured on the VERO cell line (ATCC® CRL-1586™) using Dulbecco's Modified Eagle's Medium (DMEM) with 10% heat-inactivated Fetal Bovine Serum (FBS). The viruses were inactivated and purified as reported previously [12,20-22]. The purified Wuhan-Hu-1 strain and Alpha variant (B.1.1.7) were dialyzed against Phosphate Bovine Serum (PBS) to remove undesired salts. Then, the virus samples were passed through a 0.22 filter, and the concentration of each virus was determined by Bradford. The viruses were stored at -70º*C* until use. All procedures on the virus culture and treatments were performed in a BSL-III facility in compliance with the biosafety guidelines of the Razi Vaccine and Serum Research Institute of Iran.

Formulation of inactivated SARS-CoV-2 in MF-59 and Alum adjuvants

Inactivated SARS-CoV-2 (Wuhan-Hu-1 strain) was formulated in MF-59 (SEPPIC, France), and/or Alum hydroxide (Brenntag Biosector, Denmark) adjuvants. To formulate in the MF-59 adjuvant, 4 μg of inactivated SARS-CoV-2 in 50 μl PBS buffer was mixed with 50 μl of MF-59 (at a *v/v* ratio of 50/50) (Vaccine Formulation Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran) for 30 *min* using a vortex at 3000 *rpm* and a homogenizer until a homogeneous product developed. To formulate the inactivated SARS-CoV-2-Alum vaccine, according to our laboratory set up, 4 μg of the virus was admixed with 200 μg of Alum and shaken at 100 *rpm* for 60 *min* at room temperature to make adsorption of the viral particles on the surface of Alum gel [23].

Immunization and study design

The Balb/c mice were assigned to three experimental groups by simple randomization, each one consisting of 10 mice. Groups 1 to 3 were immunized subcutaneously with 100 μl of inactivated SARS-CoV-2-MF59 (4 μg), inactivated SARS-CoV-2-Alum and PBS, respectively. The experimental mice were immunized three times on days 0, 21, and 42, followed by evaluation of immune responses three weeks after the final immunization [12].

Spleen cell harvesting

Twenty-one days post the last immunization, the mice of all experimental groups were euthanized by cervical dislocation and the spleens were removed and put in cold PBS/FBS 2% under the laminar hood and dissected mechanically. The single cell suspension was provided by vigorously pipetting and centrifuging at 300 *g/5min/4°C* and the RBCs were lysed using am-

monium chloride as mouse RBC lysis buffer. The single-cell suspension was centrifuged at 300 *g/5min/4°C* and then resuspended in RPMI-1640 (Gibco, Germany) which was supplemented with 10% FBS, 2 *mM* L-glutamine, 1 *mM* sodium pyruvate, 1% non-essential amino acids, 100 *µg/ml* streptomycin and 100 *IU/ml* penicillin. The cell number and viability were checked by trypan blue dye exclusion [22].

Lymphocyte proliferation assessment by the BrdU method

Three weeks after the third immunization, 3×10^5 cells in 100 *µl* complete RPMI-1640 medium were dispensed into each well of 96-well flat-bottom culture plates (SPL, China). The cells were stimulated with 200 *ng* of inactivated SARS-CoV-2 in a total volume of 200 *µl*. Un-stimulated cells, Phytohemagglutinin-A (5 *µg/ml*, Gibco)-stimulated cells and culture medium were considered as negative control, positive control and blank, respectively. After three days of *in vitro* stimulation, 20 *µl* of BrdU (Roche, Germany) reagent was added to each well. After an 18-*hr* incubation, the plates were centrifuged at 300 *g* for 10 *min*, the supernatant was discarded, the plates were dried at 60*°C* for 30 *min*, and 200 *µl* of fixation/denaturation buffer was added to each well at room temperature. After 30 *min*, the buffer was aspirated and 100 *µl* of the anti-BrdU-HRP conjugate was added to each well. After a 2-*hr* incubation, the plates were washed 5 times with PBS, and the TMB substrate was added to the wells and incubated for 10 *min* in the dark.

Then, 100 *µl* of 2N H2SO4 was added to stop the reaction. The Optical Density (OD) of the samples was read at 450 *nm* by an ELISA plate reader (BioTek, USA). The lymphocyte proliferation response was reported as stimulation index (SI): OD_{450} of the stimulated wells/OD_{450} of the un-stimulated wells [22].

Antigen recall of lymphocytes and collecting culture supernatant

Spleen cells from the experimental mice, in a total number of 3×10^6 in 1500 *µl* of complete RPMI-1640 medium, were seeded into each well of 24-well plates (SPL, China). The cells were stimulated by adding 1500 *ng*/well of inactivated SARS-CoV-2 at 37*°C* in 5% CO_2. After 60 *hr* of the antigen recall, the culture supernatant was harvested and stored at -70*°C* for IFN-γ and IL-4 cytokine assessment.

Quantitative ELISA for IFN-γ and IL-4 cytokines

The quantity of IFN-γ and IL-4 cytokines in the culture supernatant of spleen cells was estimated by quantitative mouse IFN-γ and IL-4 cytokine ELISA Kits (Mabtech, Stockholm, Sweden) according to the manufacturer's manual. The quantity of each cytokine was reported as *pg/ml* using a standard curve of each cytokine. In addition, the IFN-γ/IL-4 ratio was reported [24].

Mouse bleeding and serum separating

Three weeks after the last immunization, the mouse bleeding was performed by retro-orbital sinus puncturing. The blood samples were centrifuged at 10000 *rpm*/10 *min* and the serum samples were harvested and frozen at -20*°C* until specific IgG response assay.

Specific total IgG response by an optimized indirect ELISA

An optimized indirect ELISA was used to assess the specific IgG response. Briefly, 500 *ng* of inactivated SARS-CoV-2 (Wuhan-Hu-1 strain and Alpha variant (B.1.1.7) in each ELISA) in 100 *µl* PBS was added into 96-well ELISA Maxisorp plates (Greiner, Germany) and incubated at 4*°C* overnight. The plates were washed with washing buffer (PBS plus 0.1% Tween 20) and blocked for 60 *min* at 37*°C* with blocking buffer (2% skimmed milk in washing buffer). The plates were washed and 100 *µl* of serial dilutions of serum samples from 1/500 up to 1/1124000 were added into each well. The plates were incubated at 37*°C* for 90 *min* and washed five times. Then, 100 *µl* of 1/10000 dilution of anti-mouse HRP-conjugate (Razirad, Iran) was added to each well and the plates were incubated for 2 *hr*. The plates were washed five times and 100 *µl* of TMB substrate was added to each well and kept in the dark. The colorimetric reaction was stopped with 100 *µl* of 2N H_2SO_4 and the optical density was measured at 450/630 *nm* with an ELISA plate reader (BioTek, USA) and IgG response was reported as endpoint titer of each individual mouse [22].

Specific IgG1 and IgG2a isotypes assessment

The assessment of the specific IgG1 and IgG2a subclasses was carried out on the 1/1000 serum dilution of experimental mice. The ISO-2 kit (Sigma, USA) was used to assess these isotypes. Goat anti-mouse HRP-conjugate (Razirad, Iran) was used for the assessment, according to the manufacturer's manual [25].

Statistical analysis

All experiments for each mouse were done in duplicate and/or triplicate. The data of all the experiments were presented as mean±standard deviation (SD). Test for normal distribution was done using Kolmogorov-Smirnov test. Data of BrdU, IFN-γ, IL-4, and IFN-γ/IL-4 ratio were analysed by One-way ANOVA followed by Tukey test (Graph Pad Prism 6.01 software, La Jolla, CA, USA) were used for statistical analysis. The data of total IgG titer, IgG1 and IgG2a isotypes and total IgG cross-binding IgG were analysed by Mann Whitney test considering to the distribution of raw data. In all of the cases, p values less than 0.05 were considered significantly different. A confidence intervals of 95% was considered for statistical analysis.

Results

Lymphocyte proliferation

Results from the lymphocyte proliferation response in the experimental groups showed that injection of the inactivated SARS-CoV-2-MF59 led to a significant increase as compared with the control group (p= 0.0012). However, the vaccine formulated in Alum led to increased proliferative responses by 9.037% as compared with the control group, but was not statistically

significant (p=0.8267). In addition, inactivated SARS-CoV-2-MF59 showed a significant increase in proliferation responses as compared with the inactivated SARS-CoV-2-Alum group (p=0.0042) (Figure 1).

IFN-γ cytokine

Results from the IFN-γ cytokine response in the experimental groups showed that injection of the inactivated SARS-CoV-2-MF59 vaccine led to a significant increase in the IFN-γ cytokine as compared with the control group (p=0.0017). In addition, the inactivated SARS-CoV-2-Alum group showed a 24.39% increase the control group but was not statistically significant (p=0.5985). Furthermore, injection of the inactivated SARS-CoV-2-MF59 group showed a significant increase in the IFN-γ cytokine response as compared with the vaccine formulated in Alum (p=0.0089) (Figure 2).

IL-4 cytokine

Results from the IL-4 cytokine response in the inactivated SARS-CoV-2-MF59 and inactivated SARS-CoV-2-Alum groups revealed a significant increase as compared with the control group (p=0.0462 and p= 0.0113, respectively). However, no significant difference was observed between the inactivated SARS-CoV-2-MF59 and inactivated SARS-CoV-2-Alum groups in the IL-4 cytokine response (p=0.7662) (Figure 3).

IFN-γ/IL-4 cytokine ratio

The analysis of the IFN-γ/IL-4 ratio in the inactivated SARS-CoV-2-MF59 group showed a 49.55% increase as compared with the control group (p=0.0741). However, immunization with the inactivated SARS-CoV-2-Alum group did not show a significant difference as compared with the control group (p=0.7707). In addition, the inactivated SARS-CoV-2-MF59 group showed a significant increase in the IFN-γ/IL-4 ratio the inactivated SARS-CoV-2-Alum group (p=0.0068) (Figure 4).

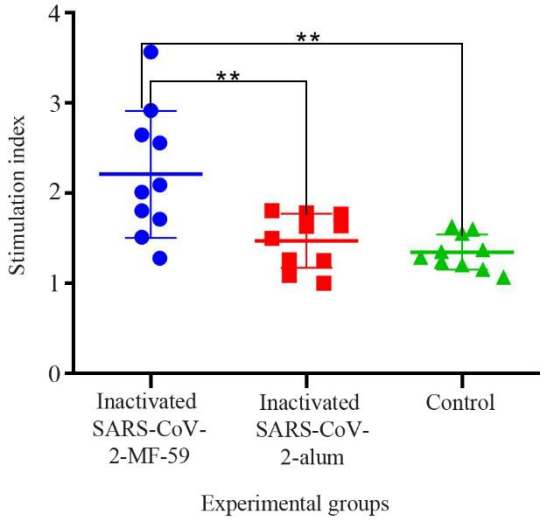

Figure 1. Results from lymphocyte proliferation response in the experimental groups (10 mice per group, data presented as mean±S.D) analyzed by one-way ANOVA followed by Tukey test. Inactivated SARS-CoV-2-MF59 group showed a significant increase compared with inactivated SARS-CoV-2-Alum group (p=0.0042).

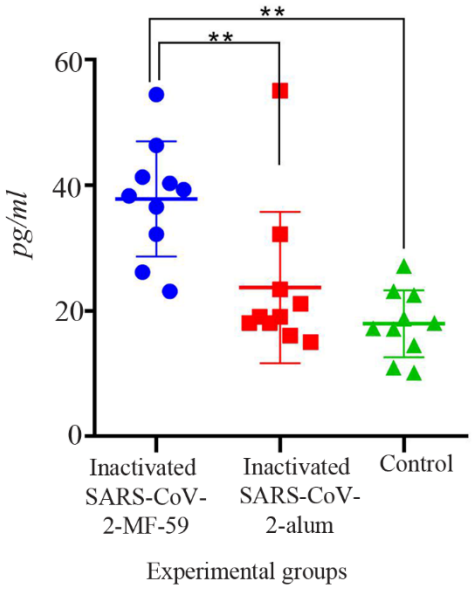

Figure 2. Results from IFN-γ cytokine response in the experimental groups (10 mice per group, data presented as mean±S.D). Statistical analysis using one-way ANOVA followed by Tukey test showed injection of inactivated SARS-CoV-2-MF59 vaccine led to a significant increase in IFN-γ cytokine response as compared with SARS-CoV-2-Alum group (p=0.0089).

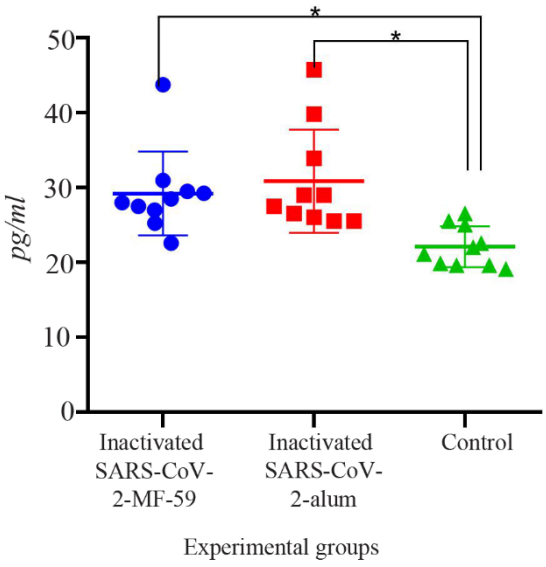

Figure 3. Results from IL-4 cytokine response (10 mice per group, data presented as mean±S.D). IL-4 cytokine secretion in the inactivated SARS-CoV-2-MF59 and inactivated SARS-CoV-2-Alum groups revealed a significant increase as compared with the control group (p=0.0462 and p=0.0113, respectively) using one-way ANOVA followed by Tukey test .In addition, no significant difference was observed between inactivated SARS-CoV-2-MF59 and inactivated SARS-CoV-2-Alum groups (p=0.7662).

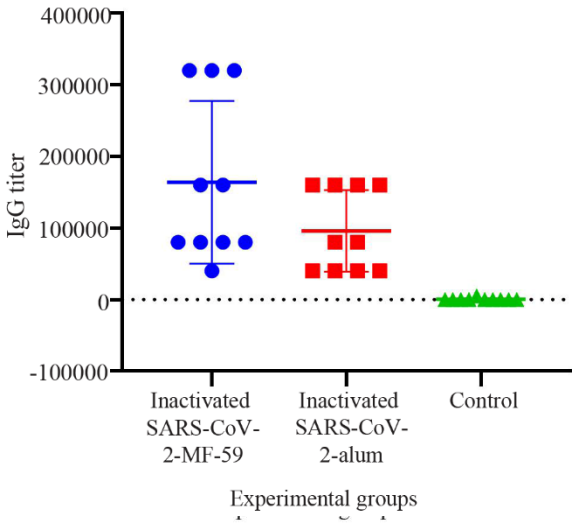

Figure 4. The analysis of the IFN-γ/IL-4 ratio in the vaccinated mice (10 mice per group, data presented as mean±S.D). Immunization with inactivated SARS-CoV-2-MF59 group showed a significant increase *vs.* inactivated SARS-CoV-2-Alum group (p=0.0068) as analyzed by one-way ANOVA followed by Tukey test.

The results from the specific IgG titer against the Wuhan strain of SARS-CoV-2

Results from the specific IgG titer against the Wuhan strain virus showed that immunization with the inactivated SARS-CoV-2-MF59 and inactivated SARS-CoV-2-Alum groups resulted in a significant increase the control group (p=0.0001). In addition, immunization with inactivated SARS-CoV-2-MF59 vaccine led to a significant increase in the specific IgG titer the inactivated SARS-CoV-2-Alum group (p=0.0270) (Figure 5).

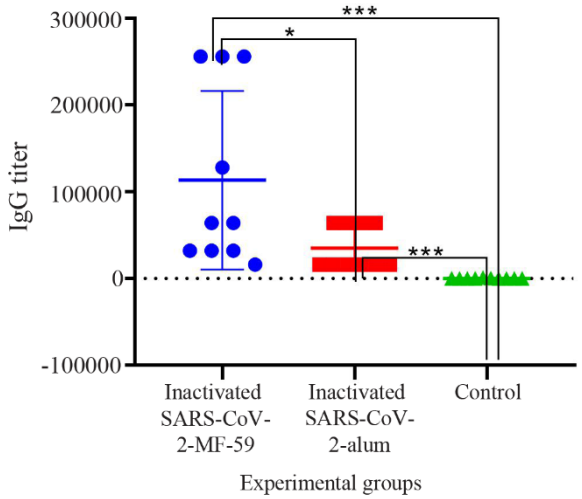

Figure 5. Results from specific IgG titer against the vaccine strain (Wuhan strain virus) (10 mice per group, data presented as mean± S.D). Immunization with inactivated SARS-CoV-2-MF59 vaccine resulted in a significant increase in the specific IgG titer *vs.* inactivated SARS-CoV-2-Alum group (p=0.0270) as analyzed by one-way ANOVA followed by Tukey test.

Specific IgG1 response

Results from the specific IgG1 isotype antibody in the inactivated SARS-CoV-2-MF59 and inactivated SARS-CoV-2-Alum groups showed a significant increase the control group (p=0.0001). In addition, immunization with the inactivated SARS-CoV-2-MF59 vaccine revealed a significant increase in the specific IgG1 response the inactivated SARS-CoV-2-Alum group (p=0.0121) (Figure 6A).

Specific IgG2a response

Results from the specific IgG2a isotype antibody in the inactivated SARS-CoV-2-MF59 showed a significant increase the control group (p=0.0079), while the

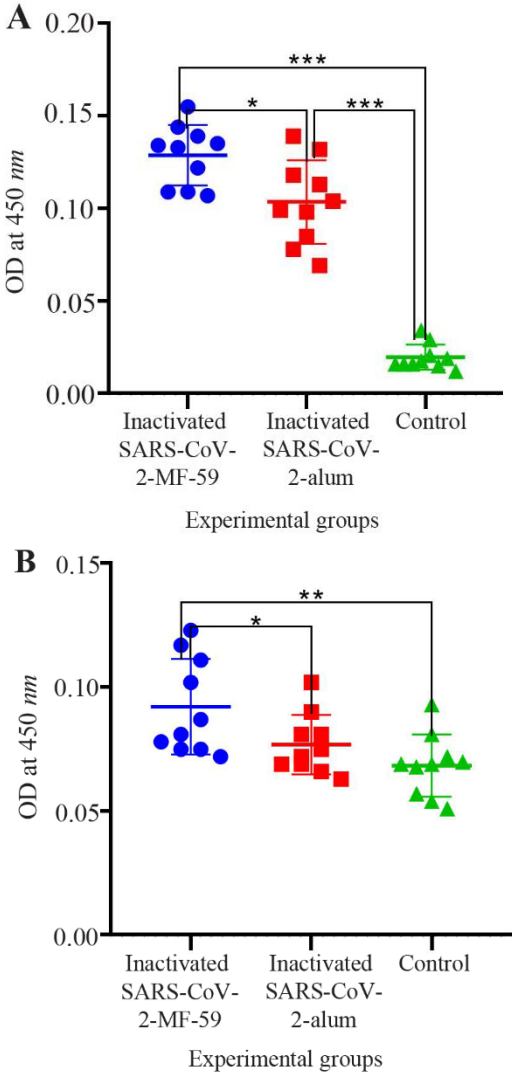

Figure 6. A), Specific IgG1 isotype antibody in mice groups (10 mice per group, data presented as mean±S.D). Inactivated SARS-CoV-2-MF59 group revealed a significant increase in the specific IgG1 response *vs.* the inactivated SARS-CoV-2-Alum and the control group (p=0.0121 and p=0.0001, respectively). B), Specific IgG2a isotype antibody in mice groups (10 mice per group, data presented as mean±S.D). Inactivated SARS-CoV-2-MF59 group showed a significant increase *vs.* the inactivated SARS-CoV-2-Alum vaccine and the control group (p=0.0493 and P=0.0079, respectively) using one-way ANOVA followed by Tukey test.

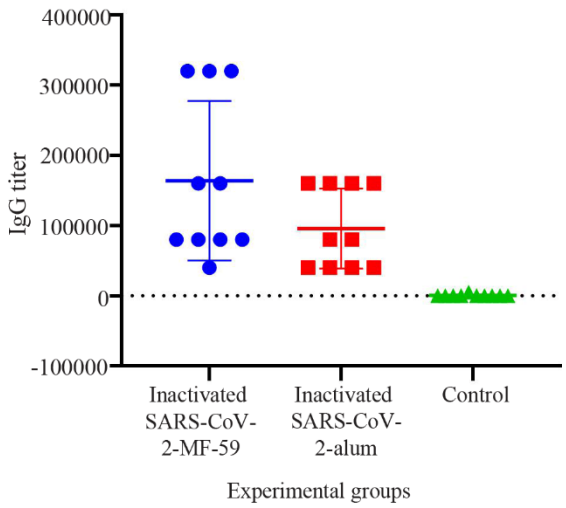

Figure 7. Results from cross-reactive IgG titer against heterologous Alpha variant (10 mice per group, data presented as mean±S.D). Inactivated SARS-CoV-2-MF59 and inactivated SARS-CoV-2-Alum groups showed a significant increase the control group (P=0.0001). In addition, immunization with inactivated SARS-CoV-2-MF59 vaccine showed a 70.83% increase in cross-reactive IgG titer inactivated SARS-CoV-2-Alum group as analyzed by one-way ANOVA followed by Tukey test.

inactivated SARS-CoV-2-Alum vaccine showed no significant difference as compared with the negative control group (p=0.2456). In addition, immunization with the inactivated SARS-CoV-2-MF59 vaccine led to a significant increase in the specific IgG2a response as compared with the inactivated SARS-CoV-2-Alum vaccine (p=0.0493) (Figure 6B).

Results from cross-binding IgG titer against Alpha variant (B.1.1.7) of SARS-CoV-2

Results from the specific IgG titer against the heterologous Alpha variant virus in the inactivated SARS-CoV-2-MF59 and inactivated SARS-CoV-2-Alum groups showed a significant increase the control group (p=0.0001). In addition, immunization with the inactivated SARS-CoV-2-MF59 vaccine showed a numerically higher (70.83%), but not statistically significant (p=0.1989), Alpha-binding IgG response compared with Alum (Figure 7).

Discussion

It is clear that some properties of a vaccine are driven by the adjuvant used in the vaccine formulation. It has been shown that the adjuvant nature has an important role in vaccine immunogenicity, and this part is less studied regarding the potency of an adjuvant on the cross-reactive response for an inactivated SARS-CoV-2 vaccine [26]. Here, we hypothesized that vaccine formulation may be involved in the cross-binding response of vaccines. Therefore, the present study used MF59 and Alum adjuvants in the inactivated SARS-CoV-2 vaccine model to compare the immunogenicity as well as cross-binding properties of these vaccines. Results from this study showed a significant increase in lymphocyte proliferation response of the MF59-inactivated SARS-CoV-2 group as compared with the Alum-formulated one. Considering that lymphocyte proliferation is a marker of cell-mediated immune response [24], this finding showed more potency of the MF59 adjuvant in the induction of cellular immune response as compared with the Alum-based vaccine. A study on recombinant H1N1 hemagglutinin formulated in MF59 and Alum adjuvants showed a stronger T cell response in the MF59-adjuvanted vaccine as compared with the Alum-based vaccine [23]. In another study, Wack *et al* showed that the influenza vaccine formulated in the MF59 adjuvant led to a better T cell response than the Alum-based vaccine [27]. These findings are parallel to our findings and confirmed that a better T cell response can be achieved when the vaccine is formulated in the MF59 adjuvant. Results from IFN-γ and IFN-γ/IL-4 ratio showed a significant increase in the MF59 adjuvant group vs. the Alum adjuvant. Nevertheless, IL-4 cytokine response in the MF59 adjuvant group showed no significant difference *vs.* the Alum group. This finding may be related to the nature of alum and MF59 adjuvants, because both adjuvants are able to induce IL-4 response. While, the MF59-inactivated SARS-CoV-2 vaccine was associated with a more IFN-γ-dominant cytokine profile.

A study on a recombinant H1N1 hemagglutinin vaccine showed a higher IFN-γ cytokine response in the MF59-formulated vaccine *vs.* the Alum-adjuvanted vaccine [23]. In addition, a study on the split influenza A/H7N9 vaccine formulated with the MF59 adjuvant showed strong IFN-γ and Th1 responses in comparison with the Alum vaccine [28]. These findings confirmed our results and showed the capability of the MF59-formulated vaccine *vs.* the Alum vaccine in the induction of the IFN-γ response. It is clear that IFN-γ through differentiation and stimulation of T cell response leads to a cellular immune response which in turn kills virus-infected cells through the function of CD8+T cells and finally leads to virus clearance [29,30]. However, interpretation about the pattern of immune response is based on a limited cytokine panel and does not directly involve phenotypic characterization of T-cell subsets or measurement of Th1-associated transcription factors and this can be considered a limitation for this study.

Next, the specific IgG titer was assessed in the experimental groups *vs.* the vaccine strain (Wuhan-Hu-1 strain) as well as a non-vaccine strain (Alpha variant (B.1.1.7) "English variant"). Results from the specific IgG response *vs.* the vaccine strain (Wuhan-Hu-1 strain) showed a significant increase in the MF-59 vaccine group *vs.* the Alum vaccine. A study carried out by Boudreau *et al* on the H5N1 avian influenza vaccine administered with MF59 and/or with alum showed a strong IgG response when MF59 was used as an adjuvant in comparison with the Alum vaccine [31]. In addi-

tion, a study on healthy and diabetic mice showed that immunization with inactivated whole-virion H5N1-MF59 vaccine enhanced the antibody responses in both healthy and diabetic mice, but the MF59-adjuvanted vaccine group showed a significant increase in IgG responses as compared with the Alum-adjuvanted group [32].

In a study conducted by Bai et al., the inactivated SARS-CoV-2-MF59 vaccine was demonstrated to induce more robust humoral immune responses as compared with the Alum adjuvant [33].

Results from these studies confirm our findings about a higher IgG response in the MF59-adjuvanted vaccine. In addition, results from IgG1 and IgG2a isotypes showed a significant increase in the SARS-CoV-2-MF59 vaccine *vs.* the Alum vaccine. It is proven that each isotype of IgG has a critical role on the bioactivity of the humoral immune response and sometimes one special isotype of IgG is correlated with protection in a host. Considering that each isotype of IgG has a distinct function, it is acceptable that poly-isotypic response is an advantage in the humoral immune response and higher IgG1 and IgG2a responses in the SARS-CoV-2-MF59 vaccine group may reflect a more potent humoral immune response occurring in this group [25,31]. An antibody response is one of the key mechanisms in the protection and prevention of infections in the body through several immunologic mechanisms. Antibodies are able to bind to the virus particles and neutralize and block the viral entry. In addition, antibodies are able to trigger viral elimination through activation of NK cells, macrophages and complement cascade.

Therefore, monitoring antibody response is an important aspect of understanding the scope of immunity. It is also one of the key factors for a successful vaccine development and also cross-protection against new variants of a virus strain [34-37]. Therefore, we assessed cross-binding response of IgG *vs.* non-vaccine strain. ELISA for cross-binding IgG response *vs.* non-vaccine strain [Alpha variant (B.1.1.7) "English variant"] showed a numerically higher in the specific IgG titer for the SARS-CoV-2-MF59 vaccine group *vs.* inactivated SARS-CoV-2-Alum group. This finding may show the potency of the MF59 adjuvant in the induction of higher cross-binding IgG response. However, testing additional variants would be necessary before making broader claims about heterologous breadth. We could not do this because of limitations in access to other variants, and this is a limitation of this study.

In addition, we couldn't confirm cross-neutralization of humoral immune response and this is another limitation of the study. A study performed by Khurana *et al* showed that the MF59-H5 influenza vaccine in a prime-boost vaccination promotes a broad H5N1 cross-clade neutralization antibody and it seems affinity maturation of the humoral response has a critical role in this effect [38]. Bai *et al* in a study in mice showed that the MF59-like adjuvanted vaccine induced higher cross-reactive T cell responses against the SARS-CoV-2 prototype and new variants than the Alum adjuvant. They concluded that vaccine adjuvant strategy is a rational strategy against SARS-CoV-2 and variants of concern in older adults [33]. This judgment is based on the cross-reactive property of their vaccine when it was formulated in the MF59 adjuvant, suggesting the potency of MF59 adjuvant in the induction of cross-reactive response [33]. Frey *et al* in a study showed the potential of an adjuvanted vaccine to provide cross-protection to novel viruses. They used MF59 in the inactivated H5N1 influenza vaccine as a model and the results revealed the potential of the inactivated H5N1 influenza-MF59 vaccine in providing protection against other H5N1 strains [39]. These reports support the claim that MF59 adjuvant could potentially be the best insurance against strain diversity of pathogens and this can be achieved by induction of cross-binding immune response [40]. This property of an adjuvant is very important in the induction of cross-reactive immune response and thereby mitigating the new wave of infections, which can arise from newly mutated variants of a virus. However, we believe that more studies are needed to prove this claim, especially the cross-neutralization test, which is a serious limitation of this study.

Conclusion

In this mouse model, the MF59-formulated vaccine enhanced selected immunogenicity endpoints, including lymphocyte proliferation, IFN-γ-associated responses, and IgG1, and IgG2a antibody responses, compared with the Alum formulation. Both formulations induced binding IgG responses to the Alpha variant; however, inactivated SARS-CoV-2 formulated in MF59 adjuvant led to a numerically higher cross-binding IgG response for Alpha variant. These findings showed that vaccine formulation may be important in inducing cross-binding humoral responses. Because neutralization and challenge assays were not performed, these findings cannot be interpreted as evidence of cross-neutralization or protection.

Acknowledgement

We thank Dr. Louiza Chitour, former Regional Sales & Marketing Manager from the Business Unit of SEPPIC Company for providing MF59 adjuvant as a gift. We also thank the staffs of Vaccine Formulation Laboratory of Razi Vaccine and Serum Research Institute of Iran for vaccine formulation. All the experiments on the mice were carried out in accordance with the Creature Care and Use Convention of Razi Vaccine and Serum Research Institute of Iran and also the Ethics Committee of Semnan University of Medical Sciences of Iran (Ethics code: IR.SEMUMS.REC.1400.338).

Conflict of 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.

References

1. Alizon S, Sofonea MT. SARS-CoV-2 epidemiology, kinetics, and evolution: A narrative review. Virulence 2025;16(1):2480633.
2. Zhao F, Xia W, Xu Y, Wu X, Hu S, Zhang JZH. Predicting Nirmatrelvir Resistance in SARS-CoV-2 M(pro) Mutants with an Integrated Computational Framework. J Phys Chem B 2026;130(28):7096-112.
3. Sankaranarayanan S, Mohkhedkar M, Janakiraman V. Mutations in spike protein T cell epitopes of SARS-COV-2 variants: Plausible influence on vaccine efficacy. Biochim Biophys Acta Mol Basis Dis 2022;1868(9): 166432.
4. Zhao R, Zang W, Xing H, Huang Y, Lu M. Engineered delivery systems for vaccine adjuvants. Int Immunopharmacol 2026;180:116720.
5. Shao F, Zhu X, Yi M, Gao H, Wu J, Fang R, et al. TLR agonists as adjuvants for viral vaccines: mechanisms, applications, and future directions. Front Microbiol 2025; 16:1740572.
6. Nan L, Zhang X, Ziyi L, Li S, Xia N. Montanide ISA-51: a promising adjuvant in cancer vaccine immunotherapy. Expert Rev Vaccines 2025;24(1):958-71.
7. Hojecki CE, Tursi NJ, Livingston C, Weiner DB, Gary EN. Advances in molecular adjuvants for nucleic acid vaccines. Front Immunol 2025;16:1646800.
8. Zhang W, Cui H, Xu J, Shi M, Bian L, Cui L, et al. Biodistribution and mechanisms of action of MF59 and MF59-like adjuvants. J Control Release 2025;378:573-87.
9. Liu X, Gong S, Xu J, Wei Y, Chen X, Wu Y, et al. Effect of Different Adjuvants on the Immunogenicity of a Recombinant Herpes Zoster Vaccine in Mice, Rats and Non-Human Primates. Vaccines (Basel) 2025;13(11): 1124.
10. Seubert A, Monaci E, Pizza M, O'Hagan DT, Wack A. The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. J Immunol 2008;180(8):5402-12.
11. Xu K, Li J, Lu X, Ge X, Wang K, Wang J, et al. The Immunogenicity of CpG, MF59-like, and Alum Adjuvant Delta Strain Inactivated SARS-CoV-2 Vaccines in Mice. Vaccines (Basel) 2024;12(1):60.
12. Haghighi M, Khorasani A, Karimi P, Keshavarz R, Mahdavi M. Different Formulations of Inactivated SARS-CoV-2 Vaccine Candidates in Human Compatible Adjuvants: Potency Studies in Mice Showed Different Platforms of Immune Responses. Viral Immunol 2022;35 (10):663-72.
13. Yamaya A, Sonoda K, Kawakita T, Koshiishi Y, Shingai M, Matsumoto M, et al. ARNAX, but not conventional adjuvants, alum and squalene, induces antigen-specific CD8+ T cell proliferation in vaccination with influenza split vaccine. Int Immunopharmacol 2025;159:114895.
14. Bettini E, Locci M. SARS-CoV-2 mRNA vaccines: immunological mechanism and beyond. Vaccines (Basel) 2021;9(2):147.
15. Bekker LG, Ntusi NAB. Lessons from two SARS-CoV-2 waves in South Africa. Lancet Glob Health 2021;9(9): e1177-e8.
16. Thakur S, Sasi S, Pillai SG, Nag A, Shukla D, Singhal R, et al. SARS-CoV-2 Mutations and Their Impact on Diagnostics, Therapeutics and Vaccines. Front Med (Lausanne) 2022;9:815389.
17. Liu Y, Liu J, Zou J, Kalveram B, Machado RRG, Ren P, et al. Cross-neutralization and cross-protection among SARS-CoV-2 viruses bearing different variant spikes. Signal Transduct Target Ther 2022;7(1):285.
18. Menezes F, Chen S, Grunenberg N, Malahleha M, Mngadi K, Muyoyeta M, et al. AS01(B)-, MF59-, and alum-based adjuvants and HIV vaccine immunogenicity: a post-hoc cross-protocol comparison of results from HVTN 100, 107, 120, and 702. Front Immunol 2026;17: 1768201.
19. Wu L, Yang R, Wu H, Yang B, Zhang X, Tao Y, et al. Comparison of Adjuvant Potency of Alum, AddaVax, and ISA 71 VG on the Seasonal Split Influenza Vaccine in Mice. Microorganisms 2025;13(11):2542.
20. Kaffashi A, Huang J, Bairami A, Fallah Mehrabadi MH, Yaslianifard S, Bashashati M, et al. Complete genome sequencing and molecular characterization of SARS-COV-2 from COVID-19 cases in Alborz province in Iran. Heliyon 2021;7(9):e08027.
21. Banihashemi SR, Es-Haghi A, Fallah Mehrabadi MH, Nofeli M, Mokarram AR, Ranjbar A, et al. Safety and Efficacy of Combined Intramuscular/Intranasal RAZI-COV PARS Vaccine Candidate Against SARS-CoV-2: A Preclinical Study in Several Animal Models. Front Immunol 2022;13:836745.
22. Kalanaky S, Fakharzadeh S, Karimi P, Hafizi M, Jamaati H, Hassanzadeh SM, et al. Nanoadjuvants Produced by Advanced Nanochelating Technology in the Inactivated-Severe Acute Respiratory Syndrome Coronavirus-2 Vaccine Formulation: Preliminary Results on Cytokines and IgG Responses. Viral Immunol 2023;36(6):409-23.
23. Sepasi A, Ghafourian M, Taghizadeh M, Mahdavi M. Formulation of Recombinant H1N1 Hemagglutinin in MF59 and Alum Adjuvants: A Comparison of the Vaccines Potency and Efficacy in BALB/C Mice. Viral Immunol 2023;36(6):401-8.
24. Mahdavi M, Mavandadnejad F, Yazdi MH, Faghfuri E, Hashemi H, Homayouni-Oreh S, et al. Oral administration of synthetic selenium nanoparticles induced robust Th1 cytokine pattern after HBs antigen vaccination in mouse model. J Infect Public Health 2017;10(1):102-9.
25. Velashjerdi Farahani S, Reza Aghasadeghi M, Memarnejadian A, Faezi S, Shahosseini Z, Mahdavi M. Naloxone/alum mixture a potent adjuvant for HIV-1 vaccine: induction of cellular and poly-isotypic humoral immune responses. Pathog Glob Health 2016;110(2):39-47.

26. Bodas-Pinedo A, Lafuente EM, Pelaez-Prestel HF, Ras-Carmona A, Subiza JL, Reche PA. Corrigendum: Combining different bacteria in vaccine formulations enhances the chance for antiviral cross-reactive immunity: a detailed in silico analysis for influenza A virus. Front Immunol 2023;14:1284628.
27. Wack A, Baudner BC, Hilbert AK, Manini I, Nuti S, Tavarini S, et al. Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice. Vaccine 2008;26(4):552-61.
28. Ou H, Yao H, Yao W, Wu N, Wu X, Han C, et al. Analysis of the immunogenicity and bioactivities of a split influenza A/H7N9 vaccine mixed with MF59 adjuvant in BALB/c mice. Vaccine2016;34(20):2362-70.
29. Takahama S, Yoshio S, Masuta Y, Murakami H, Sakamori R, Kaneko S, et al. Hepatitis B surface antigen reduction is associated with hepatitis B core-specific CD8(+) T cell quality. Frontiers Immunol 2023;14: 1257113.
30. Jung KI, McKenna S, Vijayamahantesh V, He Y, Hahm B. Protective versus Pathogenic Type I Interferon Responses during Virus Infections. Viruses. 2023;15(9): 1916.
31. Boudreau CM, Yu WH, Suscovich TJ, Talbot HK, Edwards KM, Alter G. Selective induction of antibody effector functional responses using MF59-adjuvanted vaccination. J Clin Invest 2020;130(2):662-72.
32. Wu J, Wang F, Fang F, Zhang W, Chang H, Zheng L, et al. Superior protection provided by a single dose of MF59-adjuvanted whole inactivated H5N1 influenza vaccine in type 1 diabetic mice. Arch Virol 2011;156 (3):387-95.
33. Bai S, Kang Y, Chen W, Xie H, Zhang L, Lv M, et al. Comparison of Immunogenicity of Alum and MF59-Like Adjuvant Inactivated SARS-CoV-2 Vaccines Against SARS-CoV-2 Variants in Elderly Mice. Viral Immunol 2023;36(8):526-33.
34. Alzaabi AH, Ahmed LA, Rabooy AE, Zaabi AA, Alkaabi M, AlMahmoud F, et al. Longitudinal changes in IgG levels among COVID-19 recovered patients: A prospective cohort study. PLoS One 2021;16(6):e0251159.
35. Masood KI, Qaiser S, Abidi SH, Khan E, Mahmood SF, Hussain A, et al. Humoral and T cell responses to SARS-CoV-2 reveal insights into immunity during the early pandemic period in Pakistan. BMC Infect Dis 2023;23 (1):846.
36. Alrubayyi A, Touizer E, Hameiri-Bowen D, Charlton B, Gea-Mallorquí E, Hussain N, et al. Natural killer cell responses during SARS-CoV-2 infection and vaccination in people living with HIV-1. Sci Rep 2023;13(1):18994.
37. Cao Y, Yisimayi A, Bai Y, Huang W, Li X, Zhang Z, et al. Humoral immune response to circulating SARS-CoV-2 variants elicited by inactivated and RBD-subunit vaccines. Cell Res 2021;31(7):732-41.
38. Khurana S, Coyle EM, Dimitrova M, Castellino F, Nicholson K, Del Giudice G, et al. Heterologous prime-boost vaccination with MF59-adjuvanted H5 vaccines promotes antibody affinity maturation towards the hemagglutinin HA1 domain and broad H5N1 cross-clade neutralization. PLoS One 2014;9(4):e95496.
39. Frey SS, Versage E, Van Twuijver E, Hohenboken M. Antibody responses against heterologous H5N1 strains for an MF59-adjuvanted cell culture-derived H5N1 (aH5n1c) influenza vaccine in adults and older adults. Hum Vaccin Immunother 2023;19(1):2193119.
40. O'Hagan DT, Rappuoli R, De Gregorio E, Tsai T, Del Giudice G. MF59 adjuvant: the best insurance against influenza strain diversity. Expert Rev Vaccines 2011;10(4): 447-62.