Different formulations of inactivated SARS-CoV-2 vaccine candidates in human compatible adjuvants: Potency studies in mice showed different platforms of immune responses

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Abstract

Several inactivated SARS-CoV-2 vaccines were approved for human use but are not highly potent. Here, different formulations of inactivated SARS-CoV-2 virus in Alum, Montanide 51VG and Montanide ISA720VG adjuvants were developed and then immune responses were assessed. SARS-CoV-2 virus was inactivated with formalin and formulated in the adjuvants. BALB/c mice were immunized subcutaneously with 4µg of experimental vaccines on days 0 and 14 and two weeks after the final immunization, IL-4 and IFN-γ cytokines, CTL activity and specific IgG titer and IgG1, IgG2a, IgG2a/IgG1 ratio and also anti-RBD IgG response were assessed. Immunization with SARS-CoV-2-Montanide ISA51VG showed a significant increase in IFN-γ cytokine versus SARS-CoV-2-Alum, SARS-CoV-2-Montanide ISA720VG and control groups ($P<0.0033$). Cytokine IL-4 response in SARS-CoV-2-Alum group showed a significant increase versus SARS-CoV-2-Montanide ISA51VG, SARS-CoV-2-Montanide ISA720VG and control groups ($P<0.0206$). In addition, SARS-CoV-2-Montanide ISA51VG vaccine induced the highest IFN-γ/IL-4 cytokine ratio versus other groups ($P<0.0004$). CTL activity in SARS-CoV-2-Montanide ISA51 VG and SARS-CoV-2-Montanide ISA720 VG groups showed a significant increase versus SARS-CoV-2-Alum and control groups ($P<0.0075$). Specific IgG titer in SARS-CoV-2-Montanide ISA51 VG and SARS-CoV-2-Montanide ISA720VG showed significant increase versus SARS-CoV-2-Alum and control groups ($P<0.0143$). Results of specific IgG1 and IgG2a level in SARS-CoV-2-Alum, SARS-CoV-2-Montanide ISA51VG and SARS-CoV-2-Montanide ISA720VG vaccine showed a significant increase versus the control group ($P<0.0001$) but SARS-CoV-2-Montanide ISA51VG and SARS-CoV-2-Montanide ISA 720VG groups showed highest IgG2a/IgG1 ratio and a significant increase versus SARS-CoV-2-Alum group ($P<0.0379$). Results of anti-RBD IgG response showed that inactivated SARS-CoV-2+Alum and SARS-CoV-2-Montanide ISA 720VG vaccine groups demonstrated a significant increase versus SARS-CoV-2-Montanide ISA51VG group.

It seems that the type of vaccine formulation is a critical parameter that effect on immunologic patterns and vaccine potency. Here, results showed that human compatible oil-based adjuvants were more potent than Alum adjuvant in the induction of cellular and humoral immune responses versus SARS-CoV-2 virus.

Introduction

The COVID-19 pandemic started in December 19 in Wuhan (China). The new SARS-CoV-2 is the seventh member of the Coronaviridae of the order Nidovirales with RNA genome that infects humans and mammals. The World Health Organization named the epidemic a Public Health Emergency of Global Importance in January 2020 and a pandemic in March 2020 (Jeyanathan, Afkhami et al. 2020).

Vaccination is the most effective way to prevent viral infections and it seems that it is important to have a global vaccination to prevent the pandemic (Mahdavi, Ebtekar et al. 2011). SARS CoV-2 transmission occurs with high effectiveness and infectivity, as with other respiratory viruses, predominantly along the respiratory pathway. The most known route is droplet delivery, while aerosols can constitute another significant route (Ciotti, Ciccozzi et al. 2020). As well, financial problem, lost personal freedom and widespread emotional distress caused the increased of mental problems (Pfefferbaum and North 2020).

Thus, different countries and governments have been paid a high price to prevent the spread and treatment of this disease and also development an efficient vaccine (Wang, Shi et al. 2006). Analysis of the genomes and comparing with the previously identified genomes of coronaviruses show that SARS-CoV-2 has distinct characteristics that separate it from the other coronaviruses (Nao, Yamagishi et al. 2017, Andersen, Rambaut et al. 2020). It is believed that SARS-CoV-2 is able to transmit to another species (Shi, Wang et al. 2020). It is clear that both humoral and cellular immune responses are needed to combat and clear the viral infections, so these properties for a successful vaccine should be considered in the vaccine design and also vaccine formulation (Jamali, Mahdavi et al. 2007, Zhu, Li et al. 2020). Different platform of SARS-CoV-2 vaccines are developed such as mRNA vaccine, DNA vaccine, viral vectors, recombinant vaccine and inactivated vaccines which some of these platforms are commercialized and are using in different countries (Frederiksen, Zhang et al. 2020, Kaur and Gupta 2020). Vaccine formulation with adjuvants and other excipients is so critical and effect on the vaccine potency. And these factors influence on vaccine stability and also the type of triggered immune responses which in turn effect on the vaccine efficacy (Fakharzadeh, Kalanaky et al. 2013, Rostami, Ebtekar et al. 2017, Mojarab, Shahbazzadeh et al. 2020). One of the commercialized vaccines for COVID-19 is inactivated viral particles which now is using in different countries. Naturally, immunogenicity and potency of inactivated virus is low and formulation in an adjuvant makes it more immunogen (Hotez, Corry et al. 2020, Logunov, Dolzhikova et al. 2020, Wang, Peng et al. 2020).

In this study, we developed different formulations of inactivated SARS-CoV-2 viral particles with different classes of adjuvants and then cellular and humoral immune responses against each formulation of vaccine was assessed in the experimental mice. We have shown that different formulations of vaccine triggered different levels and platforms of immune responses.

Materials And Methods

Virus isolation, propagation

SARS-CoV-2 virus was isolated from throat/nasal swab sample of a patient (positive in real time PCR) from Karaj, Iran (SARS-CoV-2/human/IRN/Alborz-IR/2020 surface glycoprotein (S) gene, complete cds 3,822 bp linear RNA MW709393.1 GI: 1998262910). The virus replication was performed on Vero cell line which were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS) and then confirmed by real-time PCR and gene sequencing (MW709393). For efficient growth in Vero cells, the virus strain was purified by plaque assay. The first purified clone passaged three times on Vero cells to obtain an efficient virus stock. This stock virus shows more efficiency with more than 90% CPE in 48 hours post infection and a titer 6.5 to 7 TCID₅₀/ml. The SARS-CoV-2 propagation carried out in biosafety level-III (BSL-III) facilities.

Virus inactivation and quantification

A SARS-CoV-2 strain was cultivated in Vero cell line for propagation, and the supernatant of the infected cells was inactivated with formalin 0.04% vol/vol at RT for 30 hours (finally 4:10000). Following

clarification of cell debris concentration were performed by ultrafiltration and 8% PEG-6000. Purification performed using column chromatography. The purified and inactivated viruses dialyzed versus PBS and passed through 0.22 filter and finally quantified using Bradford method. The quantified virus aliquots stored at -70 °C until use.

Vaccines formulation

Inactivated SARS-COV-2 virus was formulated in Montanide ISA 720VG, Montanide ISA51VG (SEPPIC, France), and /or Alum hydroxide (Pasteur Institute of Iran) adjuvants. In order to formulate in oil-based adjuvants, inactivated SARS-COV-2 virus in PBS buffer was admixed with Montanide ISA 720VG (at the ratio of 30/70) and Montanide ISA 51VG (at the ratio of 50/50) by vigorously shaking to develop a milky white suspension and homogenized using homogenizer to develop a homogen suspension. In order to formulate Alum-based vaccine, 4µg of inactivated SARS-COV-2 virus in PBS buffer was mixed with 200µg of Alum hydroxide adjuvant (for each dose of vaccine formulation) and shaken at 100 RPM for 60 minutes at room temperature and allowed that the viral particles adsorb on the surface of Alum gel. All vaccine formulations were performed in the clean room of Department of FmD Vaccine formulation of Razi Vaccine and Serum Institute of Iran (Karaj, Iran). After final vaccine formulation, each 200 µl of all vaccines contained 4µg of inactivated SARS-COV-2 virus that used for immunization.

Mice and immunization

Six-to eight-week-old male BALB/c mice (N=40) were purchased from Royan Institute of Iran (Tehran, Iran). The mice were housed for one week before the experiments, given free access to the food and water and maintained in a 12 hrs light\and 12 hrs dark cycles. The experimental mice were handled with an expert technician and in accordance with the Animal Care and Use Protocol of Razi Vaccine and Serum Research Institute of Iran. Experimental mice were randomly divided into four groups and each one consisting of 10 mice as below:

Group 1

Mice were immunized subcutaneously with 4µg of inactivated SARS-COV-2-Alum vaccine.

Group 2

Mice were immunized subcutaneously with 4µg of inactivated SARS-COV-2-Montanide ISA-51VG vaccine.

Group 3

Mice were immunized subcutaneously with 4µg of inactivated SARS-COV-2-Montanide ISA-720VG vaccine.

Group 4

Mice were immunized with PBS as control group.

Experimental groups of mice were immunized subcutaneously two times with 14-day-intervals and two weeks after the final immunization immunologic parameters were assessed.

In vitro antigen recall of spleen cells

Two weeks after the second immunization, the spleen of the immunized mice was removed under sterile conditions and mechanically dissected and suspended in the sterile cold PBS containing penicillin and streptomycin. The cell suspension centrifuged at 300 g/ 5min and RBCs were lysed using lysis buffer and single-cell suspension was washed three times and adjusted to 3×10^6 cells/ml in RPMI-1640 (Gibco, Germany) supplemented with 10% FBS, 4 mM L-glutamine, 1 mM sodium pyruvate, 100 µg/ml streptomycin and 100 IU/ml penicillin. One milliliter of cell suspension containing a total number of 3×10^6 spleen cells were seeded in each well of 24-well plates. The spleen cells were then stimulated in vitro with adding 1 µg/ml of inactivated SARS-COV-2 virus and the plates incubated at 37°C in 5% CO₂. After 48 hrs post antigen recall, the culture supernatant were centrifuged at 5000 RPM/10 min and the supernatants were harvested and stored at -70°C for cytokines assay.

IFN-γ and IL-4 cytokines ELISA

The quantity of IFN-γ and IL-4 cytokines was estimated by quantitative mouse IFN-γ and IL-4 cytokine ELISA Kits (Mabtech, Stockholm, Sweden) according to the manufacturer's instruction. The ELISA of IFN-γ and IL-4 cytokines were performed in the presence of cytokines standards and using the standard curve of each cytokine. The pg/ml of each sample was reported and also for calculating the IFN-γ/IL-4 ratio, the quantity of IFN-γ of each mouse was divided into that one of IL-4 cytokine.

CTL assay

The CTL activity of experimental groups was assessed by Granzyme B (Gr-B) release. Briefly, 1.5×10^6 spleen cells of experimental mice were cultured in 96-well plate and stimulated with 1 µg/ml of inactivated SARS-COV-2 virus. As negative control for each mouse, some wells were cultured without antigen stimulation. The plates were incubated at 37°C in 5% CO₂ and after 48 hrs of antigen stimulation; culture supernatants were harvested and were used for Gr-B assay by ELISA according to the manufacturer's instruction (eBioscience, USA). The quantity of Gr-B of stimulated wells was subtracted with unstimulated one of each mouse and used as net Gr-B release as a criterion of CTL activity.

ELISA of specific IgG antibodies titer and their isotypes

Specific antibodies were determined by an optimized indirect ELISA method. Briefly, 100 µl of 5 µg/ml of inactivated SARS-COV-2 in PBS were added into 96-well ELISA Maxisorp plates (Greiner, Germany) and incubated overnight at 4°C. The wells washed with PBS containing 0.1% Tween 20 (washing buffer) and blocked 1 hr at 37°C with 2% skimmed milk in PBS + 0.1% Tween 20 (blocking buffer). The plates were washed with washing buffer and 100 µl of 1/25 diluted sera up to 16 serial dilutions were added into each wells and incubated at 37°C for 90 min. The wells washed five times with washing buffer and

incubated for 2 hrs with 100 µl of 1/8000 dilution of anti-mouse conjugated to HRP (Razirad, Iran). The wells washed five times and incubated 30 min with 100 µl of TMB substrate in the dark and the reaction was stopped with 100 µl of 2N H₂SO₄ and colour density was measured at 450 nm with ELISA plate reader. The cut off of ELISA for each titer was calculated on 10 sera samples of sham group by formula: Mean+3SD of these samples and based on the cut off the titer of each mouse of the experiments was earned. The final results of antibody titer for each mouse presented as Log₁₀ of antibody titer. In addition, detection of specific IgG1 and IgG2a subclasses were carried out using goat anti-mouse IgG1 and IgG2a secondary antibodies (Sigma, USA) according to the manufacture's instruction.

Assessment of specific IgG anti-RBD protein

The specific IgG antibody response against RBD protein was determined by a laboratory-optimized indirect ELISA. At the first for antigen coating, 100 µl of 2µg/ml of RBD protein (The Native Antigen Company, UK) in carbonate-bicarbonate buffer with pH 9.6 was added into 96-well ELISA Maxisorp plates (Greiner, Germany) and incubated overnight at 4°C. The wells were washed with PBS containing 0.1% tween 20 (washing buffer) and then blocked 1 hr at 37°C with 1.5% BSA in PBS + 0.05% tween 20 (blocking buffer). The plates were washed 5 times with washing buffer and 100 µl of 1/50 diluted sera of experimental vaccinated and sham group of mice were added into each well and incubated at 37°C for 90 min. The wells were washed 5 times with washing buffer and incubated for 90 min with 100 µl of 1/8000 dilution of anti-mouse HRP-conjugate (Razirad, Iran). The wells washed 5 times and incubated 10min with 100 µl of TMB substrate in the dark and the reaction was stopped by adding 100 µl of 2N HCL and colour density was measured at 450 nm with ELISA plate reader. The cutoff of RBD-ELISA was calculated on the sera samples of sham group by formula: Mean+3SD and then IgG response to RBD for individual mouse was reported by formula; OD of RBD ELISA of individual mouse/cutoff.

Statistical analysis

The data were presented as mean ± standard deviation (SD) of each experiment. The statistical significance of differences was analysed using two-way ANOVA followed by Tukey test (Graph Pad Prism 6.01 software, Graph Pad Software, La Jolla, CA). Statistical analysis of antibody titers and IgG isotypes were carried out by the Mann-Whitney U test. In all of the cases, P values less than 0.05 was considered significant difference.

Results

IFN-γ cytokine

The results of IFN-γ cytokine in the experimental groups show that only the injection of SARS-COV-2 vaccine formulated in Montanide ISA51VG adjuvant resulted to a significant difference as compared with the control group (P= 0.0003). In addition, immunization with vaccine-Montanide ISA51VG showed a significant increase in IFN-γ response as compared with the vaccines that formulated in Alum and Montanide ISA 720VG adjuvants (P=0.0004 and P=0.0033, respectively) (Fig. 1).

IL-4 cytokine

Immunization with SARS-COV-2-Alum vaccine and SARS-COV-2-Montanide ISA 51VG vaccine showed a significant increase in IL-4 cytokine secretion versus control group ($P=0.0001$ and $P=0.0288$, respectively). In different vaccine formulations, immunization with SARS-COV-2-Alum vaccine showed a significant increase of IL-4 cytokine response in comparison with SARS-COV-2-Montanide ISA 51VG and SARS-COV-2-Montanide ISA 720VG vaccines ($P=0.0206$ and $P=0.0005$, respectively) (Fig. 2).

IFN- γ /IL-4 cytokine ratio

The results of IFN- γ /IL-4 cytokine ratio in the experimental groups revealed that immunization with SARS-COV-2-Montanide ISA51VG vaccine induced the highest IFN- γ /IL-4 cytokine ratio and in comparison with SARS-COV-2-Alum vaccine, SARS-COV-2-Montanide ISA 720VG and control groups showed significant differences ($P=0.0001$, $P=0.0004$ and $P=0.0001$, respectively) (Fig. 3).

CTL response

The results of CTL activity based on Gr-B secretion in the mice which immunized with SARS-COV-2-Montanide ISA51VG, SARS-COV-2-Alum and SARS-COV-2-Montanide ISA 720VG vaccines showed a significant increase versus control group ($P=0.0001$, $P=0.0017$ and $P=0.0001$, respectively). In addition, immunization with SARS-COV-2-Montanide ISA51VG and SARS-COV-2-Montanide ISA 720VG vaccines showed a significant increase in comparison with SARS-COV-2-Alum group ($P=0.0001$ and $P=0.0075$, respectively). But no significant difference in CTL activity was observed between SARS-COV-2 Montanide ISA51VG and SARS-COV-2 Montanide ISA720VG groups ($P=0.2628$) (Fig. 4).

Specific IgG titer

The results of specific IgG titer in the experimental groups demonstrated that immunization with SARS-COV-2-Montanide ISA51VG, SARS-COV-2-Alum and SARS-COV-2-Montanide ISA 720VG vaccines showed a significant increase versus control group ($P<0.0001$). Furthermore, immunization with SARS-COV-2-Montanide ISA51VG and SARS-COV-2-Montanide ISA 720VG vaccines induced a significant increase of IgG titer in comparison with SARS-COV-2-Alum group ($P=0.0143$ and $P=0.0048$, respectively). There was no significant difference in specific IgG titer between SARS-COV-2-Montanide ISA51VG and SARS-COV-2-Montanide ISA720VG groups ($P=0.6867$) (Fig. 5).

Specific IgG1 antibody

The results of specific IgG1 isotype demonstrated that immunization with SARS-COV-2-Montanide ISA51VG, SARS-COV-2-Alum and SARS-COV-2-Montanide ISA 720VG vaccines showed a significant increase versus control group ($P<0.0001$). The group immunized with SARS-COV-2-Alum induced a significant increase of IgG1 isotype in comparison with SARS-COV-2-Montanide ISA51VG and SARS-COV-2-Montanide ISA 720VG vaccines ($P=0.0043$ and $P=0.0041$, respectively). There was no significant

difference in specific IgG1 response between SARS-COV-2 Montanide ISA51VG and SARS-COV-2 Montanide ISA720VG groups ($P=0.4488$) (Fig. 6).

Specific IgG2a antibody

The results of specific IgG2a isotype antibody in the experimental groups demonstrated that immunization with SARS-COV-2-Montanide ISA51VG, SARS-COV-2-Alum and SARS-COV-2-Montanide ISA 720VG vaccines showed a small increase versus control group but no significant differences was observed among experimental groups ($P>0.1550$) (Fig. 7).

IgG2a/IgG1 ratio

The results of IgG2a/IgG1 ratio in the experimental groups demonstrated that immunization with SARS-COV-2-Montanide ISA51VG and SARS-COV-2-Montanide ISA 720VG vaccines resulted to a significant increase versus SARS-COV-2-Alum group ($P=0.0379$ and $P=0.0175$, respectively). While there was no significant difference in IgG2a/IgG1 ratio between SARS-COV-2-Montanide ISA51VG and SARS-COV-2-Montanide ISA720VG groups ($P=0.4458$) (Fig. 8).

Specific IgG antibodies against RBD protein

Results from anti-RBD IgG response demonstrated that immunization with SARS-COV-2-Montanide ISA51VG, SARS-COV-2-Alum and SARS-COV-2-Montanide ISA 720VG vaccines resulted in a significant increase versus control group ($P<0.0185$). In addition, immunization with inactivated SARS-COV-2+Alum and SARS-COV-2-Montanide ISA 720VG vaccines showed a significant increase versus SARS-COV-2-Montanide ISA51VG group ($P<0.0001$).

While SARS-COV-2-Alum and SARS-COV-2-Montanide ISA 720VG groups showed a comparable IgG response versus RBD protein and did not show a significant difference versus each other ($P=0.9999$) (Fig. 9).

Discussion

With emerging the pandemic of COVID-19 and high mortality rate and fast dissemination, much efforts was put into the development an efficient vaccine (Bos, Rutten et al. 2020). Finally several vaccines for COVID-19 have been developed by a variety of organizations and academic centers, ranging from the inactivated virus and protein-based vaccines to DNA and mRNA-based vaccines with high efficacy in human clinical trials. And some of these vaccines have been approved for human use. One important issue regarding the vaccine potency is the type of adjuvant in the vaccine formulation and its relation to the immunologic patterns and vaccine potency (Peng, Cao et al. 2020, Wang, Peng et al. 2020, Crommelin, Volkin et al. 2021).

Herein, we have developed three different formulations of inactivated SARS-COV-2 vaccines in human compatible adjuvants and focused on the nature of immune responses which triggered by each

formulation of vaccine to show the critical role of adjuvant and also vaccine formulation in the SARS-COV-2 vaccine model. Here, IFN- γ cytokine result shows that inactivated SARS-COV-2-Montanide ISA-51VG triggered the highest cytokine IFN- γ response as compared with the alum- and Montanide ISA-720VG-based vaccines. Several studies have shown the potency of Montanide ISA-51VG adjuvant in the polarization toward a strong IFN- γ and Th1 response (Atsmon, Kate-Ilovitz et al. 2012, Soema, van Riet et al. 2015, Savoji, Sereshgi et al. 2021) as we achieved in SARS-COV-2 vaccine model. The role of IFN- γ cytokine in the controlling viral infections is well known (Noh 2020). Infected patients with SARS-CoV-2 had a complex trend of IFN- α , medium levels of IFN- γ , and undetectable IFN- β and λ cytokines (Trouillet-Assant, Viel et al. 2020). Several studies have shown that vaccines that induced higher level of IFN- γ cytokine, was more protective (Bos, Rutten et al. 2020, Ramasamy, Minassian et al. 2021, Rasmussen, Kelley et al. 2021). Cytokine IL-4 response in the different vaccine formulations showed a strong and dramatic response in Alum-based vaccine and even higher than the oil-based vaccines. It is so-long that the potency of Alum-based vaccines in the induction of IL-4 cytokine response is well known and confirming Th2 response pattern (Bungener, Geeraedts et al. 2008, Serre, Mohr et al. 2010). While formulation of inactivated SARS-COV-2 virus in oil-based adjuvants suppressed IL-4 cytokine response and confirming Th1 pattern in Montanide adjuvanted groups. In addition, IFN- γ /IL-4 cytokine ratio representing the Th1 cytokine network (Maspi, Ghaffarifar et al. 2017, de la Cruz, Villanueva-Lizama et al. 2019) that considers cytokine Th1 pattern has also increased significantly in Montanide ISA51VG adjuvanted group versus Alum-and Montanide ISA720VG-formulated vaccines. And is another confirm for Th1 polarization in Montanide ISA51VG adjuvanted vaccine group.

In this study, we also used the secretion of Granzyme B as a marker of CTL activity. The results showed that inactivated SARS-COV-2 vaccine based on Montanide ISA-51 VG formulation showed the highest Gr-B release meaning the highest CTL activity among the experimental groups. Gr-B triggers a cascade of events within the target cells that infected with virus that lead to induction the apoptosis and cell death (Lord, Rajotte et al. 2003) as well as clearance of viral infection from the lungs (Doherty, Topham et al. 1997). It is well-known that early decrease in TCD8⁺ population is an important factor in the pathogenesis of COVID-19 infection and improvement of TCD8⁺ lymphocyte response in the vaccination would be an important mechanism for resistance against COVID-19 infection (Elsaesser, Sauer et al. 2009, Ganji, Farahani et al. 2020, Zheng, Zhang et al. 2020). Here, oil-based adjuvants were more successful than Alum-based vaccine in the induction of CTL response against SARS-COV-2 antigens.

In the next step of study we have focused on the humoral immune responses triggered by each formulation of vaccine. Results from IgG responses based on the end point in ELISA showed that immunization with SARS-COV-2-Montanide ISA-51VG and SARS-COV-2-Montanide ISA-720VG vaccines induced a significant increase in the SARS-COV-2 specific IgG titer in comparison with SARS-COV-2-Alum group. This property of oil-based vaccines is so critical because the basis of protection in COVID-19 infection relies mainly on the specific antibodies, however T cells response is important, too (Juno, Tan et al. 2020, Carrillo, Izquierdo-Useros et al. 2021).

Various studies have shown that oil-based adjuvanted vaccines are more potent in the induction of antibody response and even T cell responses than Alum-based vaccines (Khorasani, Madadgar et al. 2016, Savoji, Haghighat et al. 2019, Savoji, Sereshgi et al. 2021). Stronger humoral immune responses in the oil-based adjuvanted SARS-COV-2 vaccines versus Alum-based SARS-COV-2 vaccine may be due to the nature of the oil-based adjuvants, because Montanide family adjuvants are able to protect the antigen from denaturation by protease enzymes and free radicals at the injection site. These agents through denaturation the structure of protein decrease the vaccine potency. In addition, several studies have shown the potency of Montanide adjuvant family in the induction of follicular helper T cell that has critical role in the differentiation of plasma cells and humoral immune responses (Aucouturier, Dupuis et al. 2002, Riteau, Radtke et al. 2016, Savoji, Sereshgi et al. 2021).

In addition, IgG isotypes analysis showed that formulation of inactivated SARS-COV-2 virus in Alum adjuvant triggered highest IgG1 response while immunization with SARS-COV-2-Montanide ISA-51VG and SARS-COV-2-Montanide ISA-720VG vaccines showed a tiny increase in IgG2a response and a significant increase in IgG2a/IgG1 ratio versus Alum-based vaccine. Considering that IgG1 as a symbol of Th2 response and IgG2a and IgG2a/IgG1 ratio as a symbol of Th1 response (Mahdavi, Mavandadnejad et al. 2017, Mahdavi, Tajik et al. 2017), this finding shows the potency of oil-based vaccines in the polarization toward Th1 response and also a change in the type of IgG isotypes responses. Furthermore, due to the critical role of RBD protein in the attachment to ACE2 receptor of host cells, and anti-RBD IgG antibody which is considered as a potentially neutralizing factor against SARS-CoV-2 virus, in the next IgG response against RBD protein was assessed which potentially can show neutralization activity (Khoury, Cromer et al. 2021, Starr, Czudnochowski et al. 2021). Results show that inactivated SARS-COV-2+Alum and SARS-COV-2-Montanide ISA 720VG vaccine groups showed a comparable IgG response versus each other against RBD protein and an increase versus SARS-COV-2-Montanide ISA51VG group. This finding shows that Montanide-based vaccine is able to trigger anti-RBD response like as Alum-based vaccine but oil-based vaccines were superior to Alum-based vaccine in the induction of Th1 cytokine and CTL activity which is critical in the controlling SARS-COV-2 infection.

Conclusion

In conclusion the results of the present study provide evidences that the type of adjuvant in the vaccine formulation can modifies the type of immune responses and also potency of humoral immune responses. This finding encourages the SARS-COV-2 vaccinologists to focus on the different formulations of vaccine to achieve a more potent and safe vaccine especially for inactivated SARS-COV-2 based vaccines which has fewer efficacies than mRNA-based vaccines.

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Figures

Figure 1

The results of IFN- γ cytokine in the experimental groups. Immunization with SARS-COV-2-Montanide ISA-51VG shows a significant increase versus control group ($P=0.0003$). In addition, immunization with SARS-COV-2-Montanide ISA-51VG showed a significant increase in IFN- γ response as compared with SARS-COV-2-Alum and SARS-COV-2-Montanide ISA-720VG groups ($P=0.0004$ and $P=0.0033$, respectively).

Figure 2

Immunization with SARS-COV-2-Alum and SARS-COV-2-Montanide ISA-51VG vaccine showed a significant increase in IL-4 cytokine response versus control group ($P=0.0001$ and $P=0.0288$, respectively). Furthermore, immunization with SARS-COV-2-Alum vaccine showed a significant increase of IL-4 cytokine response in comparison with SARS-COV-2-Montanide ISA-51VG and SARS-COV-2-Montanide ISA-720VG vaccines ($P=0.0206$ and $P=0.0005$, respectively).

Figure 3

The results of IFN- γ /IL-4 cytokine ratio in the experimental groups. Immunization with SARS-COV-2-Montanide ISA-51VG vaccine showed a significant increase in IFN- γ /IL-4 cytokine ratio in comparison with SARS-COV-2-Alum, SARS-COV-2-Montanide ISA-720VG and control group ($P=0.0001$, $P=0.0004$ and $P=0.0001$, respectively).

Figure 4

The results of CTL activity based on Gr-B secretion in the mice. Immunization with SARS-COV-2-Montanide ISA-51VG, SARS-COV-2-Alum and SARS-COV-2-Montanide ISA-720VG vaccines showed a significant increase versus control group ($P=0.0001$, $P=0.0017$ and $P=0.0001$, respectively). In addition, immunization with SARS-COV-2-Montanide ISA51VG and SARS-COV-2-Montanide ISA 720VG vaccines showed a significant increase in comparison with SARS-COV-2-Alum group ($P=0.0001$ and $P=0.0075$,

respectively). But no significant difference in CTL activity was observed between SARS-COV-2 Montanide ISA-51VG and SARS-COV-2 Montanide ISA-720VG groups ($P=0.2628$).

Figure 5

The results of specific IgG titer in the experimental groups based on the end-point in ELISA. Immunization with SARS-COV-2-Montanide ISA-51VG, SARS-COV-2-Alum and SARS-COV-2-Montanide ISA -720VG vaccines showed a significant increase versus control group ($P<0.0001$). Furthermore, immunization with SARS-COV-2-Montanide ISA-51VG and SARS-COV-2-Montanide ISA-720VG vaccines demonstrated a

significant increase in specific IgG titer in comparison with SARS-COV-2-Alum group ($P=0.0143$ and $P=0.0048$, respectively). There was no significant difference in specific IgG titer between SARS-COV-2 Montanide ISA-51VG and SARS-COV-2 Montanide ISA-720VG groups ($P=0.6867$).

Figure 6

The results of specific IgG1 isotype antibody in the experimental groups. Immunization with SARS-COV-2-Montanide ISA-51VG, SARS-COV-2-Alum and SARS-COV-2-Montanide ISA-720VG vaccines showed a

significant increase versus control group ($P<0.0001$). The group immunized with SARS-COV-2-Alum showed a significant increase in IgG1 isotype response in comparison with SARS-COV-2-Montanide ISA-51VG and SARS-COV-2-Montanide ISA-720VG vaccines ($P=0.0043$ and $P=0.0041$, respectively).

Figure 7

The results of specific IgG2a isotype antibody in the experimental groups. Immunization with SARS-COV-2-Montanide ISA51VG, SARS-COV-2-Alum and SARS-COV-2-Montanide ISA 720VG vaccines showed a

small increase versus control group but no significant differences was observed among experimental groups ($P>0.1550$).

Figure 8

The results of IgG2a/IgG1 ratio in the experimental groups. Immunization with SARS-COV-2-Montanide ISA-51VG and SARS-COV-2-Montanide ISA-720VG vaccines resulted to a significant increase in IgG2a/IgG1 ratio versus SARS-COV-2-Alum group ($P=0.0379$ and $P=0.0175$, respectively). While there was

no significant difference in IgG2a/IgG1 ratio between SARS-COV-2 Montanide ISA51VG and SARS-COV-2 Montanide ISA720VG groups (P=0.4458).

Figure 9

Results from specific IgG antibodies against RBD protein which reported as ratio of OD of RBD ELISA of individual mouse/cutoff. Immunization with inactivated SARS-COV-2+Alum and SARS-COV-2-Montanide ISA 720VG vaccines showed a significant increase versus SARS-COV-2-Montanide ISA51VG group

($P < 0.0001$). While SARS-COV-2-Alum and SARS-COV-2-Montanide ISA 720VG groups showed a comparable IgG response in anti-RBD IgG response and did not show a significant difference versus each other ($P = 0.9999$).