Improvement of the inactivated SARS-CoV-2 vaccine potency through formulation in alum/naloxone adjuvant; Robust T cell and anti-RBD IgG responses

Melika Haghighi 1, 2*, Akbar Khorasani 3*, Pegah Karimi 1, 2, Mehdi Mahdavi 1, 2, 4*

1 Advanced Therapy Medicinal Product (ATMP) Department, Breast Cancer Research Center, Motamed Cancer Institute, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran
2 Recombinant Vaccine Research Center, Tehran University of Medical Sciences, Tehran, Iran
3 Department of FMD Vaccine Production, Razi Vaccine & Serum Research Institute, Agricultural Research, Education & Extension Organization (AREEO), Karaj, Iran
4 Immunotherapy Group, The Institute of Pharmaceutical Science (TIPS), Tehran University of Medical Science, Tehran, Iran

A R T I C L E  I N F O
Article type: Original

Article history:
Received: Feb 7, 2022
Accepted: Apr 27, 2022

Keywords:
Alum Adjuvant
Immune responses
Inactivated SARS-CoV-2 - virus
Naloxone
Vaccine formulation

A B S T R A C T
Objective(s): SARS-CoV-2, emerging as a major threat to public health, has to be controlled through vaccination. Naloxone (NLX), an opioid receptor antagonist, demonstrated its adjuvant activity for microbial vaccines. In this study, inactivated SARS-CoV-2 was developed in the Alum/NLX adjuvant to increase the potency of the inactivated SARS-CoV-2 vaccine.

Materials and Methods: BALB/c mice were immunized on days 0 and 14 with inactivated SARS-CoV-2-Alum, -Alum + NLX 3 mg/kg, -Alum + NLX 10 mg/kg, and -Freund adjuvant, as well as PBS. IFN-γ and IL-4 cytokines and Granzyme-B release were assessed with ELISA. In addition, specific total IgG, IgG1/IgG2a isotypes, and ratio as well as anti-RBD IgG responses were assessed with an optimized ELISA.

Results: SARS-CoV-2-Alum-NLX10 group showed a significant increase in the IFN-γ cytokine response versus SARS-CoV-2-Alum, SARS-CoV-2-Alum-NLX3, and PBS groups. The SARS-CoV-2-Alum-NLX3 group exhibited a significant decrease in IL-4 cytokine versus SARS-CoV-2-Alum. The mice immunized with SARS-CoV-2-Alum-NLX10 showed a significant increase in CTL activity versus SARS-CoV-2-Alum and PBS. In addition, mice immunized with SARS-CoV-2-Alum-NLX3, SARS-CoV-2-Alum-NLX10 and SARS-CoV-2-Freund demonstrated an increase in IgG response, as compared with SARS-CoV-2-Alum and PBS group. Furthermore, all formulations of SARS-CoV-2 vaccines could induce both IgG1 and IgG2a isotypes. But, the IgG2a/IgG1 ratio in SARS-CoV-2-Freund and SARS-CoV-2-Alum-NLX10 revealed an increase as compared with that of the SARS-CoV-2-Alum group.

Anti-RBD IgG response in the SARS-CoV-2-Alum-NLX10 group showed a significant increase as compared with the Alum-based vaccine.

Conclusion: Formulation of inactivated SARS-CoV-2 virus in NLX/alum adjuvant improved the potency of humoral and, especially, cellular responses.

Introduction
SARS-CoV-2, a novel coronavirus, has become a major concern for public health worldwide. The major sources of the disease are currently wild animal hosts and infected patients (1). The genome of the virus was sequenced by researchers in which 86.9% of the genome was the same as the SARS-CoV genome (2). The name was later changed to Corona Virus-2 Severe Acute Respiratory Syndrome (SARS-CoV-2) (3). The virus exhibits less pathogenesis but higher dissemination relative to diseases induced by a previously-identified human coronavirus (4). The global spread of SARS-CoV-2, mainly through respiratory droplets and direct contact, has led to development of several vaccines; however, there are doubts about the potency and safety of some currently-used vaccines (5-8). In order to combat the high transmission risk of a virus, it is crucial to identify the most appropriate targets for vaccine formulation. Spike, appearing to be the most appropriate target, is used in several vaccines as an immunogen (6). The vaccination strategy was successful in the prevention of some infectious diseases in communities (9, 10). Despite previous coronavirus epidemics, there is a need for a safe and effective vaccine capable of inducing protective and long-lasting immune responses (11, 12). In vaccine development, selection of an immunogen is critical to combat and eliminate the pathogen in a successful immune response. There is accumulating evidence suggesting that the spike protein, as a surface protein of the SARS-CoV-2 virus, is a suitable choice for vaccine development. Several studies demonstrated that humoral responses against the spike protein with neutralization activity are protective in the experimental infections as well as in the people who

*Corresponding author: Mehdi Mahdavi. ATMP Department, Breast Cancer Research Center, Motamed Cancer Institute, Tehran, Iran. NO.146, South Gandi Ave, Vanak Sq. Tehran, Iran; Recombinant Vaccine Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran; Immunotherapy Group, The Institute of Pharmaceutical Science (TIPS), Tehran University of Medical Science, Tehran, Iran. Tel/Fax: +98-21-88203915; Email: Mahdavivac@gmail.com

*These authors contributed equally to this work.
recovered from the infection (13, 14). Therefore, induction of humoral immune responses, based on the function of B lymphocytes, is a basis for the development of efficient vaccines.

Importantly, T cell responses are also important for the induction of other aspects of immune responses. Indeed, T cells can serve as a helper to improve the quality and quantity of humoral immune responses (13, 15, 16). One of the most important components of vaccines is adjuvants that influence the quantity, quality, and pattern of immune responses. In fact, adjuvants are molecules or compounds which have inherent immunomodulatory properties and effectively potentiate host antigen-specific immune responses, when administered in conjunction with an antigen (17, 18).

Naloxone (NLX), an opioid receptor antagonist approved by the FDA, is administered to people with opioid peptide-induced respiratory toxicity (19, 20). A variety of studies demonstrated the adjuvant activity of NLX for microbial vaccines (21, 22). It is demonstrated that NLX, alone or in combination with alum, is able to not only induce strong humoral immune responses but also improve Th1 and IFN-γ cytokine responses (18, 23, 24). NLX seems to improve the immunogenicity and efficacy of vaccines by improving the function of T cells (23, 24). Currently, several vaccines have been approved against COVID-19 infection and are being used in the populations that mainly focused on the neutralization antibodies against the spike protein. Although B lymphocytes are responsible for humoral immune responses, the role of T cells, as a helper for humoral immune responses, is not deniable.

The present study hypothesized that formulation of inactivated SARS-CoV-2 virus, with a modulating agent influencing T cell functions, may improve the quality and quantity of antibody responses. In this regard, the inactivated SARS-CoV-2 virus was prepared in the alum adjuvant and formulated with two doses of 3 and 10 mg/kg of NLX. After immunization of the experimental mice, different aspects of immune responses were analyzed.

Materials and Methods

**SARS-CoV-2 virus isolation, propagation, inactivation, and quantification**

A throat swab specimen was prepared from a patient who was positive in real-time PCR (Karaj, Alborz province, Iran) for the SARS-CoV-2 virus. In order to isolate the virus, the sample was transferred to the Vero cell-specific for Coronavirus (CCL-18) in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The virus strain was purified by the plaque assay and the first purified clone was passaged three times to obtain an efficient stock. The stock virus, with a modulating agent, was prepared in the alum adjuvant and formulated with two doses of 3 and 10 mg/kg of NLX. The final product of the virus was stored at -70 °C until use (24).

**Vaccine formulations**

The inactivated SARS-CoV-2 virus was used for vaccine formulation in alum hydroxide (Pasteur Institute of Iran) and Freund adjuvants (Sigma, USA; Complete and incomplete Freund adjuvants for the first and second immunizations, respectively). Briefly, 4 µg of the virus in PBS buffer was admixed with 200 µg of alum (for one dose vaccine formulation) and shaken at 100-110 RPM for one hour at room temperature to adsorb on the alum adjuvant. To add NLX to the vaccine formulation, one part of the alum-formulated vaccine was mixed with 200 µg of NLX for each dose (10 mg/kg), as an inactivated SARS-CoV-2 Alum-NLX10 vaccine; for another vaccine formulation, one part of the alum-formulated vaccine was mixed with 60 µg of NLX for each dose (3 mg/kg), as an inactivated SARS-CoV-2 Alum-NLX3 vaccine.

In addition, inactivated SARS-CoV-2 virus in PBS buffer was mixed with the Freund adjuvant (at v/v of 50/50) and homogenized using a homogenizer to achieve a homogenized suspension. In the end, 200 µl of each vaccine formulation contained 4 µg of the virus.

**Mice**

The male BALB/c mice (six- to 8-week-old, N=50) were provided from Royan Institute of Iran (Tehran, Iran). The mice (20 g body weight at the beginning of the study) were housed for 7 days before the immunization, and allowed access to food and drink ad libitum with 12-hr light/dark cycles. All mice handling, immunization, and sampling were in accordance with the Animal Care and Use Protocol of the Razi Vaccine and Serum Research Institute of Iran.

**Experimental groups and immunization**

The mice were randomly assigned to five experimental groups and each one consisted of 10 mice. Mice in groups 1–5 were immunized two times, subcutaneously on days 0 and 14 with 4 µg of inactivated SARS-CoV-2-Alum, inactivated SARS-CoV-2-Alum-NLX3 vaccine, inactivated SARS-CoV-2-Alum-NLX10 vaccine, and inactivated SARS-CoV-2-Freund adjuvant (25, 26), as well as PBS as a control group, respectively. Two weeks after the last immunization, cellular and humoral aspects of immune responses were assessed.

**Spleen cell culture and in vitro stimulation with inactivated SARS-CoV-2 virus**

Fourteen days after the last immunization, the spleens of the experimental mice were aseptically removed and dissected mechanically in sterile cold wash buffer (PBS + FBS 2%). The cell suspension was provided by vigorous pipetting and the samples were centrifuged at 300 g for 5 min, and RBCs were lysed using lysis buffer (0.16M ammonium chloride and 0.17M Tris base). After three-time washing, the cell suspension was adjusted to 3×10⁷ cells/ml in RPMI-1640 (Gibco, Germany) supplemented with 5% FBS, 1mM sodium pyruvate, 4mM L-glutamine, 100 µg/ml streptomycin, and 100IU/ml penicillin. The spleen cell suspension was adjusted to 3×10⁷ cells/ml and one milliliter was seeded into 24-well plates and stimulated with 1 µg/ml of inactivated SARS-CoV-2 virus for 48 hr at 37 °C in 5% CO₂. Afterward, the culture supernatant was harvested by
ELISA for IFN-γ and IL-4 cytokines

The supernatant from antigen recalled spleen cells was used for IFN-γ and IL-4 cytokines assay. Commercial ELISA Kits for mouse IFN-γ and IL-4 cytokines (Mabtech, Stockholm, Sweden) were used for the assay. ELISA for IFN-γ and IL-4 cytokines was performed according to the manufacturer's instructions. The quantity of the cytokines of each individual mouse was presented as pg/ml. In addition, the IFN-γ/IL-4 cytokine ratio of each mouse was calculated by dividing the IFN-γ to IL-4 from each mouse.

Cytotoxic T lymphocyte (CTL) activity

The CTL activity was measured by Granzyme B (Gr-B) release (12, 27). Briefly, 1.5x10^6 spleen cells in complete medium were cultured in 96-well plates and recalled with 0.2 µg of the inactivated SARS-CoV-2 virus. Some wells were considered without antigen as a negative control for each mouse and the total volume for each well was 200 µl. The plates were then incubated at 37 °C in 5% CO_2 for 48 hr and then the culture supernatants were harvested for Gr-B assay by commercial ELISA kits according to the company manual (eBioscience, USA). For each individual mouse, the pg/ml of stimulated wells was subtracted from the those of unstimulated wells and considered as net Gr-B release which is a criterion of CTL activity.

ELISA for specific total IgG and IgG1/IgG2a isotypes

Specific total IgG antibody responses were determined by an optimized indirect ELISA for SARS-CoV-2, which was developed in our laboratory. Briefly, 100 µl of 0.5 µg of inactivated SARS-CoV-2 in PBS was added into each well of 96-well ELISA Maxisorp plates (Greiner, Germany), and put overnight at 4 °C. The wells were washed with washing buffer (PBS containing 0.1% Tween 20) three times and blocked for 1 hr at 37 °C with blocking buffer (2% skimmed milk in washing buffer). The plates were then washed five times with washing buffer, and 100 µl of 1/25 of diluted serum samples (up to 16 serial dilutions) was added into each well and incubated at 37 °C for 2 hr. The wells were washed five times with washing buffer and incubated for 90 min with 100 µl of 1/8000 dilution of Rabbit anti-mouse IgG conjugated to HRP (Razirad, Iran). The wells were washed five times and incubated with 100 µl of TMB substrate in the dark for 10 min. The plates were washed 6 times and incubated with 100 µl of the TMB substrate in the dark for 10 min and the reaction was stopped using 100 µl of 2N HCL. The color density of the plates was measured at A_450 nm with an ELISA reader. The row data of serum samples of the sham group was used to calculate the cutoff of RBD-ELISA by the equation: Mean + 3SD. The specific IgG response to RBD was presented as OD of RBD ELISA of individual mouse/cutoff.

Statistical analysis

The data of immunoassay was presented as mean ± standard deviation (SD). The statistical analysis among the experimental groups was performed using ordinary one-way ANOVA followed by the Tukey test (Graph Pad Prism 6.01 software, La Jolla, CA, USA). In addition, statistical analysis of IgG1, IgG2a isotypes antibodies, as well as the IgG2a/IgG1 ratio, was performed by the Mann-Whitney U test. Among the experimental groups, P-values less than 0.05 were considered a significant difference.

Results

IFN-γ cytokine response

Inactivated SARS-CoV-2-Freund group, as well as SARS-CoV-2-Alum-NLX10, showed a significant increase in the IFN-γ cytokine response versus the control group (P=0.0001). However, mice immunized with the SARS-CoV-2-Alum vaccine and SARS-CoV-2-Alum-NLX3 did not show a significant difference versus the control group (P=0.8384). In addition, mice immunized with SARS-CoV-2-Alum-NLX10 showed a significant increase in the IFN-γ cytokine secretion as compared with the SARS-CoV-2-Alum group (P=0.0001), while SARS-CoV-2-Alum-NLX3 showed a tiny increase, as compared with the SARS-CoV-2-Alum group (P=0.9998). Immunization with SARS-CoV-2-Freund showed a significant increase as compared with SARS-CoV-2-Alum and SARS-CoV-2-Alum-NLX3 groups (P=0.0001); however, a comparable IFN-γ response was observed in SARS-CoV-2-Alum-NLX10 and SARS-CoV-2-Freund groups (P=0.6195) (Figure 1).

IL-4 cytokine response

Mice immunized with inactivated SARS-CoV-2-Freund
Inactivated SARS-CoV-2 formulation in alum/naloxone

Haghighi et al.

as well as SARS-CoV-2-Alum showed a significant increase in the IL-4 cytokine response versus the control group ($P=0.0109$ and $P=0.0010$, respectively). Immunization with SARS-CoV-2-Alum-NLX3 showed a significant increase versus the SARS-CoV-2-Alum group ($P=0.0133$), while the SARS-CoV-2-Alum-NLX10 group showed a slight decrease versus the SARS-CoV-2-Alum group ($P=0.5631$) (Figure 2).

IFN-γ/IL-4 ratio

Results from the IFN-γ/IL-4 ratio (Figure 3) demonstrated that immunization with SARS-CoV-2-Freund resulted in a significant increase versus SARS-CoV-2-Alum, SARS-CoV-2-Alum-NLX3, and PBS groups ($P=0.0001$). Furthermore, mice immunized with SARS-CoV-2-Alum-NLX10 revealed a significant increase, as compared with SARS-CoV-2-Alum, SARS-CoV-2-Alum-NLX3, and PBS groups ($P<0.001$, while the SARS-CoV-2-Alum-NLX3 group showed a 21.46% increase as compared with the SARS-CoV-2-Alum group ($P=0.9519$).

Granzyme-B release

Results from CTL activity based on Granzyme-B release (Figure 4) showed that mice injected with SARS-CoV-2-Alum-NLX10 and SARS-CoV-2-Freund showed a significant increase in Gr-B release versus the SARS-CoV-2-Alum group ($P=0.0216$ and $P=0.0002$, respectively). Mice immunized with SARS-CoV-2-Freund and SARS-CoV-2-Alum-NLX10 indicated increased Gr-B release, as compared with SARS-CoV-2-Alum-NLX3 groups ($P=0.0706$ and $P=0.7986$, respectively).

Specific total IgG response

As shown in Figure 5, the mice immunized with SARS-CoV-2-Alum-NLX3 and SARS-CoV-2-Alum showed a significant increase in IgG response versus the control group ($P<0.0129$). In addition, injection with SARS-CoV-2-Alum-NLX3 vaccine revealed a significant increase in specific IgG versus SARS-CoV-2-Alum at dilutions of 1/25 up to 1/800 ($P=0.0129$). In addition, injection with SARS-CoV-2-Alum-NLX10 vaccine revealed a significant increase versus the SARS-CoV-2-Freund group at dilutions of 1/25 up to 1/800 ($P<0.0011$). Naloxone formulated in the vaccine resulted in a significant IgG response in the SARS-CoV-2-Alum-NLX10 group versus the SARS-CoV-2-Alum group at dilutions of 1/25 up to 1/800 ($P=0.0354$). Furthermore, the SARS-CoV-2-Alum-NLX3 group revealed a borderline increase versus SARS-CoV-2-Alum ($P=0.0527$).

**Figure 2.** IL-4 response in the vaccinated groups. Naloxone formulated in the vaccine resulted in a significant decrease in the IL-4 response in the SARS-CoV-2-Alum-NLX3 group versus SARS-CoV-2-Alum ($P=0.0133$), while SARS-CoV-2-Alum-NLX10 group showed a slight decrease in the IL-4 response versus SARS-CoV-2-Alum ($P=0.5631$).

**Figure 3.** IFN-γ/IL-4 ratio after vaccination of the study groups. Mice immunized with SARS-CoV-2-Alum-NLX10 showed a significant increase in the IFN-γ/IL-4 ratio, as compared with those immunized with SARS-CoV-2-Alum and SARS-CoV-2-Alum-NLX3, as well as PBS groups ($P<0.0001$). However, the SARS-CoV-2-Alum-NLX3 group revealed a 21.46% increase in the IFN-γ/IL-4 ratio versus the SARS-CoV-2-Alum group ($P=0.9519$).

**Figure 4.** Granzyme-B release of vaccinated mice as a criterion of CTL activity. Mice injected with SARS-CoV-2-Alum-NLX10 and SARS-CoV-2-Freund showed a significant increase in Gr-B release versus the SARS-CoV-2-Alum group ($P=0.0216$ and $P=0.0002$, respectively).

**Figure 5.** Specific IgG response in the vaccinated mice after two times immunization. Injection with SARS-CoV-2-Alum and also SARS-CoV-2-Alum-NLX3 vaccine revealed a significant increase in specific IgG versus SARS-CoV-2-Freund at dilutions of 1/25 up to 1/200 ($P=0.0129$). In addition, injection with SARS-CoV-2-Alum-NLX10 revealed a significant increase versus the SARS-CoV-2-Freund group at dilutions of 1/25 up to 1/800 ($P<0.0001$). Naloxone formulated in the vaccine resulted in a significant IgG response in the SARS-CoV-2-Alum-NLX10 group versus the SARS-CoV-2-Alum group at dilutions of 1/25 up to 1/800 ($P=0.0354$). Furthermore, the SARS-CoV-2-Alum-NLX3 group revealed a borderline increase versus SARS-CoV-2-Alum ($P=0.0527$).
group at dilutions of 1/25 up to 1/1600 ($P<0.0063$), while SARS-CoV-2-Alum-NLX10 and SARS-CoV-2-Freund groups exhibited a significant IgG response at dilutions of 1/25 up to 1/3200, as compared with the control group ($P<0.0463$). Mice immunized with SARS-CoV-2-Alum and SARS-CoV-2-Alum-NLX3 showed a significant increase, as compared with the SARS-CoV-2-Freund group at dilutions of 1/25 up to 1/200 ($P<0.0129$). In addition, SARS-CoV-2-Alum-NLX10 showed a significant IgG response, as compared with the SARS-CoV-2-Freund group at dilutions of 1/25 up to 1/800 ($P<0.0011$). Furthermore, the SARS-CoV-2-Alum-NLX10 group revealed a significant increase, as compared with SARS-CoV-2-Alum at dilutions of 1/25 up to 1/800 ($P<0.0354$), while SARS-CoV-2-Alum-NLX3 group exhibited no significant differences versus SARS-CoV-2-Alum and SARS-CoV-2-Alum-NLX10 groups in any dilutions ($P>0.0527$).

**Specific IgG1 isotype**

Results from specific IgG1 isotype antibodies (Figure 6) demonstrated that SARS-CoV-2-Freund, SARS-CoV-2-Alum, SARS-CoV-2-Alum-NLX3, and SARS-CoV-2-Alum-NLX10 revealed a significant increase, as compared with the control group ($P<0.0001$). Mice immunized with SARS-CoV-2-Alum, SARS-CoV-2-Alum-NLX3, and SARS-CoV-2-Alum-NLX10 revealed a significant increase, as compared with the SARS-CoV-2-Freund group ($P<0.0002$). Mice immunized with SARS-CoV-2-Alum-NLX10 and SARS-CoV-2-Alum-NLX3 did not show significant differences versus SARS-CoV-2-Alum ($P>0.4181$).

**Specific IgG2a isotype**

Results from specific IgG2a isotype antibodies in SARS-CoV-2-Freund, SARS-CoV-2-Alum, SARS-CoV-2-Alum-NLX3, and SARS-CoV-2-Alum-NLX10 showed an increase in the IgG2a versus the control group ($P=0.0157$, $P=0.0664$, $P=0.0309$, and $P=0.0064$, respectively). Mice immunized with SARS-CoV-2-Alum-NLX3 and SARS-CoV-2-Alum-NLX10 increased the IgG2a versus the SARS-CoV-2-Alum group; however, this was not statistically significant ($P=0.7354$ and $P=0.1941$, respectively) ($P=0.0157$, $P=0.0664$, $P=0.0309$ and $P=0.0064$, respectively). Mice immunized with SARS-CoV-2-Alum-NLX3 and SARS-CoV-2-Alum-NLX10 had increased IgG2a isotype versus SARS-CoV-2-Alum but statistically it was not significant ($P=0.7354$ and $P=0.1941$, respectively) (Figure 7).

**IgG2a/IgG1 ratio**

The results from the IgG2a/IgG1 ratio in SARS-CoV-2-Freund, SARS-CoV-2-Alum, SARS-CoV-2-Alum-NLX3, and SARS-CoV-2-Alum-NLX10 exhibited an increase in the IgG2a/IgG1 ratio versus the SARS-CoV-2-Alum group (67.13%, 13.45% and 20.96%, respectively; $P=0.0157$, $P=0.0664$, $P=0.0309$ and $P=0.0064$, respectively) (Figure 8). Mice immunized with SARS-CoV-2-Alum-NLX3 and SARS-CoV-2-Alum-NLX10 had increased IgG2a isotype versus SARS-CoV-2-Alum but statistically it was not significant ($P=0.7354$ and $P=0.1941$, respectively) (Figure 7).

**Specific IgG against the RBD protein**

The results in SARS-CoV-2-Freund, SARS-CoV-2-Alum, SARS-CoV-2-Alum-NLX3, and SARS-CoV-2-Alum-NLX10 groups showed a significant increase against the RBD protein, as compared with the control group ($P<0.0001$). In addition, the SARS-CoV-2-Alum-NLX3 group showed a significant decrease in anti-RBD IgG response, versus the
SARS-CoV-2-Alum group \( (P=0.0001) \). Nevertheless, the SARS-CoV-2-Alum-NLX10 group revealed a significant increase, as compared with those immunized with SARS-CoV-2-Alum and even SARS-CoV-2-Freund \( (P<0.0001) \) (Figure 9).

**Discussion**

The unexpected appearance of SARS-CoV-2 and its rapid spread endanger the public health and the economies of all countries worldwide. Although tremendous attempts were made to curb the virus’s spread, most of them were not successful in the control of the infection \( (29, 30) \). In this light, the vaccines developed by DNA and mRNA-based technologies, viral vector, inactivated organism, live-attenuated, and recombinant vaccines have been used for prevention of the infection \( (8, 30-32) \). Eventually, several vaccines were approved and commercialized for human use and are now being used in various countries. These vaccines mainly target the spike proteins and are designated based on humoral immune responses as well as, somehow, T cell responses. Inactivated virus vaccine is one of the approved vaccines for the SARS-CoV-2 virus, which showed lower potency compared with mRNA and subunit vaccines \( (33) \). Therefore, there is an urgent need for improvement of immune responses in this type of vaccine.

In the present study, we hypothesized that changes in the vaccine formulation toward a robust T cell response may influence the vaccine potency. In this regard, NLX, as an immunomodulator, was used in this study because of the fact that NLX could modulate T cell responses in the various vaccine models \( (18, 21, 23, 24) \).

Results from IFN-γ cytokine responses in the SARS-CoV-2-Alum-NLX10 group showed a significant increase as compared with alum-based and, even, SARS-CoV-2-Alum-NLX3 vaccines. This finding showed that polarization of T cell responses toward the Th1 pattern is triggered when the vaccine is formulated with NLX. In addition, polarization toward the Th1 pattern seemed to be dose-dependent because the dose of 10 mg/kg was more potent than that of 3 mg/kg. Our previous studies on the adjuvant activity of NLX in several vaccine models confirmed this finding. Th1 polarization was first confirmed in the HSV DNA vaccine \( (18) \) and then in HPV \( (24) \), HIV-1 \( (23, 26) \), and several bacterial vaccine models \( (22, 34, 35) \). It is well-known that the Th1 response is highly critical in the control and clearance of viral infections \( (12) \), which is a characteristic of NLX in the modulation of immune responses. The results from IL-4 cytokine secretion in the SARS-CoV-2-Alum-NLX3 group showed a significant decrease, but not in SARS-CoV-2-Alum-NLX10. Suppression of the IL-4 response in the SARS-CoV-2-Alum-NLX3 group is another evidence for modulation toward the Th1 pattern when compared with the Alum-based vaccines. Consistent with our findings, previous studies revealed that NLX has the ability to suppress the IL-4 cytokine response \( (18, 23, 36) \), which is another confirmation of Th1 polarization. Furthermore, the IFN-γ/IL-4 ratio in mice immunized with SARS-CoV-2-Alum-NLX10 showed a significant increase, as compared with SARS-CoV-2-Alum, confirming strong Th1 polarization through the vaccine formulated in NLX. The cytokine ratio in mice immunized with SARS-CoV-2-Alum-NLX10 is comparable with SARS-CoV-2-Freund, showing the ability of NLX in stimulation of T cells and shifting toward the Th1 pattern as reported by previous studies \( (18, 34) \).

The activity of TCDB+3, based on Gr-B release, showed that NLX could improve the CTL activity in the SARS-CoV-2-Alum-NLX10 group, as compared with the alum-based vaccine. In addition, NLX10 seemed to be more potent than NLX3 in the vaccine formulation for the induction of CTL activity. This finding showed the potency of NLX in the improvement of CTL activity. In addition, this effect was demonstrated to be dose-dependent similar to those detected in the polarization toward the Th1 pattern. A study conducted on a murine cancer vaccine model also showed that NLX, in combination with this vaccine, improved the CTL response, which is consistent with our study \( (37) \).

Assessment of the specific IgG antibody titer in the SARS-CoV-2-Alum-NLX10 group exhibited a significant increase versus the SARS-CoV-2-Alum group, while NLX3 showed no dramatic effect. This finding showed the potency of NLX in improvement of humoral immune responses; importantly, this effect was dose-dependent because NLX10, in contrast to NLX3, was a more successful dose in the improvement of the IgG response versus the vaccine. In addition, results from IgG1 and IgG2a isotypes showed the potency of NLX in improvement of the IgG2a isotype and IgG2a/IgG1 ratio, which is a criterion of the Th1 pattern because the IFN-γ cytokine is the causative of isotype switching of IgM to IgG2a class \( (28, 38) \). Of note, this result is parallel to the IFN-γ cytokine response, confirming the improvement of the Th1 immune response.

Several studies demonstrated that NLX reinforced humoral immune responses in the vaccine formulation, as our findings achieved in the inactivated SARS-CoV-2 vaccine model \( (22, 23, 26, 39) \). It is well-known that antibodies in vaccinations and infections are produced by B cells but it is well-known that the function, as well as the quantity and quality of antibody responses, highly...
depend on the help of T cells through cytokines, receptor-ligand contacts, and growth factors (40, 41). Herein, NLX seemed to provide a helper signal for B cell responses in the vaccine formulation through improving T cell responses and thereby improved humoral immune responses (42). Antibody response is the first barrier in the SARS-CoV-2 virus neutralization and disease prevention. NLX, as an adjuvant in the vaccine formulation, resulted in a dramatic humoral response, highlighting the potency of this adjuvant in the inactivated SARS-CoV-2 vaccine formulation and encouraging human vaccine development.

Next, the anti-RBD IgG response was evaluated, which can potentially demonstrate the neutralization activity (43, 44). Our results showed that NLX10 in the vaccine formulation significantly increased the anti-RBD IgG response while NLX3 suppressed the response in comparison to the alum-based vaccine. This finding showed another potency of NLX in the induction of the antibody response against the neutralizing protein on the virus but the dose of NLX is a critical factor in the adjuvant activity.

**Conclusion**

Results from the present study provided evidence for the potency of the NLX/alum adjuvant in the inactivated vaccine model for SARS-CoV-2 which increased T cell and antibodies responses in a dose-dependent manner for NLX. Because of the lower potency of the inactivated vaccine for SARS-CoV-2 in the induction of cellular immune response, this formulation can be used to solve this problem (45, 46). Findings from the present study showed that NLX, in combination with Alum, can result in an appropriate inactivated SARS-CoV-2 vaccine, which triggered more robust immune responses in comparison to the alum-formulated vaccine.

**Acknowledgment**

This study was supported by Razi Vaccine and Serum Research Institute of Iran (Grant no. 2297/250). The authors appreciate Dr Morteza Taghizadeh and also Borna Zist Pazhohan Knowledge Company and their staff for supporting this project.

**Authors’ Contributions**

MM Conceived the study and design; MH, PK, and MM Performed animal handling, assay, data analysis, and manuscript preparation; MM and AK Revised the paper; MEM and AK Supervised the project; MH, PK, AK, and MM Approved the final version of the manuscript.

**Conflicts of Interest**

The authors declare that no conflicts of interest exist for this research.

**References**

1. Shi Y, Wang G, Cai XP, Deng JW, Zheng L, Zhu HH, et al. An overview of COVID-19. J Zhejiang Univ Sci B 2020;21:343-360.
2. Chang L, Yan Y, Wang L. Coronavirus disease 2019: Coronavirususes and blood safety. Transfus Med Rev 2020;34:75-80.
3. The Lancet Infectious D. Challenges of coronavirus disease 2019. Lancet Infect Dis 2020;20:261.
4. Dharma K, Khan S, Tiwari R, Sircar S, Bhat S, Malik YS, et al. Coronavirus disease 2019-COVID-19. Clin Microbiol Rev 2020;33:1-48.
5. Alkandari D, Herbert JA, Alkhalaif MA, Yates C, Panagiotou S, SARS-CoV-2 vaccines: Fast track versus efficacy. Lancet Microbe 2021;2:e89-e90.
6. Iqbal Yatoo M, Hamid Z, Parray OR, Wani AH, Ul Haq A, Saxena A, et al. COVID-19-recent advancements in identifying novel vaccine candidates and current status of upcoming SARS-CoV-2 vaccines. Hum Vacc Immunother 2020;16:2891-2904.
7. Dhma K, Sharun K, Tiwari R, Dadar M, Malik YS, Singh KP, et al. COVID-19, an emerging coronavirus infection: Advances and prospects in designing and developing vaccines, immunotherapeutics, and therapeutics. Human vaccines & immunotherapeutics. 2020;16:1232-1238.
8. Jafari A, Danesh Pouya F, Niknam Z, Abdollahpour-Altappeh M, Rezaei-Tavirani M, Rasmj Y. Current advances and challenges in COVID-19 vaccine development: from conventional vaccines to next-generation vaccine platforms. Mol Biol Rep 2022;1-15.
9. Najminejad H, Kalantar SM, Mokarram AR, Dabaghi M, Abdollahpour-Altappeh M, Ebrahimi SM, et al. Bordetella pertussis antigens encapsulated into N-trimethyl chitosan nanoparticulate systems as a novel intranasal pertussis vaccine. Artif Cells Nanomed Biotechnol 2019;47:2605-2611.
10. Amini Y, Tebianian M, Mosavari N, Fasihi Ramandi M, Ebrahimi SM, Najminejad H, et al. Development of an effective delivery system for intranasal immunization against Mycobacterium tuberculosis ESAT-6 antigen. Artif Cells Nanomed Biotechnol 2017;45:291-296.
11. Corey L, Mascola JR, Fauci AS, Collins FS. A strategic approach to COVID-19 vaccine R&D. Science 2020;368:948-950.
12. Mahdavi M, Ektekar M, Khoshshid HRK, Azadmankes N, Hartoonian C, Hassan ZM. ELISPOT analysis of a new CTI based DNA vaccine for HIV-1 using GM-CSF in DNA prime/peptide boost strategy: GM-CSF induced long-lived memory responses. Immunology letters 2011;140:14-20.
13. Wang J, Peng Y, Xu H, Cui Z, Williams RO. The COVID-19 vaccine race: challenges and opportunities in vaccine formulation. AAPS PharmSciTech 2020;21:1-12.
14. Kuo T-Y, Lin M-Y, Coffman RL, Campbell JD, Traquina P, Lin Y-I, et al. Development of CpG-adjuvanted stable prefusion SARS-CoV-2 spike antigen as a subunit vaccine against COVID-19. Scientific Reports 2020;10:1-10.
15. García-Arriaza J, Garaigorta U, Pérez P, Lázaro-Frias A, Zamora C, Gastañpiaza P, et al. COVID-19 vaccine candidates based on modified vaccinia virus ankara expressing the SARS-CoV-2 spike protein induce robust t-and b-cell immune responses and full efficacy in mice. J Virol 2021;95:e02260-20.
16. Cox RJ, Brokstad KA. Not just antibodies: B cells and T cells mediate immunity to COVID-19. Nat Rev Immunol 2020;20:581-582.
17. Beyer WEP, Palache AM, Reaper LA, Boulách M, Osterhau A. Association between vaccine adjuvant effect and pre-seasonal immunity. Systematic review and meta-analysis of randomised immunogenicity trials comparing squalene-adjuvanted and aqueous inactivated influenza vaccines. Vaccine. 2020;38:1614-1622.
18. Jamali A, Mahdavi M, Hassan ZM, Sabahi F, Farafari MJ, Bamdad T, et al. A novel adjuvant, the general opioid antagonist naloxone, elicits a robust cellular immune response for a DNA vaccine. Int Immunol 2009;21:217-225.
19. Burris S, Norland J, Edlin BR. Legal aspects of providing naloxone to heroin users in the United States. International Journal of Drug Policy 2001;12:237-248.
20. Yasaghi M, Mahdavi M. Potentiation of human papilloma virus vaccine candidate using naloxone/alum mixture as an adjuvant: increasing immunogenicity of HPV-16E7&d vaccine. Iran J Basic Med Sci 2016;19:1003-1009.
21. Jamali A, Mahdavi M, Shahabi S, Hassan ZM, Sabahi F, Javan M, et al. Naloxone, an opioid receptor antagonist, enhances induction of protective immunity against HSV-1 infection in BALB/c mice. Microb Pathog 2007;43:217-223.
22. Jazani NH, Karimzad M, Mazloomi E, Sohrabpour M, Hassan ZM, Ghasemnejad H, et al. Evaluation of the adjuvant activity of naloxone, an opioid receptor antagonist, in combination with heat-killed Listeria monocytogenes vaccine. Microbes Infect 2010;12:382-388.

23. Velashjerdi Farahani S, Reza Aghasadeghi M, Memarnejadian A, Faezi S, Shahosseini Z, Mahdavi M, et al. Naloxone/alum mixture a potent adjuvant for HIV-1 vaccine: induction of cellular and poly-isotypic humoral immune responses. Pathog Glob Health 2016;110:39-47.

24. 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:e08027.

25. Fathi M, Nezamzadeh R, Abdollahpour-Altappeh M, Yazdi MH, Khoramabadi N, Mahdavi M. Formulation of a recombinant HIV-1 polytope candidate vaccine with naloxone/alum mixture: Induction of multi-cytokine responses with a higher regulatory mechanism. APIMS 2021;129:480-488.

26. Mojrab S, Shahbazzadeh D, Moghibi M, Eshraghi Y, Bagheri KP, Rahimi R, et al. Immune responses to HIV-1 polytope vaccine candidate formulated in aqueous and alcoholic extracts of Propolis: Comparable immune responses to Alum and Freund adjuvants. Microbial Pathogenesis 2020;140:103932.

27. Mahdavi M, Tadjik AH, Ebtekar M, Rahimi R, Adibzadeh MM, Moozarmpour HR, et al. Granulocyte-macrophage colony-stimulating factor, a potent adjuvant for polarization to Th-17 pattern: an experience on HIV-1 vaccine model. Apmis. 2017;125:596-603.

28. Pi-Estopiñan F, Pérez MT, Fraga A, Bergado G, Díaz GD, Orosa I, et al. A cell-based ELISA as surrogate of virus neutralization assay for RBD SARS-CoV-2 specific antibodies. Vaccine 2022;40:1958-1967.

29. Hellewell J, Abbott S, Gimma A, Bosse NI, Jarvis CI, Russell TW, et al. Feasibility of controlling COVID-19 outbreaks by isolation of cases and contacts. Lancet Global Health 2020;8:e488-e96.

30. Forni G, Mantovani A. Covid-19 vaccines: Where we stand and challenges ahead. Cell Death Differ 2021;28:626-639.

31. Liu X, Liu C, Liu G, Luo W, Xia N. Covid-19: Progress in diagnostics, therapy and vaccination. Theranostics 2020;10:7821-7835.

32. Lazarus JV, Ratzan SC, Palayew A, Gostin LO, Larson HJ, Rabin K, et al. A global survey of potential acceptance of a Covid-19 vaccine. Nat Med 2021;27:225-228.

33. Kim JH, Marks F, Clemens JD. Looking beyond Covid-19 vaccine phase 3 trials. Nat Med 2021;27:205-211.

34. Jazani NH, Parsania S, Sohrabpour M, Mazloomi E, Karimzad M, Shahabi S. Naloxone and alum synergistically augment adjuvant activities of each other in a mouse vaccine model of Salmonella typhimurium infection. Immunobiology 2011;216:744-751.

35. Khorsheidvand Z, Shahabi S, Mohammazzadeh H, Daryani A, Tapkeh KH. Mixture of alun–naloxone and alum–naltrixone as a novel adjuvant elicits immune responses for Toxoplasma gondii lysate antigen in BALB/c mice. Experimental Parasitology 2016;162:28-34.

36. Sacerdoti P, Gaspani L, Panerai AE. The opioid antagonist naloxone induces a shift from type 2 to type 1 cytokine pattern in normal and skin-grafted mice. Ann Y Acad Sci 2000;917:755-763.

37. Hassan ATM, Hassan ZM, Moazzeni SM, Mostafaie A, Shahabi S, Ebtekar M, et al. Naloxone can improve the anti-tumor immunity by reducing the CD4+ CD25+ Foxp3+ regulatory T cells in BALB/c mice. Int Immunopharmacol 2009;9:1381-1386.

38. Rostami H, Ebtekar M, Ardestani MS, Yazdi MH, Mahdavi M. Co-utilization of a TLR5 agonist and nano-formulation of HIV-1 vaccine candidate leads to increased vaccine immunogenicity and decreased immunogenic dose: A preliminary study. Immunol Lett 2017;187:19-26.

39. Jazani NH, Sohrabpour M, Mazloomi E, Shahabi SJF, Microbiology M. A novel adjuvant, a mixture of alum and the general opioid antagonist naloxone, elicits both humoral and cellular immune responses for heat-killled Salmonella typhimurium vaccine. FEMS Immunol Med Microbiol 2011;61:54-62.

40. Caili S, Veiga DT, Balasubramanian P, Athale S, Domic B, Murat E, et al. A CD4+ T cell population expanded in lupus blood provides B cell help through interleukin-10 and succinate. Nat Med 2019;25:75-81.

41. Kim ST, Choi J-Y, Lainez B, Schulz VP, Karas DE, Baum ED, et al. Human extracellular CD4+ Th cells help memory B cells produce Igs. J Immunol 2018;201:1359-1372.

42. Jazani NH, Parsania S, Sohrabpour M, Mazloomi E, Karimzad M, Shahabi SJF. Naloxone and alun synergistically augment adjuvant activities of each other in a mouse vaccine model of Salmonella typhimurium infection. Immunobiology 2011;216:744-751.

43. Khoury DS, Cromer D, Reynaldi A, Schlub TE, Wheatley AK, Juno JA, et al. Neutralizing antibody levels are highly predictive of immune protection from symptomatic SARS-CoV-2 infection. Nat Med 2021;27:1205-1211.

44. Starr TN, Czudnochowski N, Liu Z, Zatta F, Park YJ, Addetia A, et al. SARS-CoV-2 RBD antibodies that maximize breadth and resistance to escape. Nature 2021 Sep;597:97-102.

45. Sauer K, Harris T. An Effective COVID-19 Vaccine Needs to Resist to Escape. Nature 2021 Sep;597:97-102.

46. Cañete PF, Vinuesa CG. COVID-19 makes B cells forget, but T cells remember. Cell 2020;183:13-15.