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Research Paper

In Vitro Evaluation of the Long-term Immune Status of Razi-Cov-Pars Vaccine

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ABSTRACT

Background: The Razi-Cov-Pars vaccine, a recombinant protein vaccine developed in Iran, has undergone evaluation in three dosage strengths across three clinical trial phases. In phase I, volunteers received one of three dosages (5, 10, and 20 µg) corresponding to low, medium, and high strengths.

Materials and Methods: This study assessed the long-term in vitro immune status of the Razi-Cov-Pars vaccine by transferring lymphocyte supernatants (SN) from individuals vaccinated for 8 months to lymphocytes from individuals vaccinated for 16 months and non-vaccinated healthy individuals. Immunological parameters were evaluated using the 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide assay for stimulation index and reverse transcription polymerase chain reaction for interleukin 10, transforming growth factor-ß (TGF-ß) and TGF-α levels.

Results: The findings revealed that lymphocyte SN from individuals vaccinated for 8 months significantly enhanced the proliferation of lymphocytes from individuals vaccinated for 16 months with the licensed 10 µg strength vaccine. This suggests that the Razi-Cov-Pars vaccine may have long-term effects on lymphocyte proliferation and immune response. Notably, there was a significant increase in TGF-α levels across all vaccine strengths, hinting at the potential role of TGF-α in long-term immune processes following vaccination with Razi-Cov-Pars. Additionally, there was a marked increase in interleukin-10 levels in all vaccinated groups, indicating active pro-inflammatory elements in the SN. TGF-β expression significantly increased in the high-strength vaccine group, whereas no notable difference was observed in the low and medium-strength groups.

Conclusion: We detected a significant stimulation in the lymphocytes isolated from vaccinated individuals after 16 months by measurement of proliferative potential and cytokine gene expression thereby assessing the long-term effect of the vaccine.

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Introduction

Several vaccines were developed a few months into the COVID-19 pandemic, with various platforms, including messenger ribonucleic acid (mRNA), whole, deactivated, recombinant protein, and vector-based. All these platforms share a common goal which is to trigger the human immune system to initiate a rapid and effective response against the invading virus. COVID-19 vaccines are divided into two categories as follows: Vaccines based on immunogenic components of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and vaccines that use the whole virus [1]. Vaccines based on immunogenic components of SARS-CoV-2 are based on the following five different mechanisms: Protein subunits, virus-like particles, DNA-based or RNA-based vaccines, non-replicated viral vectors, and replicating viral vectors [2]. Protein subunit-based vaccines consist of isolated and purified proteins from SARS-CoV-2 that exhibit immunogenic properties and stimulate humoral immune responses. Virus-like particle-based vaccines are made from viral proteins that imitate the structure of SARS-CoV-2, but they do not contain any genetic material. On the other hand, DNA-based or RNA-based vaccines serve as carriers for introducing viral genetic material, either in the form of DNA or mRNA [3]. This approach leads to efficient production of viral proteins in the recipient leading to immune reactions against the produced viral proteins.

In Iran, seven vaccines were developed based on the know-how and experience of the producers some were taken to the third phase of clinical trial and received emergency use approval [4]. Among them, the Razi Vaccine & Serum Research Institute with a decade-long history, developed a recombinant protein-based vaccine (Razi-Cov-Pars) which successfully entered the third phase of clinical trials and received emergency use approval from the Iranian Food and Drug Organization (FDA) [5]. This study presents a follow-up on the immune response in recipients of Iran's first recombinant protein-based vaccine. The vaccine platform was built on the recombinant SARS-CoV-2 spike protein, both in its monomeric form (covering amino acids 1-674 for S1 and 685-1211 for S2 subunits) and as a trimer (S-Trimer), and was formulated with the oil-in-water adjuvant system RAS-01 (Razi Adjuvant System-01). The safety and immunogenicity of the Razi-Cov-Pars vaccine were previously assessed in Syrian hamsters, BALB/c mice, Pirbright guinea pigs, and New Zealand white rabbits [5]. Furthermore, clinical trials for Razi-Cov-Pars have been conducted through phases one, two, and three [6, 7]. They evaluated the immunogenicity and safety of a combined intramuscular/intranasal recombinant spike protein COVID-19 vaccine in healthy adults aged 18 to 55 years in a randomized, double-blind, placebo-controlled, phase I trial [6].

Follow-up studies of vaccines are an important dimension of clinical evaluation of the efficacy. Adverse events of vaccines are important and need to be studied since they could cause long-term health problems. An adverse event post-immunization is anything medically related that transpires after vaccination.

Currently, there is an online website and database to report an adverse event (vaccine adverse event reporting system) [8]. During clinical trials, the U.S. Food and Drug Administration collected data on each of the authorized COVID-19 vaccines for a minimum of two months (eight weeks) after the final dose [8]. Currently, the Centers for Disease Control and Prevention, Food and Drug Administration, and other federal agencies continue to monitor the safety of COVID-19 vaccines. Also in Iran, the FDA is collecting data on the adverse effects of vaccines administered.

This study is a prospective blind clinical in vitro evaluation to assess the immunological response of people immunized with the Razi-Cov-Pars vaccine by measuring the lymphocyte responses to the S1 antigen from vaccinated individuals in three strengths of the vaccine and adjuvant alone 8 months after vaccination.

Materials and Methods

Study groups (inclusion and exclusion criteria)

A total of 100 hundred healthy volunteers between 17-70 years of age male and female, were included in this study at Razi Vaccine and Serum Research Institute. The participants were excluded if they did not receive the Razi-Cov-Pars vaccine. The main inclusion and exclusion criteria for clinical trial participants were as follows. Volunteers needed to be healthy according to clinical, psychological, and laboratory standards. They had to sign a written informed consent form and could not have any active, symptomatic, acute, or chronic illness requiring medical or surgical treatment on the day of vaccination. The participants were also required to test negative for human immunodeficiency virus, hepatitis C, and hepatitis B via enzyme-linked immunosorbent assay tests. Additionally, a negative β-human chorionic gonadotropin (βHCG) pregnancy test was required both on the screening day and the vaccination day. The participants had to agree to use an effec-

tive contraception method for three months after the final vaccine dose. Meanwhile, the exclusion criteria included working in a high-risk COVID-19 exposure job, having a history of long-term immunosuppressive medication use within the four months before screening, and having a history of allergic conditions like angioedema, anaphylactic reactions, or allergies to drugs or vaccines. Participants with underlying conditions such as cancer, autoimmune diseases, hematological disorders, or diabetes were also excluded. In addition, all participants resided in Tehran City, Iran.

Research sample, sample size, and sampling method

This was a double-blind randomized clinical trial. In this study, 100 healthy individuals who received the Razi-Cov-Pars vaccine in 3 doses, 2 vaccinated individuals with 3 doses of vaccine (10 µg and 16 months after), and one healthy non-vaccinated individual (control) were included in the study. The participants received two shots from one of the three strengths of this vaccine (20 µg/200 µL, 10 µg/200 µL, 5 µg/200 µL; in three groups; 14 days between each dose) and one intranasal shot. After 8 months, blood samples were taken and white blood cells from vaccinated people were separated and primed with S1 antigen and incubated for 72 h. Subsequently, the supernatants (SN) were collected to be used for further studies to detect the long-term impact on immune responses or side effects. Initially, we performed a titration test to determine the suitable concentration of SN to be used (dilutions [1/2 - 1/4 - 1/8 - 1/16], positive control [S1 and negative control]).

Isolation of lymphocytes

A total of 10 mL of anti-coagulated blood (acid citrate dextrose) was overlaid on Ficoll-Paque (Hi-sep, Himedia, India, specific gravity =1.077) band centrifuged at 400 g for 20 min (37 ˚C). The peripheral blood mononuclear cells were carefully removed from the plasma-ficoll interface and centrifuged at 1500 rounds per min for 10 min. Lymphocytes were washed three times with phosphate buffer saline. Trypan blue staining (Gibco, Auckland, New Zealand) was used to determine each cell's viability, and cells were counted using a hemocytometer following standard practice. Finally, cells were suspended in 1 mL of RPMI-1640 that had 20% fetal calf serum (Gibco, Auckland, New Zealand). In 96-well tissue culture plates with a flat bottom, counted cells were plated. To accurately quantify cell proliferation, 3×10^4 to 5×10^4 cells/well were plated.

3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay

Evaluation of cell viability/proliferation was done by 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay. The MTT assay was performed according to standard protocol. In brief, in separate 96-well plates, lymphocyte cells (5×10^4 cells/well) were added to each well in 100 µL RPMI. Cells were treated with different concentrations of SN and incubated for 72 h. Then, 20 µL of MTT solution containing 5 mg/mL of MTT powder was added to each well and incubated for 3 h in a 5% CO_2 atmosphere in a 37 ˚C incubator. The SN was removed from all the wells, and 100 µL of dimethyl sulfoxide was added. Afterward, the absorption was measured at 540 nm by a microplate reader. All the samples were tested in triplicate, and the survival rate (%) was calculated using Equation 1.

1.

Survival rate (%)=OD (treatment group)/OD (control group) ×100

Reverse transcription polymerase chain reaction

RNA extraction, cDNA synthesis, and quantitative RT-PCR total RNA was extracted from THP-1 and K562 cell lines through the RNX Plus kit (Cinnagen, Iran). RNA integrity was measured using the Nanodrop (Thermo Scientific, USA) and the gel electrophoresis method was used to determine the purity and integrity of RNA (260/280 >1.8). Subsequently, cDNA was synthesized using a reverse transcription kit (Parstous, Iran) as instructed by the manufacturer. Then, real-time quantitative PCR was used to quantify the resulting transcripts on a Step One Plus™ real-time DNA amplification system (Applied Biosystem, USA) along with SYBER Green qPCR master mix kit (AMPLIQON, Denmark) and specific primers for each sample. As a reference gene, β-2-microglobulin (*B2M*) was used to optimize T-cell immunoglobulin and mucin-domain containing-3 (*TIM-3*), hypoxia-inducible factor-1 α (*HIF-1α*), and tumor necrosis factor-α (*TNF-α*) gene expression. The quantitative RT-PCR assay was accomplished in 10 µL final reaction volume using 1.0 µL template target cDNA, 1.0 µL forward and reverse primer, 5 µL Master Mix Green-high ROX, and 3 µL water to reach total volume. Thermal cycling for each reaction (*TIM-3*, *HIF-1α*, and *TNF-α*) included an initial hold at 95 ˚C for 15 min. The denaturation, annealing, and extension cycle temperatures were 9 ˚C for 30 s, 60 ˚C for 30 s, and 72 ˚C for 30 s, respectively, for 40 times. The PCR efficiency

mean for each primer pair sample was calculated using LinRegPCR 11.0 software. The data were analyzed using the relative quantification ($2^{\Delta\Delta Ct}$) method.

Statistical analyses

The statistical comparison between groups was performed with a one-way analysis of variances (one-way analysis of variance) followed by the Tukey post hoc test. Meanwhile, $P<0.05$ are considered statistically significant.

Results

Razi-Cov-Pars vaccine (S1 peptide) had been prepared for phase I trial. In phase I, they used three doses 5, 10, and 20 µg and estimated the proper dose for injection to the healthy persons and estimated the proper dose for use as a vaccine. To evaluate the protection time of vaccine time, they evaluated the efficacy of the vaccine in the eighth and 16th months following vaccination. After 8 months, group lymphocytes from all samples were treated with three doses of S1 antigen. The SN of the 8-month stimulated lymphocytes was collected and frozen. The SN was used for further studies as a source of S1 peptide (5, 10, and 20/200 µL) as compared to S1 peptide. Also, this study examined whether the SN can suppress or stimulate lymphocyte activity through MTT test and cytokine levels measurements.

Evaluation of treatment of the lymphocytes of 16 months vaccinated persons with S1 antigen and SN

To evaluate the response of lymphocytes after 16 months of vaccination, lymphocyte SN of 8 months of vaccination and S1 antigen were used. Lymphocytes of 16 months of vaccination were collected and treated with SN of the three doses (T15 µg SN, T2 with 10 µg SN, and T3 µg SN. Positive control of lymphocytes was stimulated with 10 µg strength S1 antigen. Negative control of lymphocytes collected from unvaccinated healthy persons, not exposed to COVID-19 was tested with 10 µg S1 antigen. The results in Table 1 showed a significant increase in the stimulation index of the lymphocyte treated with S1 antigen and SN compared to the lymphocytes of unvaccinated individuals. No significant differences between S1 and SN groups were observed.

To evaluate the optimum dose of SN and S1 antigen to be used in this study, we chose four dilutions (1/2, 1/4, 1/8, 1/16) and 10 µg of S1 Ag. S1Ag and SNs (T1, T2, and T3) were added to the lymphocytes of individuals collected after 16 months of vaccination. Negative control of lymphocytes collected from unvaccinated healthy persons, not exposed to COVID-19 was tested with 10 µg S1 antigen. The results in Figure 1 indicated that the S1 antigen peptide group and T1, T2, and T3 groups showed a significant difference as compared to the negative group. We chose the titration ¼ dilution (25 µL of the SN) to be used in this study.

Evaluating the response of lymphocytes to SN on 90 vaccinated people

To confirm our study, we randomly tested 90 persons' lymphocytes with an SN of the ¼ dilution of the three doses of SN (5 µg, 10 µg, and 20 µg), and adjuvant as a negative control. The results in Figure 2 indicate a significant increase ($P<0.001$) in the stimulation index in the 20 µg group compared to 10 µg, 5 µg, and adjuvant. Also, a significant ($P<0.001$) increase in the level of proliferation index was observed in a group of 10 µg compared to the adjuvant group.

Cytokine evaluation

To evaluate the status of cytokine profiles in lymphocytes from individuals after 16 months of vaccination and stimulated with SNs. The levels of cytokine gene expression in lymphocytes from the three strengths of the vaccine and adjuvant were estimated by RT-PCR to evaluate the expression of transforming growth factor β (*TGF-β)*, *IL-10*, and *TNF-α*. The results in Figure 3 show the expression of the *IL-10* gene is significantly higher in the 20 µg,10 µg, and 5 µg groups compared to the adjuvant group. The results for the 20 µg and 10 µg are significantly higher than the 5 µg group ($P<0.003$).

Also, we noticed a statistically significant difference between the expression of the *TGF-β* gene in the lymphocytes treated with high strength (20 µg) vaccinated individuals as compared with 10 µg ($P<0.0001$), and with the 5 µg ($P<0.0001$), and adjuvant (Figure 4).

We measured the level of *TNF-α* in the lymphocytes of individuals vaccinated with high strength 20 µg/µL, moderate 10 µg/µL, and low strength 5 µg/µL as compared to the adjuvant. Adjuvant showed low expression of *TNF-α* in the RT-PCR assay compared to other groups.

There is no significant difference between the expression of *TNF-α* in the lymphocytes treated with moderate and low strength groups ($P=0.95$).

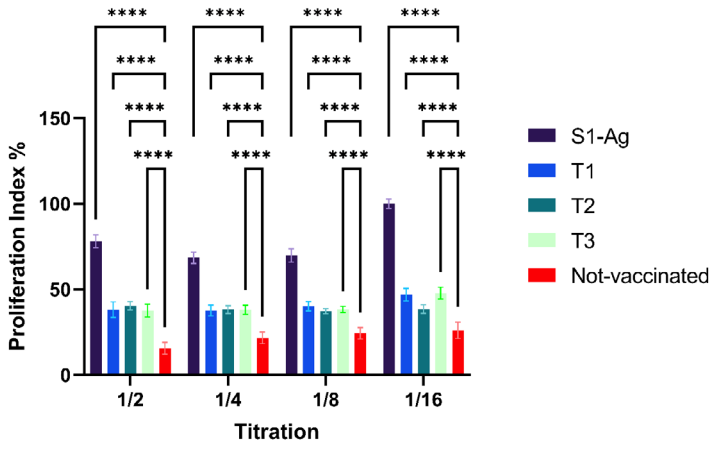

Figure 1. Proliferation index for serial dilution of SN

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Notes: T1=5 µg, T2=10 µg,T3=20 µg and S1 antigen. The significant difference is measured by P<0.0001. The highly significant was noticed in 1/16.

However, if we compare the three groups with the adjuvant group, we notice a statistically significant difference between the high strength compared to adjuvant (P<0.008) and the between the moderate strength and adjuvant show significant statistical difference (P<0.008) and there is a significant statistical difference between the low strength and adjuvant (P<0.01) (Figure 5).

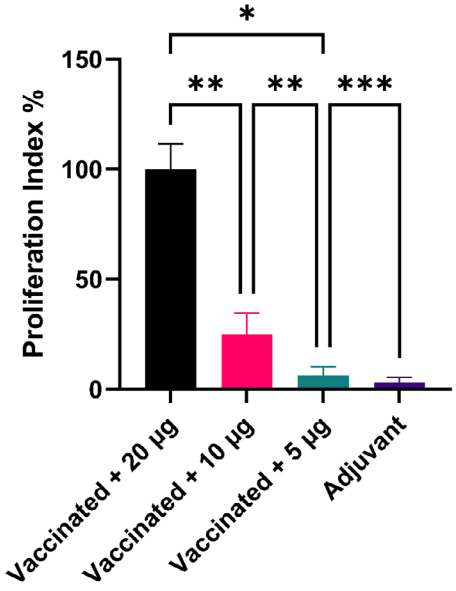

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Figure 2. Proliferation index of 1/16 dilution of SN

Notes: T1=5 µg, T2=10 µg, and T3=20 µg and S1 antigen on 90 lymphocytes after 16 months of vaccination. The mean value of 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay of the lymphocytes treated with different strengths of the vaccine in phase I trial. *P<0.05, **P<0.01, and ***P<0.001.

Discussion

This study evaluated several questions, the duration of protection of the Razi- CoV- Pars vaccine, the safety as well as the suitable dose of injection, and the status of cytokine gene expression in lymphocytes from vaccinated persons.

An outbreak of a novel Coronavirus disease nominated as COVID-19 caused by SARS-CoV-2- 2 was reported in Wuhan, China, in late December 2019. The

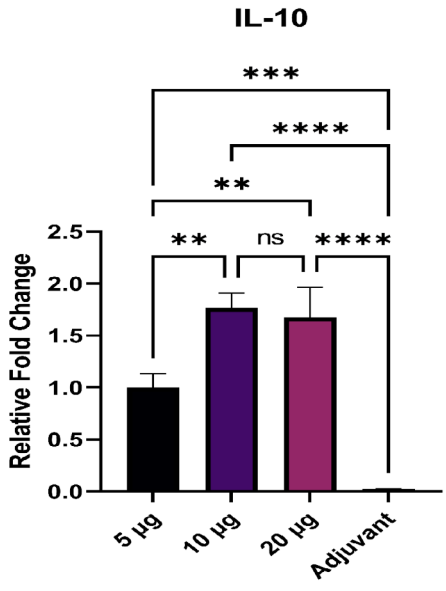

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Figure 3. The expression of *IL-10* gene in lymphocytes from people vaccinated with three strengths of the vaccine (20 µg/200 µL, 10 µg/200 µL, and 5 µg/200 µL) or adjuvant only

Notes: **P<0.003, ***P<0.001, ****P<0.0001.

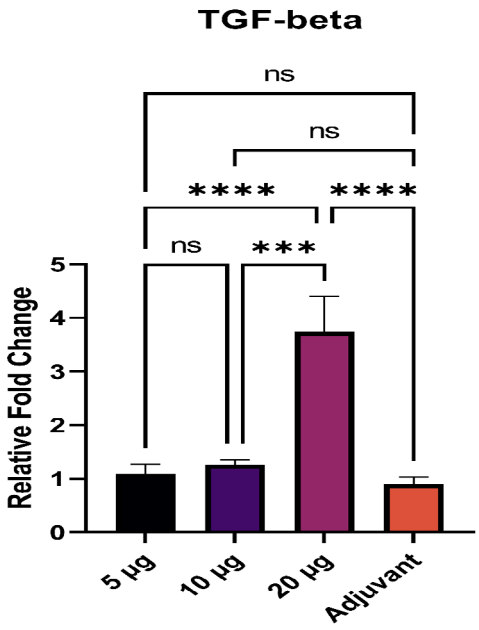

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Figure 4. The expression of *TGF-ß* gene in lymphocytes from people vaccinated with three strengths of vaccine (20 µg/200 µL, 10 µg/200 µL, 5 µg/200 µL) or adjuvant only

Notes: ***P<0.001, ****P<0.0001.

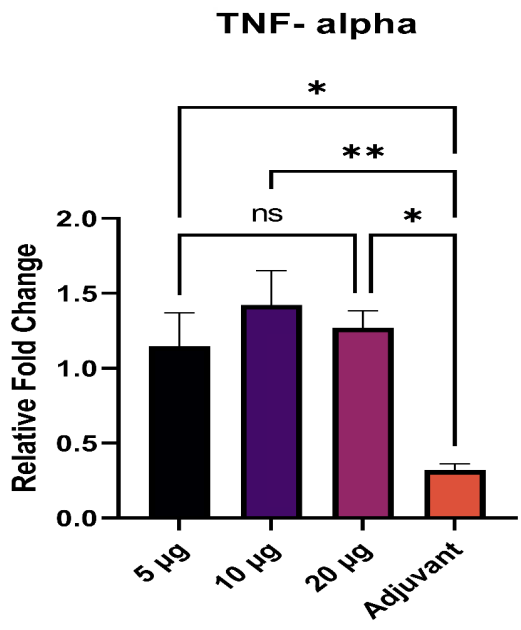

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Figure 5. The expression of *TNF-a* gene in lymphocytes from people vaccinated with three strengths of vaccine (20 µg/200 µL, 10 µg/200 µL, and 5 µg/200 µL) or adjuvant only

Notes: *P<0.01, **P<0.008.

virus subsequently affected many countries worldwide and World Health Organization (WHO) declared it a pandemic disease. SARS-CoV-2 uses a spike receptor-binding domain (RBD) protein that, by binding to angiotensin-converting enzyme 2 (ACE2) on the target cell facilitates its entrance into the target. Once it has entered the cell SARS-CoV-2 replicates via its RNA polymerase [9]. Considering the high fatality rates and the need to protect humans from this virus, scientists during the past years, have studied and discovered many vaccines. One of the COVID-19 vaccines produced in Iran is the Razi-CoV-Pars vaccine. This is a protein-based vaccine that employs recombinant versions of the spike protein to stimulate the immune system against the virus through cell-mediated immunity [9]. Previous clinical trials demonstrated that the recombinant spike protein COVID-19 vaccine is safe and well-tolerated, with no serious adverse reactions reported in healthy adult volunteers. The rate of solicited adverse reactions was comparable across all study groups, and no side effects were attributed to the vaccine. The most common adverse reaction was headache and tenderness at the injection site. Antibody responses to both the S and RBD antigens were observed in the 10 µg and 20 µg vaccine

Table 1. Evaluation of the activity of the lymphocytes of individuals 16 months after vaccination and stimulation with S1 antigen peptide

Samples	% Stimulation
S1 peptide + lymphocyte	105±4.1
T1 + lymphocyte	105±2.1
T2 + lymphocyte	128±2.1
T3 + lymphocyte	129.6±2.5
S1 antigen + lymphocyte (unvaccinated individual)	-5

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Notes: The percentage of stimulation in 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide assay for T1, T2, T3 SN are shown. SN were separated from lymphocytes eight months after vaccination and stimulated with S1 antigen peptide.

groups by day 35, showing levels four and six times higher than those in the placebo group [6].

In this study, we endeavored to evaluate lymphocytes obtained from individuals vaccinated with the Razi-Cov-Pars vaccine after 8 months in three strengths and lymphocytes taken from. vaccinated individuals after 16 months by detection of proliferative potential and cytokine gene expression to assess the long-term effect of the vaccine.

In the MTT assay, we noticed that the SN from the lymphocytes separated from the group that received only adjuvant showed low proliferation, also the (10 µL, 5 µL) strength groups demonstrated low stimulation, and the high strength (20 µL) caused higher stimulation of lymphocytes. This indicates that the higher concentration resulted in better stimulation and proliferation of lymphocytes.

Numerous studies have highlighted that antibody levels induced by vaccines remain present for up to six months after the second dose. Additionally, other research has shown that mRNA vaccines, like BNT162b2 from BioNTech-Pfizer, generate strong immunoglobulin G and neutralizing antibody responses, particularly following the second 30 µg dose. However, long-term studies have revealed a decline in serum antibodies against SARS-CoV-2, raising concerns about the duration of immunity. This reduction has been linked to breakthrough infections, leading to discussions about the need for additional booster vaccine doses [10]. Another related study demonstrates that strong anti-spike protein antibody responses are evoked in almost all individuals that receive two doses of Oxford-AstraZeneca vaccine, and which largely persist beyond six months after the first vaccination dose [11]. Our study indicated that the proliferative capacity of lymphocytes against the S1 antigen has persisted over 8 months in individuals vaccinated with the Razi-Cov-Pars vaccine.

Asadi-Pooya et al. discusses how COVID-19 vaccination may affect long COVID-19 symptoms [12]. Another research indicated that long COVID-19 post-viral syndrome may be related to genetic predisposition. Single nucleotide polymorphisms of cytokine genes *IL-6*, *TNF-α*, *IFN-γ*, and *IL-10* correlate with post-viral fatigue, pain, neurocognitive difficulties [13]. Receiving the COVID-19 vaccine had a significant association with prolonged symptoms of long COVID-19 for more than one year after the initial infection [13].

In this study, we evaluated the gene expression of three cytokines (as suppressor or inflammatory). As a preliminary study to estimate the status of immune response (suppression or stimulation) after being treated with S1 antigen and SN, this was part of an ongoing research project at Razi Vaccine & Serum Research Institute, including evaluation of the protein expression in a wide range in a of cytokines and significant number of vaccinated persons.

TNFα is an inflammatory cytokine secreted by macrophages and monocytes during acute inflammation. It plays a key role in various intracellular signaling processes that can result in necrosis or apoptosis. Additionally, TNFα is crucial for combating infections and cancers [14]. The biology of TNF-α has features that make it a promising target for therapy in patients with COVID-19. TNF has an important role in the initiation of the inflammatory cascade and is elevated early in the disease process; on this basis, early intervention may be more beneficial than late intervention [15]. It coordinates cell recruitment via regulation of chemokine and adhesion molecules [16]. In COVID-19, TNF-α-mediated inflammation can cause detrimental tissue damage and gradually promotes lung fibrosis, which later results in pneumonia, pulmonary edema, and acute respiratory distress syndrome [17]. Our studies showed a significant increase in the level of TNF-α in low, medium, and high-strength vaccinated groups as compared with the control group. This indicates the possible role of TNF-α in the long-term processes after Razi-Cov-Pars vaccination, leading to protection against the causative agent of COVID-19 [17].

IL-10 is an anti-inflammatory cytokine, over-expressed in some viral and bacterial infections which can down-regulate macrophages. IL-10 is a pleiotropic cytokine recognized for its strong anti-inflammatory and immunosuppressive properties. Initially identified as being produced by T helper 2 cells, IL-10 is currently known to be produced by a range of immune cells derived from both myeloid and lymphoid lineages, playing roles in both innate and adaptive immunity [18]. A key role of IL-10 during infection is to suppress the host's immune response to pathogens and microbiota, helping to reduce tissue damage and immunopathology. IL-10 achieves this by inhibiting the production of pro-inflammatory cytokines and antigen presentation in activated monocytes, macrophages, and dendritic cells, while also curbing excessive T-cell activation and proliferation [18, 19]. The dramatic early rise in IL-10, canonically classified as an anti-inflammatory cytokine, is a distinguishing feature of hyperinflammation during severe SARS-CoV-2 infection and several studies indicate that IL-10 levels predict poor outcomes in patients

with COVID-19 [20]. In this study, we noticed a significant increase in the level of IL-10 in low, medium, and high-strength vaccinated groups as compared with the control group. This indicates that anti-inflammatory elements are also active in the SN of cells from vaccinated individuals 8 months after vaccination with Razi-Cov-Pars. This may be an indication of long COVID-19 symptoms in certain patients who may have had reinfection and warrants further follow-up studies.

TGF-ß inhibits T cell proliferation and activation. TGF-ß was elevated during the early phase of SARS, a phenomenon that may be associated with lung infiltration and proliferation [21]. In our study, the results of TGF-ß showed a significant increase in the group receiving the high-strength vaccine while no significant difference was noticed in the low and medium-strength group compared with the control group. Considering the immune-suppressive features of TGF-ß, further studies on the long-term effect of the vaccine are necessary to evaluate the dynamics of these cytokines.

Conclusion

The results in previous clinical studies and our study showed that the recombinant spike protein COVID-19 vaccine is safe and well-tolerated. Moreover, it does not cause serious adverse reactions in healthy adult volunteers. We detected a significant stimulation in the lymphocytes isolated from vaccinated individuals after 16 months by measurement of proliferative potential and cytokine gene expression thereby assessing the long-term effect of the vaccine.

Ethical Considerations

Compliance with ethical guidelines

This research was approved by the Institutional Ethics Board of Razi Vaccine & Serum Research Institute (Code: IR.NREC.1399.005).

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Authors' contributions

All authors equally contribute to preparing all parts of the research.

Conflicts of interest

The authors declared no conflict of interest.

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