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Full Length Article

Infection with SARS-CoV-2 primes immunological memory in human nasal-associated lymphoid tissue

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ABSTRACT

Background: The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, has resulted in considerable morbidity and mortality in humans. Little is known regarding the development of immunological memory following SARS-CoV-2 infection or whether immunological memory can provide long-lasting protection against reinfection. Urgent need for vaccines is a considerable issue for all governments worldwide.

Methods: A total of 39 patients were recruited in this study. Tonsillar mononuclear cells (MNCs) were co-cultured in RPMI medium and stimulated with the full-length SARS-CoV-2 spike protein in the presence and absence of a CpG-DNA adjuvant. An enzyme-linked immunosorbent assay (ELISA) was utilised to measure the specific antibody response to the spike protein in the cell culture supernatants.

Results: The SARS-CoV-2 spike protein primed a potent memory B cell-mediated immune response in nasal-associated lymphoid tissue (NALT) from patients previously infected with the virus. Additionally, spike protein combined with the CpG-DNA adjuvant induced a significantly increased level of specific anti-spike protein IgG antibody compared with the spike protein alone ($p < 0.0001$, $n = 24$). We also showed a strong positive correlation between the specific anti-spike protein IgG antibody level in a serum samples and that produced by MNCs derived from the same COVID-19-recovered patients following stimulation ($r = 0.76$, $p = 0.0002$, $n = 24$).

Conclusion: Individuals with serological evidence of previous SARS-CoV-2 exposure showed a significant anti-spike protein-specific memory humoral immune response to the viral spike protein upon stimulation. Additionally, our results demonstrated the functional response of NALT-derived MNCs to the viral spike protein. CpG-DNA adjuvant combined with spike protein induced significantly stronger humoral immune responses than the spike protein alone. These data indicate that the S protein antigen combined with CpG-DNA adjuvant could be used as a future vaccine candidate.

1. Introduction

The outbreak of the COVID-19 has rapidly evolved into an international pandemic [14]. Development and designing of vaccines effectively beneficial to control the spread of infection is crucial therefore, governmental global attentions have focused huge efforts on vaccine development [38]. Spike protein is currently an optimal immunological target for vaccines design and development against the SARS-CoV-2 [45].

Mucosal immunity is an essential component of the immune system [24]. The nasal cavity is the entrance point for mucosally administered vaccines and is comprised of a vast and vascularised epithelial layer with

a large surface area at the nasal opening, representing a suitable route for vaccine delivery [21]. NALT is a central inductive site for immune responses following both natural infections and vaccination. It is recognised to serve as a vital immune niche for mucosal and systemic immunity against upper respiratory tract pathogens [5,24,48]. The adenoids and tonsils are components of the mucosal immune system, primarily composed of B cells (65%), in addition to approximately 30% CD3⁺ T cells and 5% macrophages [7,44].

Mucosal vaccination has previously been used against several respiratory tract infections, such as the influenza virus, in the form of nasally delivered live-attenuated influenza vaccines [27]. It typically requires the use of a live-attenuated vaccine, an inactivated virus, or a

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subunit vaccine antigen constructed with adjuvant [4]. CpG-DNA is a commonly used adjuvant in vaccine development. It improves the function of specialised antigen-presenting cells (APCs) and enhances the generation of humoral and cellular vaccine-specific immune responses. Clinical trials have shown that CpG-DNA adjuvants are associated with safe outcomes and result in the increased immunogenicity of the co-administered vaccine [6].

Following the recent declaration of COVID-19 vaccine effectiveness during clinical trials by various companies, resulting in protection against SARS-CoV-2, a widespread post-efficacy plan for subsequent phases of vaccination for the global population has become essential [23].

The development of a safe and efficient SARS-CoV-2 vaccine required 6–18 months. Currently over 50 candidate vaccines undergoing human clinical trials and 18 undergoing efficiency analyses [39]. Based on recent studies, the vaccine developed by Pfizer/BioNTech has stated an effectiveness of 95% [42]; Gamaleya has publicised an efficiency of 92%; Moderna has reported an efficacy of 94.5%; and AstraZeneca has declared an effectiveness of 70% [47]. Therefore, the abilities of these vaccines to protect against this deadly virus are likely to represent the primary preventive measure used to protect individuals from infection.

Madinah is one of the most SARS-CoV-2 prevalent cities in Saudi Arabia. Therefore, Madinah is expected to show higher levels of disease prevalence compared with other parts of the country [32].

In our recent study, we successfully used the current model to evaluate the humoral immune responses to SARS-CoV-2 spike proteins and sowed the ability of NALT derived MNCs to be suitable human model for testing the mucosal humoral immune response [31]. In that study, we designed, developed and optimized the cell culture model using S1, S2 and full-length S proteins antigen for MNCs stimulation. In addition, we used different inclusion and exclusion criteria of the patients to design and perform that study. Therefore, neither the subjects nor the results in that study have been used and included in the current study by any mean except the methodologies.

In the current study, we inspected the human MNCs as a cell culture model from the tonsils to test the priming of the humoral immunological memory response to the full-length S antigen protein of SARS-CoV-2. We moreover tested the effect of the CpG-DNA adjuvant in combination with the S protein to induce antibody response with and without the adjuvant. We used ELISA to measure the anti-S antibody levels in serum and cell culture supernatants following MNCs stimulation with the S protein.

2. Materials and methods

2.1. Tonsillar samples

Only those patients who suffered from snoring or obstructive sleep apnoea were included in this study. Patients with recurrent tonsillitis were excluded. Patients who were immunocompromised in any way were excluded. Recruited patients were divided into two groups based on ELISA results and Ministry of Health (MOH) records for confirmed RT-PCR results of viral detection. First, those who were previously infected with SARS-CoV-2 (seropositive for anti-S IgG antibody). Second, those with no history of infection (seronegative for anti-S IgG antibody).

Tonsillar tissue samples were collected from 39 patients who underwent a tonsillectomy at the department of Ear, Nose and Throat (ENT) at Saudi Germany Hospital, Madinah Region, Saudi Arabia, between 20 September 2020 and 30 February 2021. A venous blood sample was obtained before admission. All adult patients signed informed consent, and parents of children patients signed consent before conducting the study. The patients ranged in age from 1.5 to 38 years and included 20 male and 19 female. Among those COVID-19-recovered participants, there were six asymptomatic and eighteen mild to moderate patients. The disease severity was classified as the MOH protocol, which obtained from the hospital. <https://www.moh.gov.sa/Minist>

[ry/MediaCenter/Publications/Documents/MOH-therapeutic-protocol-for-COVID-19.pdf](#) (accessed on 23.08. 2021).

2.2. Isolation of tonsillar mononuclear cells (MNCs)

Tonsillar MNCs were prepared via an adapted cell suspension procedure based on a previously described method [33]. Briefly, the tonsillar samples were treated immediately following surgery under sterile conditions to obtain fresh cultures. The tissues were shredded into small pieces using a scalpel and transferred to Petri dish. The tissues were then lifted to release cells into the medium (RPMI 1640 medium containing HEPES and supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin, referred to as RPMI complete medium). The cell suspension was then passed through a 70-µm nylon mesh to eliminate all tissue fragments. MNCs were isolated using Ficoll-Paque gradient centrifugation (Premium GE Healthcare, UK) at 400 ×g for 30 min. The cells were rinsed twice using phosphate-buffered saline (PBS) and then resuspended in 5 ml of RPMI complete medium. Lastly, MNCs were adjusted to reach the optimal cell concentration at 4×10^6 cells/mL.

2.3. Recombinant SARS-CoV-2 S protein

2.3.1. SARS-CoV-2 full-length S protein

The recombinant SARS-CoV-2 full-length S protein (S1 + S2 ectodomain), consisting of Val 16–Pro 1213, was expressed with a poly-histidine tag at the C-terminus (Sino Biological, Beijing, China) in HEK293 cells. The recombinant protein is 1209 amino acids. It was reconstituted in sterile Phosphate-Buffered Saline (PBS) RNase-free (Thermo Fisher, USA).

2.4. CpG oligonucleotides (CpG-DNA)

CpG oligonucleotides (CpG-ODN2006, InvivoGen, San Diego, CA, USA) are synthetic oligonucleotides that contain unmethylated CpG dinucleotides in specific sequence contexts (CpG motifs). CpG-DNA was freshly reconstituted in sterile endotoxin-free water before use.

2.5. Cell culture and NALT MNCs

2.5.1. Stimulation of NALT MNCs for antibody production

All patients' samples were divided into two groups. First, those who were previously diagnosed with COVID-19 and recovered ($n = 24$). Second, those who had no history of COVID-19 and showed no evidence of previous infection ($n = 15$), as assessed by ELISA and the MOH database. All procedures described here were applied to the tonsillar tissues from both groups. Following NALT derived MNCs isolation, the cell suspension was adjusted to a density of 4×10^6 cells/mL. We have performed several titrations of the spike antigen and CpG to reach the optimal concentration for each of them. CpG-DNA adjuvant was reconstituted in sterile endotoxin-free water before use, and the full-length S protein (20 µg/ml) was mixed with reconstituted CpG-DNA (5 µg/ml). The MNCs were cultured in RPMI complete medium in the presence of S protein with and without the adjuvant to stimulate MNCs. Unstimulated MNCs were used as a negative control. A volume of 250 µL of cell suspension was cultured in a sterile 96-well cell culture plate (Costar) and placed in incubator in a humidified 5% CO₂ atmosphere at 37 °C. Cell culture supernatants were collected after 10 days and stored at –70 °C until assayed by ELISA to measure the antibodies induced in response to stimulation by the viral S protein.

2.5.2. Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed using a previously described assay [30]. In brief, a 96-well ELISA plate (Costar; Corning, Corning, NY, USA) was coated with 2 µg/mL of SARS-CoV-2 S protein (purchased from Sino Biological, Beijing, China) using a volume of 100 µL/well. The plate was

incubated overnight at 4 °C, and then washed five times with PBS containing 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA). The plate was blocked with 150 µL/well blocking buffer for an hour at room temperature. The cell culture supernatant samples were diluted in blocking buffer at a dilution of (1:5) whereas serum samples diluted at (1:100), before being added to the plate at 100 µL/well. Following that, the plate was incubated for 30 min at room temperature, and then followed by washing five times with washing buffer. Alkaline phosphatase-conjugated goat anti-human IgG, IgM, and IgA secondary antibodies (Sigma-Aldrich) diluted at 1:1000, 1:2000, and 1:1000, respectively and then added at 100 µL/well. The plate was incubated at room temperature for 30 min before being washed five times with washing buffer. Finally, 100 µL/well of *p*-nitrophenyl phosphate substrate (*p*-NPP, Sigma-Aldrich), was added. Away from direct light the plate was kept. After 30 min, 100 µL of stopping solution was added (1.2 N sodium hydroxide, Reagecon, UK). The optical density (OD) at 405 nm was measured using a microplate reader (ELX800; BioTek). The samples were described as positive if the OD₄₀₅ value was three standard deviations (SD) above the mean of the negative controls (NCs). The designed cut-off OD₄₀₅ value (mean NCs + 3 SD) was $0.19 + (3 \times 0.033) = 0.29$.

The study was approved by the Taibah University Ethical Committee (IRB No. MLT 2020030).

2.6. Statistical analysis

GraphPad Prism statistical software (version 9, USA) used for analysis. Data are expressed as the mean ± standard error of the mean (SEM). Differences between the two groups were analysed using Student's *t*-test, and a paired *t*-test was used to compare paired samples. Associations were assessed using two-tailed and alpha = 0.05. The Mann-Whitney test was used to compare the mean of anti-s IgG antibody levels between children and adults as well as across the severity groups (asymptomatic and mild-moderate) in those previously infected individuals. Simple linear regression model was performed to explore the association between age (predictor) and anti-s IgG antibody levels (outcome). A value of $p < 0.05$ was considered significant for all tests.

3. Results

3.1. Comparison of the humoral immune responses of MNCs of previously SARS-CoV-2 infected and non-infected patients

Based on seropositivity results as an evidence of previous infection of SARS-CoV-2 (group one) and seronegativity for non-infected (group two), the S protein-stimulated tonsillar MNCs were divided into two groups. To investigate the differences in the humoral immune responses between the two groups, we used ELISA to analyse the cell culture supernatants collected from cultured MNCs following stimulation. Significant specific anti-S protein IgG antibody levels were detected following S protein stimulation of tonsillar MNCs from patients with previous SARS-CoV-2 exposure compared with those in the non-infected group (Fig. 1, $p < 0.0001$, $n = 24$). Additionally, we observed significant specific IgM and IgA antibody levels in cell culture supernatants of MNCs of COVID-19 recovered patients when compared with those from non-infected individuals. This result suggests the presence of memory B cells in tonsillar tissue that were primed upon stimulation with the viral spike protein antigen.

3.2. CpG-DNA adjuvanted-S protein induces stronger humoral immune responses than S protein

To determine the ability of the CpG-DNA adjuvant in increasing the magnitude humoral immune responses when added to S protein, we analysed the cell culture supernatants of MNCs following stimulation in the presence and absence of the CpG-DNA adjuvant. CpG-DNA adjuvant provoked for a stronger humoral immune response when added to the S

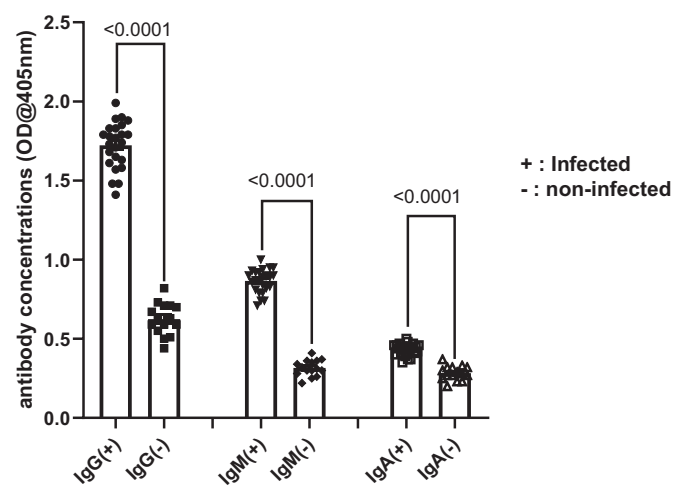


Fig. 1. Comparison between the specific anti-S protein IgG antibody levels in the cell culture supernatants following the S protein stimulation of MNCs from recovered COVID-19 and those without evidence of infection. Significantly increased IgG, IgM and IgA antibody levels were detected in MNCs derived from previously infected patients compared with those from MNCs derived from non-infected patients ($p < 0.0001$, $n = 24$). Data are expressed as the mean ± standard error of the mean (SEM).

protein. Significant anti-S antibody isotypes of the IgG, IgM and IgA (Fig. 2, $p < 0.001$, $n = 15$) in MNCs stimulated in the presence of the adjuvant when compared with that stimulated with the S protein alone of same non-infected individuals. This result supports the advantage of using CpG-DNA adjuvanted S protein as a potential candidate for intranasal administered vaccine against the novel virus.

3.3. Correlation between systemic and mucosal anti-S protein IgG antibody levels in COVID-19 recovered patients

To investigate the correlation between the systemic and mucosal humoral immune responses in COVID-19 recovered patients, we

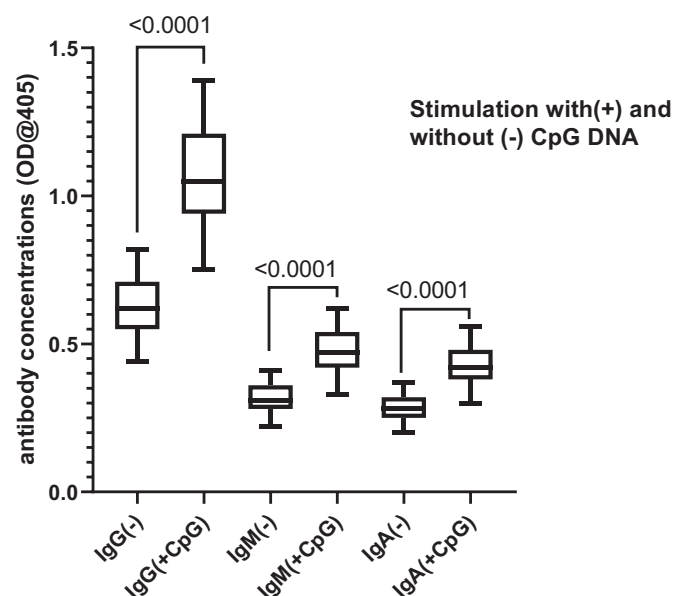


Fig. 2. The effect of S protein combined with CpG-DNA following stimulation of MNCs. The figure shows significantly increased antibody levels of MNCs stimulated with adjuvanted S protein compared with stimulation by S protein alone ($p < 0.0001$, $n = 15$). Data are expressed as the mean ± standard error of the mean (SEM).

measured the anti-S IgG antibody levels serum and the cell culture supernatants of S protein-stimulated tonsillar MNCs in paired samples using ELISA. A significant positive correlation was observed when we correlated the anti-S protein IgG antibody levels of the COVID-19 recovered patients' serum with that of the cell culture supernatant from MNCs derived from the same patient (Fig. 3, $r = 0.76$, $p = 0.0002$, $n = 15$).

3.4. Associations between mucosal anti-S IgG antibody levels, age and disease severity in COVID-19 recovered patients

We investigated the difference of anti-S IgG antibody levels between adults and children. We found that the mean of anti-S IgG antibody levels of children was significantly lower than the mean anti-S IgG antibody levels of adults (1.62 ± 0.11 vs. 1.85 ± 0.06 in unit of ODs, respectively, $p < 0.001$) Fig. 4.

Additionally, we investigated the difference between anti-S IgG antibody levels of asymptomatic and that of mild to moderate COVID-19-recovered patients. We found that no significant difference between mean of anti-S IgG antibody levels of asymptomatic and that of mild to moderate participants (1.81 ± 1.33 vs. 1.70 ± 0.15 in unit of ODs, respectively, $p = 0.102$). Interestingly, simple linear regression analysis showed that age of previously infected participants was a significant predicted levels of anti-S IgG antibody ($B = 0.011$, $SE = 0.001$ (95% confidence interval: 0.009 to 0.013), $p < 0.001$, R -square = 0.88).

4. Discussion

Tonsils are lymphoepithelial compartments involved in direct interactions with inhaled or swallowed environmental antigens.[8] The capability of tonsillar tissues to function as the induction site of local or B cell immune responses in humans has previously been studied. We have showed previously the frequencies of influenza vaccine-specific ASCs in cell suspensions derived from adenotonsillar tissues, which showed a robust priming effect for the immunological memory immune response in patients who were previously infected with the influenza virus [33].

Our current study is the first to prove a significant memory B cell response to SARS CoV-2 in human NALT one year and half after the incidence of Covid-19 pandemic. Upon viral spike antigen protein

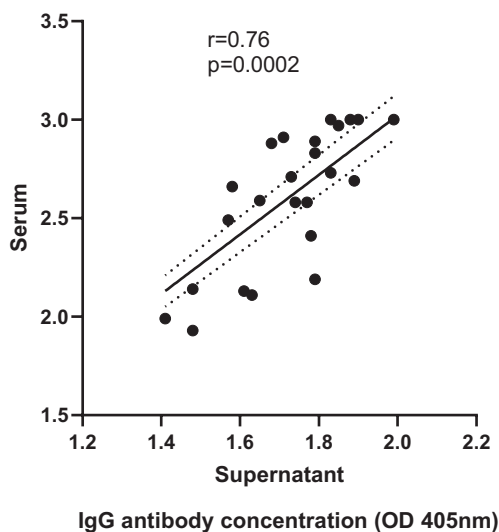


Fig. 3. Correlation between serum anti-S protein IgG antibody level and that detected in the cell culture supernatants of stimulated r MNCs derived from patients who were previously infected with SARS Co-V-2. A strong positive correlation was observed between IgG serum antibody level and that induced mucosal response of MNCs (Pearson's $r = 0.76$, $p = 0.0002$, $n = 24$).

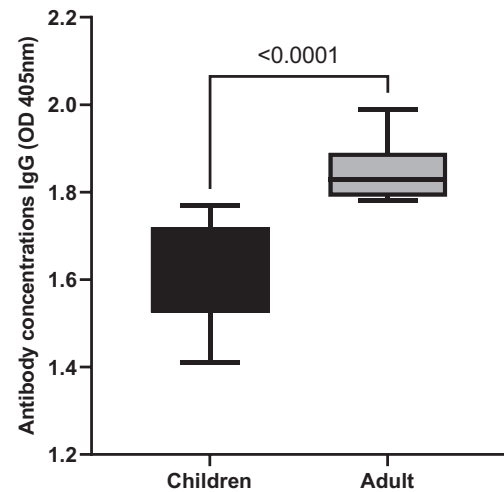


Fig. 4. Comparison between the anti-S protein IgG antibody levels in the cell culture supernatants of children compared with that in adults recovered COVID-19 and those without evidence of infection. The mean of anti-S IgG antibody levels of children ($n = 13$) was significantly lower than that of adults ($n = 11$) (1.62 ± 0.11 vs. 1.85 ± 0.06 in unit of ODs, respectively, $p < 0.001$).

stimulation, the memory B cell response in previously infected patients produced higher IgG antibody levels against the viral spike protein. Similar study on influenza viruses showed the presence of plasmablasts secreting neutralizing antibodies in patients infected with pH1N1 [11]. We presumed that SARS CoV-2 infection may activate pre-existing memory B cells targeting the viral spike protein.

The development of intranasal vaccines against COVID-19 disease is of great importance for identifying alternative routes for vaccination other than the current injectable vaccines. Intranasal vaccination procedures rely on the enriched immune system components found in the upper respiratory tract (URT), which represents the first site to react with subsequent antigen presentation. The URT contains a complex system of lymphoid tissues, containing draining lymph nodes and NALT [41].

The current results demonstrate the priming effects mediated by immunological memory cells in tonsillar MNCs, which is promising for researchers, indicate that mucosal sites can be tested to design, and develop intranasal vaccines against the emergent SARS-CoV-2 virus. Freshly differentiated memory B cells in human NALT have previously been described to be able to hastily migrate to the epithelial cell layer of NALT [26]. Our findings suggested that tonsillar MNCs from COVID-19-recovered patients were primed with an immunological memory against the S protein, which may reflect a higher activation state for ASCs in tonsils. Our results were supported by a previous study that showed a high frequency of memory B cells residing in the tonsils compared with the frequency of memory B cells in the peripheral blood [40]. The tendency of the spike protein to prime the memory immune cells activity in COVID-19-recovered patients also suggests the potential of developing an intranasal vaccine against the virus; therefore, additional investigations should be performed to investigate other immune components, such as cellular immunity.

Following a SARS-CoV-2 infection, immunological memory, in the form of antibodies as well as memory B cells, has been shown to persist for more than 8 months after symptom onset [12]. The humoral immune response to infection or vaccination results in two primary outcomes. First, antibodies are produced by ASCs, which offers rapid protective immunity. Second, the generation of long-lived memory B cells occurs, which are capable of intensifying immune recall responses [17].

Our results showed the presence of specific anti-S protein IgM and IgA isotype antibodies in COVID-19-recovered patients, but these antibodies were expressed at lower intensity levels compared with the

dominant IgG memory antibody type. IgA antibodies are commonly thought to represent the major antibody type at the mucosal level. However, whether mucosal IgA memory can be induced in humans via either natural infections or vaccination remains under debate. Several studies have reported that antigen-specific mucosal IgA responses are short-lived, and re-vaccination does not consistently provoke memory IgA responses [25,37]. Although IgA ASC numbers were described to increase in tonsillar cells following influenza immunisation, they were expected to principally characterize a primary IgA response rather than a memory response [9]. The strength of the antigen-specific IgG memory B cell responses to the SARS-CoV-2 S protein antigen in tonsillar tissues that was observed in the current study agrees with previous studies that have described a large number of IgG memory B cells that responded to influenza virus hemagglutinin in human NALT [7,33,36].

Intranasal vaccination has been shown to be effective for the stimulation of mucosal immunity [10]. Therefore, the administration of the vaccine through the nasal cavity route should be able to elicit both humoral and cell-mediated antigen-specific immune responses [49].

Our result shows the ability of the full-length SARS-CoV-2 S protein to prime a potent memory mucosal humoral immune response, resulting in a specific anti-S protein IgG antibody. The full-length S protein consists of two subunits (S1 and S2) and is, therefore, larger in size (1209 amino acid) than the separated subunits individually. Consequently, we have shown in a previous study the failure of S1 and S2 to stimulate the MNCs [31]. The addition of the CpG-DNA (known to be polyclonally stimulate memory B cells) adjuvant results in further increasing the mucosal immune response capacity and resulting in inducing higher levels of antibody production. The adjuvant commonly adds and improves the activation of the innate and adaptive immune responses, magnifying immunogenicity and increasing the effectiveness of the vaccine [43].

As we stated previously that NALT is one of the key machineries of the structured lymphoid tissue, in addition to containing all of the immunocompetent cells that are prerequisite for the generation of antigen-specific immune responses. It is hence expected to have a significant character in the development of a 'nasal vaccine' [24].

Continued progress is being made in the production of vaccines against the emergent SARS-CoV-2 virus, which are being verified by various companies; for example, Altimmune, CanSino Biologicals, Moderna, and Novavax are all in the process of producing new vaccines [45]. The intranasal vaccine delivery ChAdSARS-CoV-2 generates strong mucosal B and T cell responses and has been shown to induce high levels of SARS-CoV-2 neutralizing antibodies. Additionally, this vaccine has been shown to deter both URT and lower respiratory tract infections in mice, which may potentially protect against SARS-CoV-2 infections and transmission [19].

The CpG-DNA adjuvant works to increase the magnitude of the humoral immune response induced by vaccines against an enormous number of pathogens. It has been used in several vaccines such as, tetanus toxoid [13], *Haemophilus influenzae* type b virus [20], measles virus, and hepatitis B surface [34], which resulted in antigen-specific antibody titres that improved and expanded by up to three times in magnitude [22]. The use of the CpG-DNA adjuvant also has the capability to support follicular helper T cells (T_{FH}), enhancing antigen-induced antibody responses in NALT tissues, which be vital for forthcoming vaccination strategies against respiratory pathogens [2].

The current NALT model has been comprehensively considered to represent a successful human model for studying the diversity of responses to viral and bacterial respiratory pathogens.[1,18,50,51].

The ability of the S protein to provoke the production of anti-S protein antibodies by B cells in NALT will allow for further investigations of this human-derived cell culture model to study the response to other SARS-CoV-2 antigens such as the matrix and nucleocapsid proteins. Our study showed the predominance of an IgG antibody response over the IgM and IgA isotypes, providing evidence of previous virus exposure, and a strong correlation was observed between the anti-

S protein antibody titration levels between serum samples and MNC production from the same subjects. Our study agrees with a previous report that showed that B cells of the IgG isotype were predominant in tonsillar tissues, whereas B cells of the IgM and IgA isotypes were relatively minor [15].

The presence of a memory immune response could provide protection against reinfection [28], and the persistence of the IgG antibody has been identified over the long-term in COVID-19-recovered patients with different disease presentations [29]. Moreover, a recent study showed prolonged humoral as well as cellular immunity in recovered COVID-19 patients [3].

To the best of our knowledge, our study is the first study to use the SARS-CoV-2 S protein, with and without a CpG-DNA adjuvant, to stimulate human NALT-derived MNCs to study mucosal immunity and demonstrates the functional responsiveness of these immune cells. Additionally, our study is the first to demonstrate the recall of the memory humoral immune response in the tonsillar tissues of individuals who have recovered from a previous infection with the novel SARS-CoV-2. Therefore, additional studies focusing on the mucosal immune responses would be of a great impact on global public health.

It becomes obvious that there is a huge variation in the immune response to SARS-CoV-2 in children and adults, together at the innate as well as adaptive levels [35]. We showed that a significantly higher anti-S IgG antibody levels against SARS-CoV-2 in adults compared with children. Our finding agrees with a study found that the antibodies titration to SARS-CoV-2 in children was low compared to that of the adults [46]. Existing data proposes that the activity of innate immunity appears to be central to the primary stages of SARS-CoV-2 infection whereas adaptive memory immunity is vital to avert reinfection [16].

Further investigations remain necessary to explore other immune responses, such as the cellular immune response. Moreover, using other viral antigen proteins, such as the nucleocapsid and matrix proteins, would be useful to identify an optimal candidate mucosal vaccine, in addition to examining these various antigens in conjunction with using other safe and well-studied adjuvants, such as aluminium hydroxide and aluminium phosphate.

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