Polish Journal of Veterinary Sciences Vol. 29, No. 2 (2026), 281–289

DOI

Original article

Novel bivalent mucosal antigen formulation targeting SARS-CoV-2 and influenza: immunogenicity assessment using zan in vitro human nasal-associated lymphoid tissue model

W.H. Mahallawi

[1] Clinical Laboratory Sciences Department, College of Applied Medical Sciences, Taibah University, Prince Nayef bin Abdulaziz Road, 42353, Madinah, Saudi Arabia
[2] Health and Life Research Center, Taibah University, Prince Nayef bin Abdulaziz Road, 42353, Madinah, Saudi Arabia

Correspondence to: W.H. Mahallawi, e-mail: wmahallawi@taibahu.edu.sa

Abstract

The development of mucosal vaccines is crucial for controlling the transmission of respiratory viruses such as SARS-CoV-2 and influenza at their point of entry. This study aimed to validate a human tonsil-derived in vitro model for screening antigen formulation candidates and to evaluate the immunogenicity of a novel bivalent formulation. Mononuclear cells (MNCs) isolated from human palatine tonsils (n=20) were stimulated with full-length SARS-CoV-2 spike (S) glycoprotein, influenza A (H1N1) hemagglutinin (HA), or a combination of both (bivalent) at a concentration of 20 µg/mL. After a 10-day culture, antigen-specific IgG, IgM, and IgA antibody levels were quantified by enzyme-linked immunosorbent assay (ELISA). Stimulation with individual antigens elicited robust polyisotypic antibody responses. The S protein induced significant fold-increases over unstimulated controls for IgG (8.23, $p<0.0001$), IgM (5.77, $p<0.0001$) and IgA (4.56, $p<0.0001$). Similarly, the HA protein induced increases in IgG (7.16, $p<0.0001$), IgM (4.99, $p<0.0001$) and IgA (3.97, $p<0.0001$). Critically, bivalent stimulation resulted in superior additive responses. The anti-S component showed fold-increases of 11.09 (IgG, $p<0.0001$), 7.61 (IgM, $p<0.0001$) and 5.97 (IgA, $p<0.0001$). Similarly, the anti-HA component showed 9.66 (IgG, $p<0.0001$), 6.58 (IgM, $p<0.0001$) and 5.27 (IgA, $p<0.0001$), with no reduction in antigen-specific responses observed in the combined condition. The NALT model is a physiologically relevant platform for preclinical screening. It provides a compelling in vitro rationale for developing a bivalent intranasal antigen formulation to elicit potent mucosal immunity against major respiratory pathogens.

Keywords: bivalent formulation, influenza A, intranasal immunization, mucosal immunization, SARS-CoV-2

Introduction

The mucosal surfaces of the upper respiratory tract (URT) serve as the primary entry point for numerous pathogens, including SARS-CoV-2 and Influenza A virus, which are of major concern in both human and veterinary medicine due to their zoonotic potential and impact on public health (Iwasaki and Pillai 2014). The global impact of these viruses underscores the critical need for vaccines that can establish effective immune barriers at these sites. While intramuscular vaccines have been pivotal in reducing severe disease and mortality (Ma et al. 2024), their limited capacity to elicit robust mucosal immunity often permits breakthrough infections and ongoing viral transmission (Fiolet et al. 2022).

A key component of the URT's defensive arsenal is the nasal-associated lymphoid tissue (NALT) which, in humans, includes the palatine tonsils and adenoids (Lapuente et al. 2021). This inductive site is rich in B-cells, T-cells and antigen-presenting cells. It is responsible for generating potent mucosal immune responses, including the production of secretory IgA (SIgA) and the formation of tissue-resident memory cells (Son and Sun 2021). SIgA is particularly crucial as it can neutralize pathogens directly in the mucosal lumen without provoking inflammation, a first line of defense that is poorly engaged by systemic vaccination (Li et al. 2020).

Therefore, the development of intranasal immunization strategies that target the NALT represents a promising strategy to achieve sterilizing immunity (Rhee 2020). A significant challenge in this endeavour is the lack of predictive human models for preliminary screening. Immortalized cell lines cannot recapitulate the complex cellular interactions of organized lymphoid tissue. The use of an ex vivo human tonsil model offers a unique and ethically sound alternative to bridge this gap, providing direct insight into human mucosal immunology (Kim et al. 2022).

The utility of human tonsil-derived models for evaluating mucosal immunity is increasingly recognized. Recent studies have successfully leveraged ex vivo tonsil organoids or mononuclear cell (MNC) cultures to dissect immune responses to vaccines and pathogens, validating this system's physiological relevance for preclinical screening (Bonaiti et al. 2024). These models effectively recapitulate key features of the human mucosal immune response, including B cell activation and antibody production. Building upon this established foundation, we used a well-characterized in vitro tonsillar MNC model (Mahallawi and Aljeraisi 2021) to address a distinct and pressing question: the capacity of such a system to elicit simultaneous, robust antibody responses to two major respiratory pathogens when stimulated with a bivalent antigen formulation. This approach allows us to specifically probe the potential additive effects, a crucial consideration for next-generation multivalent mucosal vaccine design.

This study aimed to utilize a human tonsil-derived NALT model to assess the immunogenicity of a novel bivalent antigen formulation construct. We hypothesized that stimulation of tonsillar mononuclear cells with the full-length S and HA proteins would elicit robust polyisotypic antibody responses, thereby validating both the antigens and the in vitro model itself. The results provide essential preclinical data supporting the further development of this innovative mucosal antigen formulation.

Materials and Methods

Tissue acquisition and ethical statement

Human palatine tonsils were obtained from 20 patients (age range: 5-24 years) undergoing routine elective tonsillectomy for obstructive sleep apnea at Madinah Hospital, Saudi Arabia. All procedures were performed in accordance with the ethical standards of the institutional research committee and with the Helsinki Declaration. The study protocol was reviewed and approved by the Taibah University Institutional Review Board (IRB No. MLT 250221). Written informed consent was obtained from all adult participants and from the parents or legal guardians of minors. The study was conducted from February 2025 to April 2025.

Inclusion and exclusion criteria

All agreed patients and patients' guardians were included. Gender: The cohort consisted of 11 males and 9 females. Health Condition: All patients were undergoing tonsillectomy for obstructive sleep apnea. Individuals with a history of recurrent tonsillitis (≥5 episodes per year), any known immunodeficiency, chronic illness, a prior confirmed diagnosis of SARS-CoV-2 or influenza infection, as determined by patient history and medical record review, or who were smokers, were excluded from the study.

Isolation of tonsillar mononuclear cells (MNCs)

Our previous human model was used with slight modifications (Mahallawi and Aljeraisi 2021). Tonsillar tissues were immediately placed in cold Hanks' Balanced Salt Solution (HBSS; Gibco, USA) supplemented with 2 mM L-glutamine, 50 U/mL penicillin,

and 50 µg/mL streptomycin (all from Sigma-Aldrich, USA) and processed within 2 hours of excision. The tissues were mechanically dissociated using sterile scalpels and then subjected to enzymatic digestion for 30 minutes at 37°C under continuous agitation. The resulting cell suspension was passed through a 70-µm cell strainer (Falcon, USA) to obtain a single-cell suspension. Tonsillar MNCs were isolated by density gradient centrifugation using Ficoll-Paque PLUS (Cytiva, USA) at 400×g for 30 minutes at room temperature. The isolated MNCs were washed twice with PBS, counted, and resuspended at a concentration of 4×10^6 cells/mL in complete RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Antigen preparation and stimulation

Recombinant SARS-CoV-2 full-length spike glycoprotein (S1+S2 ECD, His-tagged, Cat. No. 40589-V08B1) was derived from the Wuhan-Hu-1 strain (the original wild-type strain). The influenza A H1N1 full-length hemagglutinin protein (HA; Cat. No. 11085-V08B) from Sino Biological was derived from the A/California/07/2009 (H1N1) strain, which is the prototypical A(H1N1)pdm09 virus. This is the strain that has been circulating globally since the 2009 pandemic and is included in seasonal influenza vaccines.

Lyophilized proteins were reconstituted in sterile phosphate-buffered saline (PBS) to a stock concentration of 1 mg/mL. Preliminary MTT viability assays across a concentration range of 5-40 µg/mL confirmed that 20 µg/mL was non-cytotoxic, with cell viability consistently exceeding 85% in stimulated cultures compared to unstimulated controls. An optimal, non-cytotoxic stimulatory concentration of 20 µg/mL was determined for both antigens.

For stimulation, MNCs were seeded in 96-well flat-bottom plates at a density of 1×10^6 cells per well in 250 µL of complete medium. Cells were stimulated with 20 µg/mL of S protein, 20 µg/mL of HA protein, or left unstimulated (media-only control). The plates were incubated at 37°C in a humidified atmosphere of 5% CO2 for 10 days. Cell culture supernatants were harvested on day 10, centrifuged to remove any cellular debris, and stored at -70°C until analysis. The 10-day incubation period was selected based on our established previous protocols for in vitro human tonsil cell cultures (Mahallawi and Aljeraisi 2021), which are optimized to capture the peak of B-cell activation, differentiation and antibody secretion. This timeframe allows for the full differentiation of antigen-specific B cells into antibody-secreting cells (ASCs) and the subsequent accumulation of detectable levels of immunoglobulin in the supernatant (Wagar et al. 2021, Crotty et al. 2003). This timeframe optimally captures the peak of extracellular and early germinal center responses, making it a standard for preclinical mucosal immunogenicity screening.

Quantification of antigen-specific antibodies by ELISA

Our previously established human model was used with slight modifications (Mahallawi 2020). The levels of antigen-specific IgG, IgM and IgA in the culture supernatants were measured by indirect ELISA. Supernatants from wells stimulated with the bivalent (S+HA) cocktail were analyzed in parallel ELISAs. To quantify antibodies specific for each antigen, separate plates were coated with either the S protein or the HA protein. The antibody levels for each isotype (IgG, IgM, IgA) were then calculated independently for each antigen-coated plate. This allowed for the specific measurement of the anti-S and anti-HA responses within the polyclonal mixture generated by bivalent stimulation. High-binding 96-well plates (Corning, USA) were coated with 100 µL per well of either S protein or HA protein at a concentration of 2 µg/mL in carbonate-bicarbonate coating buffer (pH 9.6) and incubated overnight at 4°C. The plates were then blocked with 200 µL per well of PBS containing 0.05% Tween-20 and 0.5% heat-inactivated FBS for 1 hour at room temperature. After washing three times with PBS containing 0.05% Tween-20 (PBST), 100 µL of undiluted or diluted (1:5 in blocking buffer) supernatant samples were added to the wells and incubated for 2 hours at room temperature. After this wash, horseradish peroxidase (HRP)-conjugated goat anti-human IgG (1:10,000 dilution; Abcam, ab98567), IgM (1:5,000 dilution; Abcam, ab98575) or IgA (1:8,000 dilution; Abcam, ab97175) were added and incubated for 1 hour at room temperature. The reaction was developed using 100 µL of tetramethylbenzidine (TMB) substrate solution (Invitrogen, USA) and stopped after 15 minutes with 50 µL of 2 M sulfuric acid. The optical density (OD) was immediately measured at 450 nm using a microplate reader (BioTek, USA). The positivity threshold for each isotype was defined as an OD value greater than the mean OD of the unstimulated control wells plus three standard deviations.

Statistical analysis

Data are presented as the Mean and Standard Deviation (SD). Statistical analysis was performed using GraphPad Prism software (version 10). Diffe-

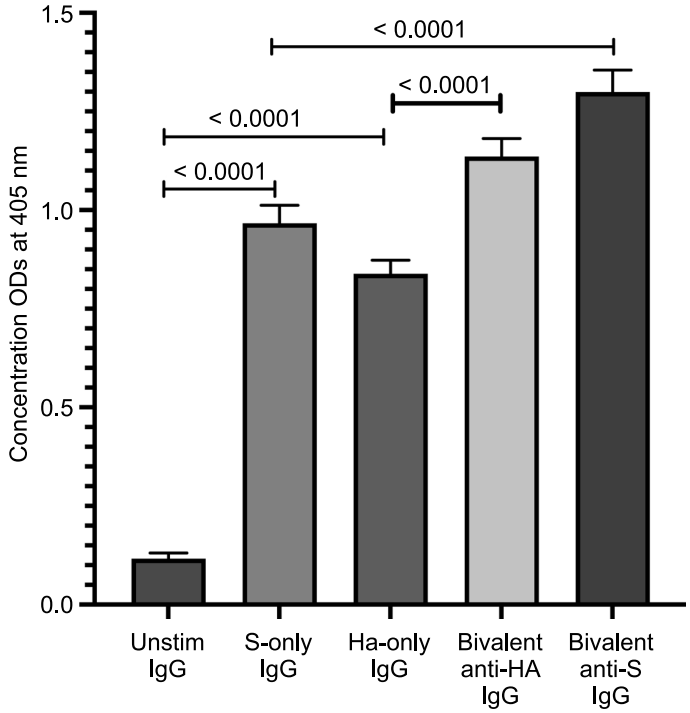

Fig. 1. Bivalent (Spike(S)+Hemagglutinin (HA)) combined viral proteins showed significant superiority in stimulating a potent anti-S IgG and anti-HA over the stimulation resulting from a single viral protein. Data are presented as mean and standard deviation (SD) ($p<0.0001$, n=20).

rences in antibody levels between antigen-stimulated and unstimulated control groups were analyzed using a paired Student's t-test. A p-value of less than 0.05 was considered statistically significant.

Results

Induction of higher anti-spike, anti-HA, and bivalent (S+HA) IgG antibody responses

Stimulation of human tonsillar MNCs with the full-length SARS-CoV-2 S protein (20 μg/mL) for 10 days induced a powerful and broad humoral immune response (Fig. 1). ELISA analysis of the culture supernatants revealed significant increases in the production of IgG antibody compared to the unstimulated control. The S protein alone triggered a significant 8.23-fold increase in anti-S IgG, while the influenza HA protein induced a 7.16-fold increase in anti-HA IgG. Bivalent (S+HA) stimulation showed significant superiority, resulting in an 11.09-fold increase in anti-S IgG and a 9.66-fold increase in anti-HA IgG over the unstimulated control ($p<0.0001$, n=20).

Higher anti-spike, anti-HA and bivalent (S+HA) IgM antibody responses

Stimulation with the individual and combined antigens also induced potent antigen-specific IgM responses, indicative of a robust primary humoral immune activation (Fig. 2). Monovalent stimulation with the SARS-CoV-2 S protein elicited a significant 5.77-fold increase in anti-S IgM over unstimulated controls. Similarly, the influenza HA protein induced a 4.99-fold increase in anti-HA IgM. Critically, the bivalent (S+HA) stimulation resulted in a superior IgM response against both viral targets, demonstrating an additive effect. The anti-S IgM levels in the bivalent condition showed a 7.61-fold increase, while the anti-HA IgM showed a 6.58-fold increase. This enhancement indicates that co-stimulation with both antigens amplifies the early, innate-like humoral immune response, consistent with broad and potent B-cell activation with no reduction in antigen-specific responses.

Higher anti-spike, anti-HA and bivalent (S+HA) IgA antibody responses

A crucial finding of this study was the potent induction of antigen-specific IgA, the primary antibody isotype responsible for mucosal immunity (Fig. 3). Stimulation with the S protein alone elicited a significant 4.56-fold increase in anti-S IgA, while the HA protein induced a 3.97-fold increase in anti-HA IgA compared to unstimulated controls. Most importantly, the bivalent condition led to enhancement of the mucosal IgA response, with increases exceeding those observed with either monovalent stimulation alone. The anti-S IgA levels increased by 5.97-fold, and the anti-HA IgA increased by 5.27-fold. This demonstrates the unique

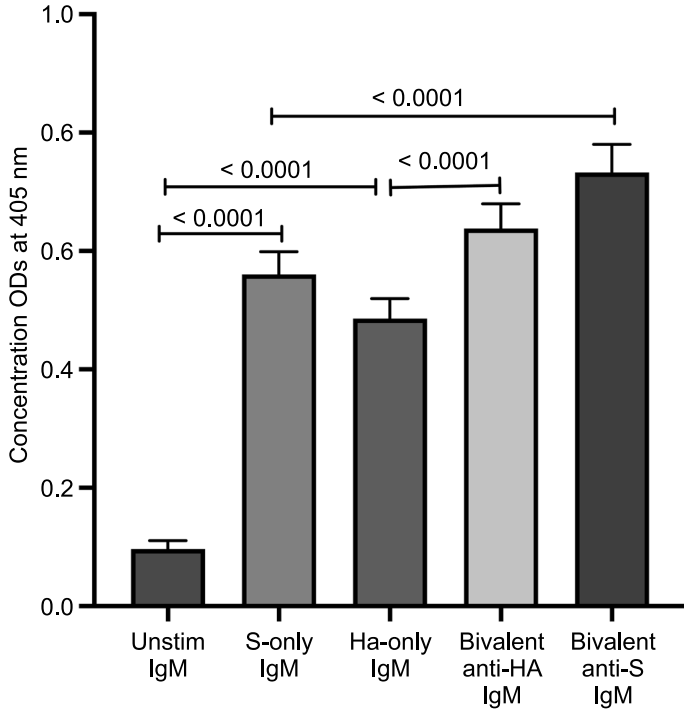

Fig. 2. Bivalent (Spike(S)+Hemagglutinin (HA)) combined viral proteins showed significant superiority in stimulating a potent anti-S and anti-HA IgM antibody over the stimulation resulting from a single viral protein. Data are presented as mean and standard deviation (SD) ($p<0.0001$, n=20).

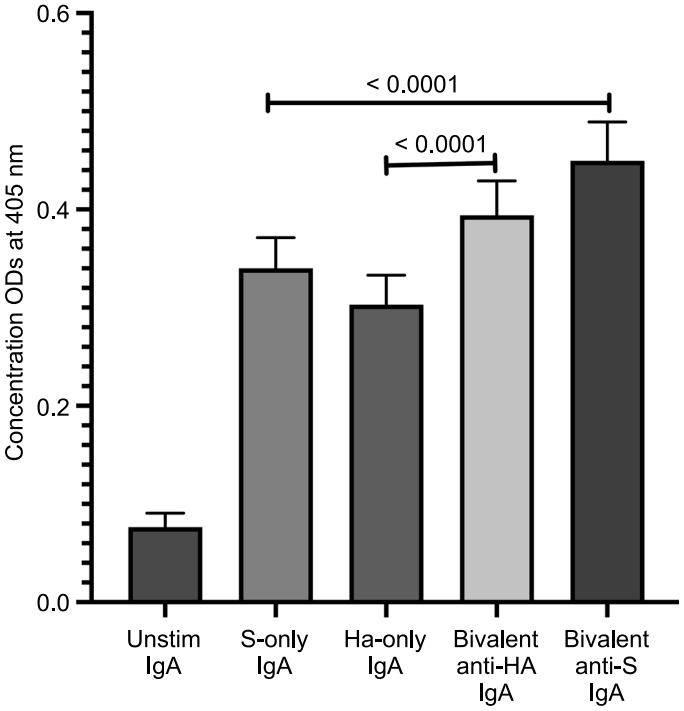

Fig. 3. Bivalent (Spike(S)+Hemagglutinin (HA)) combined viral proteins showed significant superiority in stimulating a potent anti-S and anti-HA IgA antibody over the stimulation resulting from a single viral protein. Data are presented as mean and standard deviation (SD) ($p<0.0001$, n=20).

capacity of the bivalent formulation to elicit a significantly stronger mucosal antibody response against both pathogens, which is vital for neutralizing viruses directly at their site of entry in the respiratory tract.

Additive humoral immune responses to bivalent antigen stimulation

The immunogenicity of individual and combined antigen candidates was comprehensively evaluated, with the increases summarized in Tables 1-4.

Table 1. Fold increases in antigen-specific IgG, IgM, and IgA antibody levels following stimulation of mononuclear cells with monovalent SARS-CoV-2 spike protein (20 μg/mL) for 10 days.

Antibody	Mean± SD Unstimulated	Mean± SD S-only	Fold-Increase
IgG	0.117 ± 0.015	0.963 ± 0.045	8.23x
IgM	0.097 ± 0.015	0.560 ± 0.040	5.77x
IgA	0.075 ± 0.015	0.342 ± 0.030	4.56x

Table 2. Fold increases in antigen-specific IgG, IgM, and IgA antibody levels following stimulation of mononuclear cells with monovalent influenza A (H1N1) hemagglutinin protein (20 μg/mL) for 10 days.

Antibody	Mean ± SD Unstimulated	Mean± SD HA-only	Fold-Increase
IgG	0.117 ± 0.015	0.838 ± 0.040	7.16x
IgM	0.097 ± 0.015	0.484 ± 0.040	4.99x
IgA	0.075 ± 0.015	0.298 ± 0.030	3.97x

Table 3. Fold increases in anti-SARS-CoV-2 spike protein-specific IgG, IgM, and IgA antibody levels following bivalent stimulation of mononuclear cells with S+HA (20 μg/mL each) for 10 days.

Antibody	Mean ± SD Unstimulated	Mean ± SD Bivalent anti-S	Fold-Increase
IgG	0.117 ± 0.015	1.298 ± 0.050	11.09x
IgM	0.097 ± 0.015	0.738 ± 0.040	7.61x
IgA	0.075 ± 0.015	0.448 ± 0.030	5.97x

Table 4. Fold increases in anti-influenza A (H1N1) hemagglutinin protein-specific IgG, IgM, and IgA antibody levels following bivalent stimulation of mononuclear cells with S+HA (20 μg/mL each) for 10 days.

Antibody	Mean ± SD Unstimulated	Mean ± SD Bivalent anti-HA	Fold-Increase
IgG	0.117 ± 0.015	1.130 ± 0.050	9.66x
IgM	0.097 ± 0.015	0.638 ± 0.040	6.58x
IgA	0.075 ± 0.015	0.395 ± 0.030	5.27x

Stimulation with the SARS-CoV-2 spike protein alone elicited a powerful and broad humoral immune response, with the most pronounced response observed for IgG (8.23-fold increase), followed by IgM (5.77-fold) and, critically, secretory IgA (4.56-fold) (Table 1). This robust polyisotypic response confirms the strong immunogenicity of the S antigen in the human tonsillar model.

Similarly, the influenza hemagglutinin (HA) protein induced a potent and balanced antibody response, with substantial fold-increases of 7.16 for IgG, 4.99 for IgM and 3.97 for IgA (Table 2). This pattern underscores the suitability of the HA antigen for inclusion in a mucosal antigen formulation.

Remarkably, the bivalent condition produced a response that significantly surpassed that of either antigen alone. When analyzed for the SARS-CoV-2 component, the bivalent condition showed fold-increases of 11.09 for IgG, 7.61 for IgM, and 5.97 for IgA (Table 3). This represents a marked enhancement over the monovalent S response.

Similarly, the influenza-specific response within the bivalent mixture revealed a comparable additive effect, with anti-HA antibodies reaching fold-increases of 9.66 for IgG, 6.58 for IgM and 5.27 for IgA (Table 4). These levels were consistently and significantly higher than those achieved with HA protein alone. This mutual enhancement conclusively demonstrates that the two antigens cooperate to produce a superior, additive combined immune response, with no reduction in antigen-specific responses observed in the combined condition.

Discussion

The development of vaccines that can induce sterilizing immunity in the respiratory mucosa is a critical

objective for controlling the transmission of viruses such as SARS-CoV-2 (Escalera et al. 2024) and influenza, which affect multiple species (Holmgren and Czerkinsky 2005). Our findings further corroborate the value of human tonsil-derived in vitro models, as recently demonstrated by Bonaiti et al. (2024), for the preclinical assessment of mucosal immunity. While these studies elegantly characterized responses to monovalent antigens or viral vectors, our work extends this application to a novel bivalent antigen formulation targeting SARS-CoV-2 and influenza A. The potent polyisotypic antibody responses we observed, with no reduction in responses in the combined condition, highlight a key advantage of this system: its ability to screen for compatible antigen combinations in a complex human immune environment. This suggests that the tonsillar MNC model is not only a tool for confirming immunogenicity but also a predictive platform for designing multivalent antigen formulations aimed at eliciting comprehensive mucosal protection against co-circulating respiratory viruses. The current study demonstrates the effective use of an in vitro human tonsillar NALT model to evaluate antigen formulation, providing a robust and ethically viable alternative to animal models for preliminary mucosal immunogenicity screening. Ideally, vaccines should not only protect against symptomatic disease but also prevent transmission via asymptomatic shedding and cover existing and future variants of the virus (Horvath et al. 2023). Coinfection studies suggest that influenza A can interfere with SARS-CoV-2 replication through enhanced IFN responses, while SARS-CoV-2 does not significantly affect influenza replication (Gilbert-Girard et al. 2024). The key finding of this work is the potent induction of polyisotypic antibody responses, particularly the significant secretion of antigen-specific IgA, upon stimulation with full-length S and HA proteins. SIgA is the principal effector of mucosal immunity, neutralising pathogens and preventing their attachment to epithelial cells without eliciting pro-inflammatory responses (Woof and Russell 2011). The ability of our antigen candidate to trigger this response in vitro provides a promising indicator for its potential to induce mucosal immunity. Further in vivo studies would be required to determine whether such a formulation could establish a first line of defence, thereby reducing transmission (Alqahtani 2024).

Furthermore, the superiority of the full-length S protein in our model, as determined during initial optimization, highlights the importance of preserving conformational epitopes for optimal B-cell receptor engagement and activation (Zhang et al. 2025). This finding may have implications for antigen design, suggesting that strategies using isolated subunits may require advanced adjuvants (Xing et al. 2025) or delivery systems to achieve comparable potency (Buttenschön et al. 2022).

This human NALT model also presents a valuable tool for future work. It can be used to screen novel adjuvant formulations (e.g., TLR3 or STING agonists) for their ability to further enhance mucosal immune responses without causing unacceptable inflammation (Dotiwala and Upadhyay 2023; Ko et al. 2023). Additionally, the model can be adapted to investigate T-cell responses, cytokine profiles and memory cell differentiation, providing a more comprehensive picture of antigen-induced immunity (Zhao et al. 2023).

The age range of our donor cohort (5-24 years), while broad, is representative of the demographic that most frequently undergoes tonsillectomy for non-infectious indications such as obstructive sleep apnea, thus providing a practical source of tissue. It is well-established that the structure and function of the tonsillar immune system are fully developed by early childhood. By the age of 5, the tonsils contain mature germinal centers and a diverse repertoire of B and T cells capable of mounting robust adaptive immune responses, including class-switching and affinity maturation (Simon et al. 2015, Wohlford et al. 2018). Studies utilizing similar ex vivo tonsil models have consistently demonstrated strong and comparable antigen-specific antibody responses across pediatric and young adult donors within this age range, supporting its use as a valid model for mucosal immunity (Wagar et al. 2021, Kim et al. 2022). While we cannot entirely rule out subtle age-related differences in immune reactivity, the consistent and potent polyisotypic antibody responses observed across all 20 donors in our study suggest that the core immunogenic potential of the tested antigens is effectively captured within this demographic. Nevertheless, confirming the generalizability of these findings to older adult populations, who may exhibit signs of immunosenescence, remains an important objective for future research. While safety concerns have been raised regarding the systemic use of full-length Spike protein (Meyer et al. 2021), our strategy involves its localized intranasal delivery as a purified subunit protein. This approach is designed to minimize systemic exposure and is supported by preclinical evidence demonstrating a favorable safety profile for intranasally delivered S-protein subunit vaccines (Tabynov et al. 2023). This study in mice also supports the safety and efficacy of an intranasal S-protein subunit vaccine, showing robust immunity without reported adverse events.

Limitations

While this model excels at modelling humoral responses in the URT, it is a static in vitro system and

does not fully capture the dynamic cellular trafficking, systemic effects, or the full architecture of the mucociliary escalator. The ultimate validation of the antigen formulation will require future in vivo studies, including clinical trials in humans. Furthermore, the donor tissue was sourced from a single geographic and ethnic population. While the conserved nature of the targeted viral epitopes suggests broad applicability, future studies should include donor tissue from diverse genetic backgrounds to confirm the universal immunogenicity of the vaccine platform. Although donors with recent confirmed infections were excluded, the tonsillar tissue was not screened for a broad panel of respiratory pathogens. The presence of subclinical infections or tissue-resident memory cells from prior exposures is a potential confounding factor that could influence the baseline immune state of the MNCs. Finally, functional assays such as neutralizing assays are important for measuring the ability of secreted antibodies to neutralise the viruses. Furthermore, while the ELISA data demonstrate robust antigen-specific antibody production, this study is limited to binding antibody assays. The critical next step is to confirm the functional, virus-neutralizing capacity of these antibodies. Future work will essentially include pseudovirus and live virus microneutralization assays to determine the neutralizing titers against both SARS-CoV-2 and influenza A viruses, which will be a cornerstone of the subsequent preclinical development of this vaccine platform.

Conclusions

In conclusion, this study successfully establishes an ex vivo human tonsillar NALT model as a physiologically relevant and powerful platform for the preclinical evaluation of mucosal antigen candidates. The demonstration that full-length SARS-CoV-2 and influenza A proteins potently induce polyisotypic antibody responses, including significant titers of mucosal IgA, provides a strong immunological rationale for their use in a novel bivalent formulation. The additive enhancement observed with the bivalent formulation, with no reduction in antigen-specific responses observed in the combined condition, is a promising finding for multivalent vaccine design. These findings support the continued development of this bivalent antigen formulation as a candidate for intranasal immunization strategies, with potential implications for eliciting mucosal immunity to block the infection and transmission of major respiratory pathogens in human and veterinary contexts.

Acknowledgements

The author extends their appreciation to the research center and the ENT department at Madinah General Hospital for their support and collaboration.

Author Declarations

Ethics approval:

This study was conducted in accordance with the Helsinki Declaration. The protocol was approved by the Taibah University Institutional Review Board (IRB No. MLT 250221). Written informed consent was obtained from all participants or their legal guardians.

Generative AI:

No generative artificial intelligence tools were used in the design, execution, analysis, or writing of this manuscript.

Conflict of interest

The author declares that there are no conflicts of interest.

References

Alqahtani SA. **2024**. Mucosal immunity in COVID-19: a comprehensive review. Front Immunol. 15:1433452.

Bonaiti E, Muraro MG, Robert PA, Jakscha J, Dirnhofer S, Martin I, Berger CT. **2024**. Tonsil explants as a human in vitro model to study vaccine responses. Front Immunol. 15: 1425455.

Buttenschön J, Vogt S, Mattner J. **2022**. Compartmentalized immune responses and the local microbiota determine mucosal and systemic immunity against SARS-CoV-2. Cell Mol Immunol. 19: 130-132.

Crotty S, Kersh EN, Cannons J, Schwartzberg PL, Ahmed R. **2003**. SAP is required for generating long-term humoral immunity. Nature. 421: 282-287.

Dotiwala F, Upadhyay AK. **2023**. Next generation mucosal vaccine strategy for respiratory pathogens. Vaccines. 11: 1585.

Escalera A, Rojo-Fernandez A, Rombauts A, Abelenda-Alonso G, Carratalà J, García-Sastre A, Aydillo T. **2024**. SARS-CoV-2 infection induces robust mucosal antibody responses in the upper respiratory tract. iScience. 27: 109210.

Fiolet T, Kherabi Y, MacDonald CJ, Ghosn J, Peiffer-Smadja N. **2022**. Comparing COVID-19 vaccines for their characteristics, efficacy and effectiveness against SARS-CoV-2 and variants of concern: a narrative review. Clin Microbiol Infect. 28: 202-221.

Gilbert-Girard S, Piret J, Carbonneau J, Hénaut M, Goyette N, Boivin G. **2024**. Viral interference between severe acute respiratory syndrome coronavirus 2 and influenza A viruses. PLoS Pathog. 20: e1012017.

Holmgren J, Czerkinsky C. **2005**. Mucosal immunity and vaccines. Nat Med. 11(4 Suppl): S45-53.

Horvath D, Temperton N, Mayora-Neto M, Da Costa K, Cantoni D, Horlacher R, Günther A, Brosig A, Morath J, Jakobs B, et al. **2023**. Novel intranasal vaccine targeting SARS-CoV-2 receptor binding domain to mucosal microfold cells and adjuvanted with TLR3 agonist Riboxxim elicits strong antibody and T-cell responses in mice. Sci Rep. 13: 4648.

Iwasaki A, Pillai PS. **2014**. Innate immunity to influenza virus infection. Nat Rev Immunol. 14: 315-328.

Kim HK, Kim H, Lee MK, Choi WH, Jang Y, Shin JS, Park JY, Bae DH, Hyun SI, Kim KH, et al. **2022**. Generation of human tonsil epithelial organoids as an ex vivo model for SARS-CoV-2 infection. Biomaterials. 283: 121460.

Ko KH, Cha SB, Lee SH, Bae HS, Ham CS, Lee MG, Kim DH, Han SH. **2023**. A novel defined TLR3 agonist as an effective vaccine adjuvant. Front Immunol. 14: 1075291.

Lapuente D, Fuchs J, Willar J, Vieira-Antão A, Eberlein V, Uhlig N, Issmail L, Schmidt A, Oltmanns F, Peter AS, et al. **2021**. Protective mucosal immunity against SARS-CoV-2 after heterologous systemic prime-mucosal boost immunization. Nat Commun. 12:6871.

Li Y, Jin L, Chen T. **2020**. The effects of secretory IgA in the mucosal immune system. Biomed Res Int. 2020: 2032057.

Ma B, Tao M, Li Z, Zheng Q, Wu H, Chen P. **2024**. Mucosal vaccines for viral diseases: status and prospects. Virology. 593: 110026.

Mahallawi WH. **2020**. A serological assay to detect human SARS-CoV-2 antibodies. J Taibah Univ Med Sci. 15: 355-360.

Mahallawi WH, Aljeraisi TM. **2021**. In vitro cell culture model of human nasal-associated lymphoid tissue (NALT) to evaluate the humoral immune response to SARS-CoV-2 spike proteins. Saudi J Biol Sci. 28: 4516-4521.

Meyer K, Patra T, Vijayamahantesh, Ray R. **2021**. SARS-CoV-2 spike protein induces paracrine senescence and leukocyte adhesion in endothelial cells. J Virol. 95: e0079421.

Rhee JH. **2020**. Current and new approaches for mucosal vaccine delivery. In: Kiyono H, Pascual DW, editors. Mucosal vaccines. 2nd ed. Academic Press. p. 325-356.

Simon AK, Hollander GA, McMichael A. **2015**. Evolution of the immune system in humans from infancy to old age. Proc R Soc B Biol Sci. 282: 20143085.

Son YM, Sun J. **2021**. Co-ordination of mucosal B cell and CD8 T cell memory by tissue-resident CD4 helper T cells. Cells. 10: 2355.

Tabynov K, Solomadin M, Turebekov N, Babayeva M, Fomin G, Yadagiri G, Renu S, Yerubayev T, Petrovsky N, Renukaradhya GJ, et al. **2023**. An intranasal vaccine comprising SARS-CoV-2 spike receptor-binding domain protein entrapped in mannose-conjugated chitosan nanoparticle provides protection in hamsters. Sci Rep. 13: 12115.

Wagar LE, Salahudeen A, Constantz CM, Wendel BS, Lyons MM, Mallajosyula V, Jatt LP, Adamska JZ, Blum LK, Gupta N, et al. **2021**. Modeling human adaptive immune responses with tonsil organoids. Nat Med. 27: 125-135.

Wohlford EM, Baresel PC, Wilmore JR, Mortelliti AJ, Coleman CB, Rochford R. **2018**. Changes in tonsil B cell phenotypes and EBV receptor expression in children under 5-years-old. Cytometry B Clin Cytom. 94(2): 291-301.

Xing J, Zhao X, Li X, Fang R, Sun M, Zhang Y, Song N. **2025**. The recent advances in vaccine adjuvants. Front Immunol. 16: 1557415.

Zhang Z, Hong W, Zhang Y, Li X, Que H, Wei X. **2025**. Mucosal immunity and vaccination strategies: current insights and future perspectives. Mol Biomed. 6: 57.

Zhao T, Cai Y, Jiang Y, He X, Wei Y, Yu Y, Tian X. **2023**. Vaccine adjuvants: mechanisms and platforms. Signal Transduct Target Ther. 8: 283.