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Original article

Live attenuated influenza vaccine induces broadly cross-reactive mucosal antibody responses to different influenza strains in tonsils

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ABSTRACT

Intranasal live attenuated influenza vaccine (LAIV) was used to stimulate tonsillar monocular cells (MNCs) following isolation. Haemagglutinin (HA) proteins of several influenza strains were used for the detection of HA-specific IgG, IgM and IgA antibodies using ELISA. Significant anti-sH1N1 HA IgG IgA and IgM antibody titres were detected in cell culture supernatants after stimulation (mean ± SE: 0.43 ± 0.09, mean ± SE: 0.23 ± 0.04 and mean ± SE: 0.47 ± 0.05 respectively, $p < 0.01$). LAIV stimulation of tonsillar MNCs induced significant IgG, IgA and IgM antibodies to the pH1N1 HA (mean ± SE:1.35 ± 0. 12), (mean ± SE: 0.35 ± 0.06) and (mean ± SE: 0.58 ± 0.10) respectively, $p < 0.01$. Surprisingly, LAIV was shown to induce cross-reactive anti-aH5N1 HA antibodies (mean ± SE: 0.84 ± 0.20, $p < 0.01$) to avian influenza virus (aH5N1). Anti-H2N2 HA IgG antibody was also detected in the cell culture supernatants in a significant level after LAIV stimulation (mean ± SE: 0.93 ± 0.23, $p < 0.01$). High levels of anti-sH3N2 HA IgG antibody was discovered after LAIV stimulation of tonsillar MNCs, (mean ± SE: 1.2 ± 0.23$p < 0.01$). The current model of human nasal-associated lymphoid tissue (NALT) to evaluate B cells responses to LAIV was evident that it is a successful model to study future intranasal vaccines.

1. Introduction

Influenza is a contagious disease caused by influenza viruses that infect the nose, throat, and sometimes the lungs. The virus infects host epithelia cells by binding of surface glycoprotein haemagglutinin (HA) to sialic acid receptor on the cell surface (Barbey-Martin et al., 2002; Mahallawi et al., 2020).

The pandemic (H1N1) 2009 influenza A virus (pH1N1) has previously killed over than 19,000 people globally since it emerged in April 2009 (Organization, 2012). The virus caused global pandemic in 2009 which infected an estimated 11–21% of the world population (Kelly et al., 2011). The pH1N1 virus is antigenically dissimilar from seasonal influenza (sH1N1) viruses as when it has appeared, most of human population deficiencies immunity against this virus (Girard et al., 2010; Lu et al., 2010). Previous studies showed that pH1N1 are much pathogenic in mammalian prototypes than that of sH1N1influenza viruses (Bermejo-Martin et al., 2010; Lee et al., 2009).

The pharyngeal as well as palatine tonsils are compact so far physiologically composite mucosa-associated lymphoid tissues (NALT) that make up a compartment of Waldeyer's ring. (Arambula et al., 2021) Human tonsils are major components of local mucosal immune organs, and are known to be important induction sites for both mucosal and systemic immunity against upper respiratory tract pathogens (Aljeraisi et al., 2023; Kiyono & Fukuyama, 2004; Zuercher et al., 2002).

The use of human NALT cells was shown to be a good model to test and explore the mucosal immunity to influenza viruses (Mahallawi et al., 2013). Using this novel model to assist and examine future formulation of vaccines could also help the development of vaccines against other respiratory pathogens (Aljurayyan et al., 2018).

Intranasal vaccination with LAIV has been used successfully in several countries with good efficacy. Despite having been shown to be safe and effective in humans, little research has been done in terms of the local mucosal immunity induced by the intranasal vaccine (Petukhova et al., 2009). Mucosal vaccination denotes a

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promising alternative to the commonly intramuscular vaccination routes. It is non-invasive and capable to provoke strong local as well as systemic immune responses in mucosa-associated lymphoid tissue. Thus, aiming to find a human suitable model could help in testing vaccine candidates targeting respiratory pathogens.

We aimed in the current study to investigate the mucosal antibody responses in an *in vitro* model of NALT following stimulation of tonsillar MNCs with a LAIV vaccine.

2. Methods

2.1. Patients and samples

Tonsils were obtained from patients (1–37 years) undergoing tonsillectomy. Patients formerly immunised against influenza or who were immunocompromised in any way were excluded. The research has been approved by College of Applied Medical Sciences Ethics Committee (IRB: 2022-MLT-217) written, informed consent attained from patient/parent as suitable.

2.2. Intranasal LAIV (FluMist) vaccine and recombinant hemagglutinins (HAs)

LAIV comprised the following influenza strains; A/H1N1/2009; A/H3N2 and B influenza strains,). Purified recombinant HA proteins of pH1N1 (A/California/04/2009,VR-1894DQ),sH1N1 (A/Brisbane/59/2007, VR-1742), sH3N2 (A/Brisbane/10/2007, VR-1881), aH5N1 (A/Vietnam/1203/2004, VR-1931), A/Hong Kong/8/68, VR-1679), and H7N3 (A/Canada/RV444/04, VR-1641) were from Biodefense and Emerging Infections Research Resources Repository (BEI resources ATCC (Manassas VA, USA). All the proteins were shipped at −70 °C. After reconstitution in sterile PBS, they were aliquot in separated vials and frozen at −20 °C. For each experiment we used required vials as the multi thawing was not aloud.

2.3. Tonsillar MNCs separation

The tonsils were immediately kept in HANKS transport medium (Sigma-Aldrich) following the operation. Mononuclear cells were isolated using Ficoll density centrifugation following methods described previously (Mahallawi & Aljeraisi, 2021a). In brief, tonsils were processed within one hour of surgery. Tonsils were then washed and the RPMI medium was added and then the tissues were minced thoroughly using a scalpel. The processed tissues were then lifted for 5 minutesfo MNCs release. The cell suspension was then passed through a 70-μm nylon mesh. Tonsillar MNCs were then isolated layring them on the top of Ficoll-Paque (Premium GE Healthcare, United Kingdom) and gradient centrifugation (400 × *g* for 30 min) was performed at zero break speed using refrigerated centrifuge (Thomas Scientific,USA).

2.4. Tonsillar MNCs stimulation

Tonsillar MNCs were cultured in RPMI complete medium in the presence of different influenza antigens and LAIV. Previous protocol was used with some modifications (Mahallawi et al., 2013). Briefly, following tonsillar MNCs isolation and adjusting of cell number, 250 μl of cells. MNCs were then co-cultured with the optimal concentrations of the stimulants for antibodies production in cell culture plate. Unstimulated cells were used as negative control by adding RPMI medium that similar to the stimulus volume. The plate was then incubated in 0.05 % CO2 at 37 °C and cell culture supernatants were collected at day 10 and stored −70 °C until assayed for measuring HA-specific antibodies by ELISA.

2.5. Measurement of anti-HA antibodies levels by ELISA

HA-specific IgG, IgA and IgM antibodies were analyzed following the ELISA procedure as described previously with some modifications (Mahallawi, 2020). In brief, 96-well ELISA plates (Costar) were coated after reconstitution in PBS with 100 μl/well at concentration of (2 μg/ml) of Haemagglutinin (HA). All HA proteins were tested and optimized to reach optimal coating concentration. Following that, plates were covered and then incubated overnight at 4 °C. Plates were washed 5 times with PBS containing 0.05% Tween-20 (Sigma-Aldrich). After that the plates were blocked with150 μl/well of blocking buffer PBS containing 10% FCS (Sigma-Aldrich) for 90 min. Supernatant samples were then added and plates were incubated for 190 min. Plates were washed 5 times and 50 μl/well alkaline phosphatase conjugated goat anti-human IgG, IgA and IgM (Sigma-Aldrich) were added and then incubated at room temperature for 90 min. Plates were washed 5 times and the substrate was added. The plates then were kept in the dark until colour developed. Optical densities (OD) at 405 nm then was measured using absorbance microplate reader (ELX800, Bio-Tek, Germany).

2.6. Statistical analysis

All calculations and statistical analyses were done using GraphPad Prism (V 9, USA). Data were expressed as the mean ± standard error (SE). Comparisons between two groups were performed using a paired *t*-test. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. Participant demography

A total of 15 participants were included in the study, of whom 46.6% (n = 7) were adults. The mean age for children was 8.49 ± 3. 35 years, and the mean age for adults was 27.0 ± 8.90 years. Overall, 59% of the sample were male.

3.2. LAIV induces for strong anti-pH1N1 HA antibody in tonsillar cells

We found that LAIV stimulation of tonsillar MNCs induced IgG, IgA and IgM antibodies to pH1N1 HA. Significant anti-pH1N1 IgG antibody titres were detected in cell culture supernatants after stimulation compared with unstimulated cells (Fig. 1a, (mean ± SE:1.35 ± 0.12, $p < 0.01$). LAIV stimulation also induced IgA antibody to pH1N1 HA (Fig. 1b, mean ± SE: 0.35 ± 0.06, $p < 0.01$) and IgM antibodies compared with unstimulated cells (Fig. 1c, mean ± SE: 0.58 ± 0.10, $p < 0.01$).

3.3. LAIV induces strong mucosal cross-reactive antibodies

Significant anti-sH1N1 IgG antibody titres were detected (Fig. 2a, mean ± SE: 0.43 ± 0.09, $p < 0.01$) following tonsillar MNCs stimulation with LAIV compared with unstimulated cells. In addition, LAIV was shown to induce IgA antibody (Fig. 2b**,** mean ± SE: 0.23 ± 0.04, $p < 0.01$) and IgM antibody to sH1N1 HA compared with unstimulated cells (Fig. 2c**,** mean ± SE: 0.47 ± 0.05, $p < 0.01$).

LAIV was also shown to induce avian H5N1 (aH5N1) HA antibodies. As shown in Fig. 2d, LAIV stimulation induced significant high levels of specific anti- aH5N1 HA IgG antibodies compared with unstimulated cells (mean ± SE: 0.84 ± 0.20, $p < 0.01$). Anti-H2N2 HA IgG antibodies were also detected in the cell culture supernatants after LAIV stimulation compared with unstimulated cells (Fig. 2e, mean ± SE: 0.93 ± 0.23, $p < 0.01$). As the LAIV contains

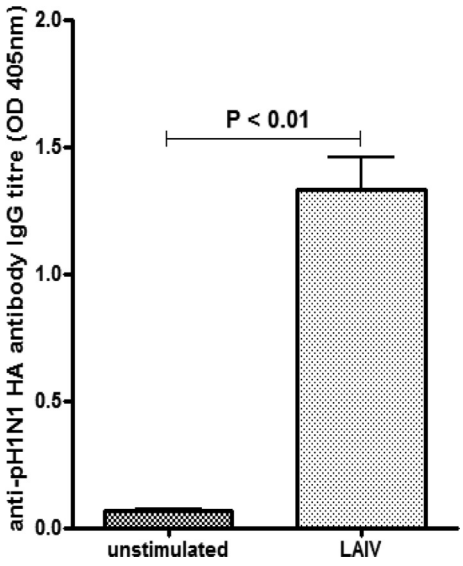

Fig. 1a. LAIV induces specific IgG anti-pH1N1 HA. LAIV induced high level of HA-specific IgG antibody to pH1N1 virus compared with unstimulated cells (n = 15, p < 0.01). Means and standard errors are shown.

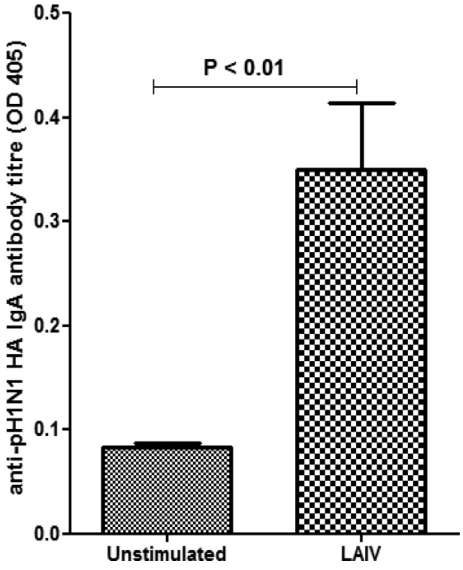

Fig. 1b. LAIV induces HA-specific IgA antibody to pH1N1. LAIV induced HA-specific IgA antibody production to pH1N1 virus compared with unstimulated cells (n = 15, p < 0.01). Means and standard errors are shown.

sH3N2 virus, expectedly we found significant high level of anti-sH3N2 HA IgG antibody after LAIV stimulation with mean ± SE: 1.2 ± 0.23 (p < 0.01, Fig. 2f). Anti-H7N3 HA antibody and there was no significant level of specific anti-HA IgG antibody to H7N3 detected compared with unstimulated cells (Fig. 2g, p > 0.05).

4. Discussion

Existing influenza vaccines principally yield antibodies targeting the viral HA and predominantly provoke strain-specific antibodies. Thus, inhibition of HA action has been the main ration of influenza vaccine effectiveness for years (Chen et al., 2018). Though, these strain-specific antibodies cannot offer protection against antigenically drifted as well as antigenically shifted strains. (Krammer & Palese, 2015).

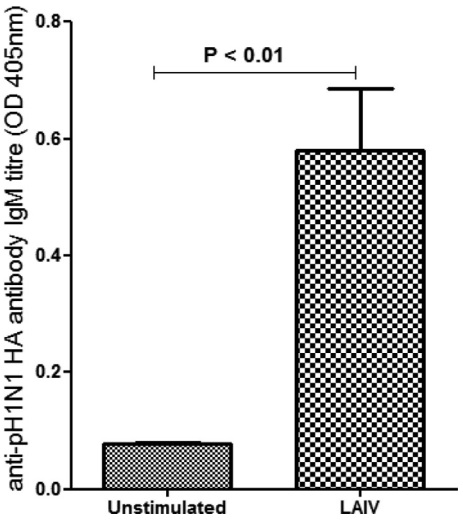

Fig. 1c. LAIV induces HA-specific IgM antibody to pH1N1. LAIV induced higher level of HA-specific IgM antibody production to pH1N1 virus compared with unstimulated cells (n = 15, p < 0.01). Means and standard errors are shown.

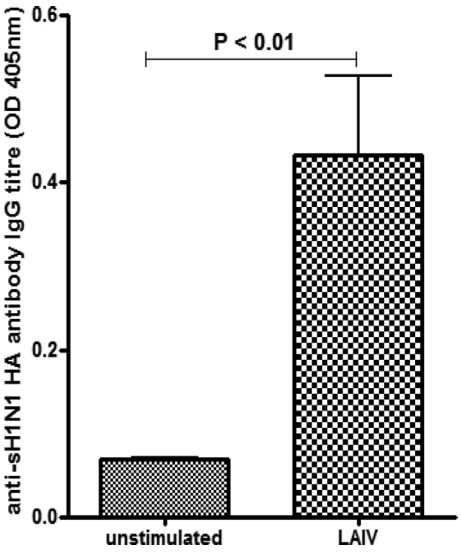

Fig. 2a. LAIV induces HA-specific anti-sH1N1 IgG. LAIV induced significantly higher levels of HA-specific IgG antibody to sH1N1 compared with unstimulated cells (n = 15, p < 0.01). Means and standard errors are shown.

In the current study, mucosal antibody responses in NALT to HAs of a number of influenza A and one influenza B viruses were investigated following *in vitro* stimulation of tonsillar cells with LAIV vaccine. Significant antibody responses of all three isotypes (IgG, IgA and IgM) to the HA of pandemic H1N1 virus were observed in tonsillar cells following LAIV stimulation. This suggests human NALT tissues are likely to be a major induction site of immune response against influenza following LAIV immunization at the nasopharynx site. It has been shown previously that LAIV intranasal vaccination induces an immune response that more closely resembles natural immunity than that elicited by injectable inactivated vaccine. (Cox et al., 2004).

As mentioned earlier that LAIV contains pH1N1 (A/H1N1 California 2009), interestingly we show that LAIV vaccine stimulation elicited cross-reactive antibodies against HAs of a number of different influenza strains including sH1N1, H2N2 and aH5N1 but not H7N3. This is consistent with our previous findings using tonsillar MNCs which were stimulated to produce HA-specific IgG antibody

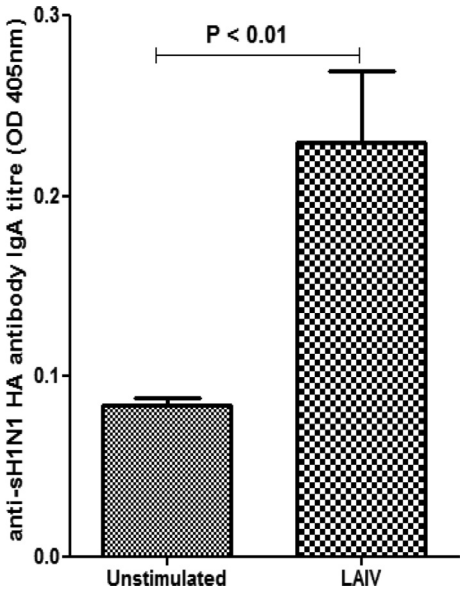

Fig. 2b. LAIV induces HA-specific IgA antibody to sH1N1. LAIV induced HA-specific IgA antibody production to sH1N1 virus compared with unstimulated cells (n = 15, p < 0.01). Means and standard errors are shown.

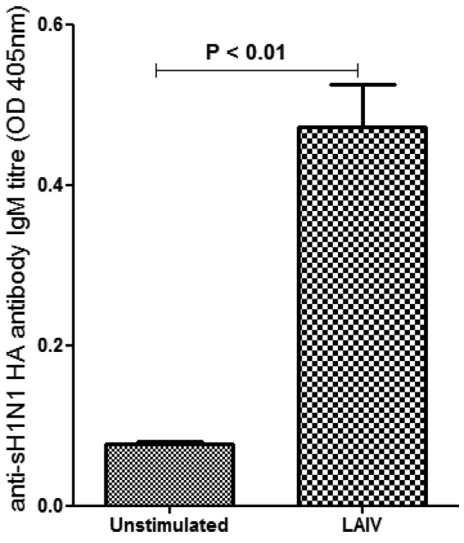

Fig. 2c. LAIV induces HA-specific IgM antibody to sH1N1. LAIV induced HA-specific IgM antibody production to sH1N1 virus compared with unstimulated cells (n = 15, p < 0.01). Means and standard errors are shown.

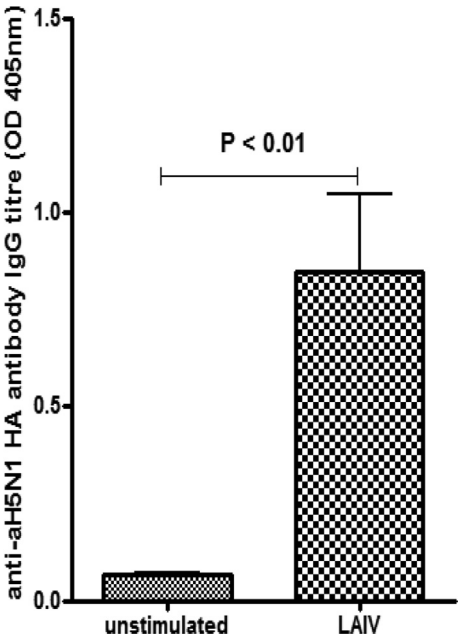

Fig. 2d. LAIV induces anti-aH5N1 HA antibody. LAIV induced significantly higher level of specific anti-HA IgG antibody to aH5N1 compared to unstimulated negative control (n = 15, p < 0.01). Means and standard errors are shown.

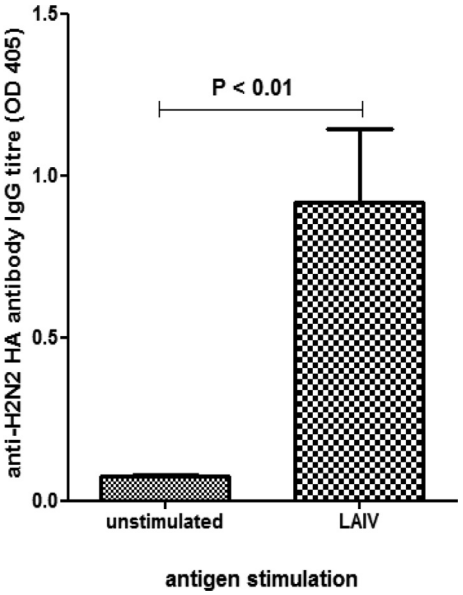

Fig. 2e. LAIV induces anti-H2N2 HA antibody. LAIV induced significantly higher specific anti-HA IgG antibody to H2N2 compared with unstimulated cells (n = 15, p < 0.001). Means and standard errors are shown.

secreting cells (ASCs) to sH1N1, H2N2 and aH5N1 by pH1N1 virus antigen (Mahallawi et al., 2013). This is likely due to the antigenic relatedness of the same influenza A group 1 viruses. However, when tonsillar MNCs were stimulated with the LAIV which contains H3N2 virus (group 2), it did not seem to induce anti-HA antibody to H7N3 virus that belongs to group 2. So we did not find evidence of cross-reactive B cell antibody response between group 2 influenza viruses in this study.

Although all three isotypes, including IgA, IgG and IgM antibodies were induced by LAIV, the highest level shown was of IgG isotype. This is also consistent with our previous results that investigate immune responses elicited via memory B cells (Mahallawi et al., 2013). It is likely that IgG antibody level represented mainly memory B cell response; whereas IgA and IgM antibody levels mainly represent a primary response. A number of studies have shown that antigen-specific mucosal IgA responses are short-lived and that re-immunization does not reliably induce memory-type IgA responses (Gianchecchi et al., 2019; Korkeila et al., 2000). The predominance of antigen-specific IgG memory B cells to influenza HA in tonsillar tissues is consistent with previous studies demonstrating the predominance of IgG memory B cell responses to protein antigens in human NALT (Boyaka et al., 2000; Zhang et al., 2000). Our recent studies using the current model were support using the tonsillar MNCs in investigating the mucosal immunity to the novel SARS CoV-2 virus (Mahallawi & Aljeraisi, 2021a; Mahallawi & Aljeraisi, 2021b). Which in turns

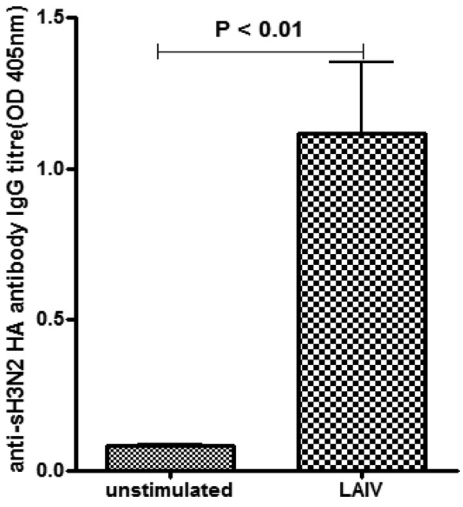

Fig. 2f. LAIV induces anti-sH3N2 HA antibody. LAIV induced higher level of anti-sH3N2 HA IgG antibody compared to unstimulated negative control (n = 15, $p < 0.01$). Means and standard errors are shown.

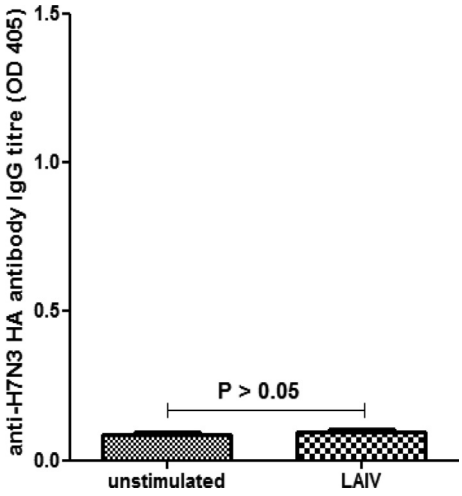

Fig. 2g. LAIV did not induce anti-H7N3 HA IgG antibody production. No significant level of specific anti-HA IgG antibody to H7N3 was detected compared with unstimulated cells (n = 15, $p > 0.05$). Means and standard errors are shown.

evident to test other respiratory pathogens and design for novel intranasal vaccines candidates.

In conclusion, we showed that LAIV has been shown to provide cross reactivity and hence possible protection against both matched and mismatched influenza strains in tonsillar MNCs. This pilot study using *in vitro* cell culture model of human NALT to assess B cells mucosal responses to LAIV has provided evidence of principle that it could be used as a tool to study future intranasal vaccines. Understanding mucosal immunity to respiratory tract infections such as influenza viruses may add considerably to the development of effective intranasal vaccines against other respiratory infections.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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