Activation and induction of antigen-specific T follicular helper cells (T*FH*) play a critical role in LAIV-induced human mucosal anti-influenza antibody response

Abdullah Aljurayyan¹, Suttida Puksuriwong¹, Muhammad Ahmed¹, Ravi Sharma², Madhan Krishnan², Salil Sood², Katherine Davies³, Devika Rajashekar³, Sam Leong⁴, Paul S McNamara⁵, Stephen Gordon⁶, Qibo Zhang¹*¹

Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, UK¹; ENT Department, Alder Hey Children’s Hospital, United Kingdom², ENT Department, Royal Liverpool and Broadgreen University Hospitals, UK³, ENT Department, Aintree University Hospital of Liverpool, UK³, Institute in the Park, Alder Hey Children’s Hospital, UK⁵, Liverpool School of Tropical Medicine, United Kingdom⁶

Short title: Activation of antigen-specific TFH cells by LAIV

*Corresponding author: Dr Qibo Zhang, MD PhD, Senior Lecturer in Immunology, Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, Ronald Ross Building, 8 West Derby Street Liverpool L69 7BE  Tel: 0044 151 7959677  Fax: 0044 151-795-5529  Email: Qibo.Zhang@liv.ac.uk

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There is increasing interest recently in developing intranasal vaccines against respiratory tract infections. Antibody response is critical in vaccine-induced protection and T<sub>FH</sub> is considered important in mediating antibody response. Most data supporting the role for T<sub>FH</sub> in antibody response are from animal studies, and direct evidence from humans is limited, apart from T<sub>FH</sub>-like cells in blood. We studied activation and induction of T<sub>FH</sub> and its role on anti-influenza antibody response by live-attenuated influenza vaccine (LAIV) in human nasopharynx-associated lymphoid tissue (NALT). T<sub>FH</sub> activation in adenotonsillar tissues were analysed by flow-cytometry, and anti-hemagglutinin (HA) antibodies examined following LAIV stimulation of tonsillar mononuclear cells (MNC). Induction of antigen-specific T<sub>FH</sub> by LAIV was studied by flow-cytometry for induced T<sub>FH</sub> and CD154 expression. LAIV induced T<sub>FH</sub> proliferation which correlated with anti-HA antibody production, and T<sub>FH</sub> was shown critical for antibody response. Induction of T<sub>FH</sub> from naïve T cells by LAIV was shown in newly induced T<sub>FH</sub> expressing BCL6 and CD21, which was followed by the detection of anti-HA antibodies. Antigen specificity of LAIV-induced T<sub>FH</sub> was demonstrated by the expression of antigen-specific T cell activation marker CD154 upon challenge by H1N1 virus antigen or HA. LAIV-induced T<sub>FH</sub> differentiation was inhibited by BCL6, IL21, ICOS and CD40 signalling blocking respectively, and that diminished anti-HA antibody production. Conclusion: We demonstrate for the first time the induction of antigen-specific T<sub>FH</sub> by LAIV in human NALT that provide critical support for anti-influenza antibody response. Promoting antigen-specific T<sub>FH</sub> in NALT by intranasal vaccines may provide an effective vaccination strategy against respiratory infections in humans.
IMPORTANCE. Airway infection such as influenza is common in humans. Intranasal vaccination has been considered a more biologically relevant and effective way of immunization against airway infection. Vaccine-induced antibody response is crucial for protection against infection. Recent data from animal studies suggest one type of T cells, named T<sub>FH</sub>, is important for the antibody response. However, data on whether this T<sub>FH</sub>-mediated help for antibody production operates in humans is limited, due to the lack of access to human immune tissue containing the T<sub>FH</sub>. In this study, we demonstrated the induction of T<sub>FH</sub> cells by an intranasal influenza vaccine in human immune tissue that provide critical support for anti-influenza antibody response. Our findings provide direct evidence that T<sub>FH</sub> cells play a critical role in vaccine-induced immunity in humans, and suggest a novel strategy to promote such cells by intranasal vaccines against respiratory infections.

Keywords: T follicular helper cell (T<sub>FH</sub>), LAIV, influenza vaccine, mucosal immunity, antibody response, nasopharynx-associated lymphoid tissue (NALT)
INTRODUCTION

Vaccination is one of the most effective preventative measures against pathogenic infection. Despite its success, there are still many infectious diseases in humans that lack effective vaccines. New strategies to improve vaccine immunogenicity are constantly being explored. Recent studies suggest a critical role for T follicular helper cells (T<sub>FH</sub>) in vaccine-induced immunity (1, 2) and promoting T<sub>FH</sub> has been considered a promising vaccination strategy. However, most of the current evidence supporting the importance of T<sub>FH</sub> in vaccination comes from animal studies, and direct evidence from humans is limited, apart from the detection of T<sub>FH</sub>-like cells from human peripheral blood samples which are thought as T<sub>FH</sub> equivalent (3, 4). Whether this T<sub>FH</sub>-mediated critical help for vaccine-induced B cell antibody response operates in humans remain largely unsubstantiated. Several recent studies have reported that the presence of “T<sub>FH</sub>-like” cells in peripheral blood following parenteral influenza vaccination appeared to correlate with an anti-hemagglutinin (HA) antibody response (5, 6).

T<sub>FH</sub> are a subset of CD4<sup>+</sup> T cells in secondary lymphoid tissue that provide help to cognate B cells for high affinity antibody production in germinal centers (GC) and for long-term humoral immunity(7). T<sub>FH</sub> express chemokine receptor CXCR5 and inducible costimulator-ICOS, IL21 and the transcription factor B-cell lymphoma 6 (BCL6) (8). Considering the importance of T<sub>FH</sub> for B cell antibody response, novel vaccines to induce/activate T<sub>FH</sub> cells may be an effective vaccination strategy for better vaccine efficacy in humans.

Influenza virus infects nasopharyngeal mucosa by binding its surface HA to sialic acid receptors on the host cell (9). Intranasal vaccination has been proposed as an effective way of immunising against influenza through induction of anti-HA antibody, which relies on the local mucosal immune tissue, i.e. nasopharynx-associated lymphoid tissue (NALT) as the induction site for immunity. Human adenoids and tonsils are major components of NALT and are known to be major induction sites for
both mucosal and systemic immunity against upper respiratory tract pathogens including influenza virus (10-13).

Live Attenuated Influenza Vaccines (LAIV) are administered as intranasal sprays and comprise of live-attenuated influenza type A (H1N1 and H3N2), and type B viruses. LAIV has been used in a number of countries including USA and Canada (FluMist®) (14), and in Europe (Fluenz™), and been shown to induce both mucosal and serum antibodies, as well as cellular immune responses (15-17).

Although LAIV has been shown to be effective against influenza (18), limited data are available on the induction of LAIV-induced immunity in humans and on how the anti-HA antibody response is regulated by T cells. We have studied the activation and induction of T\(_{\text{FH}}\) by LAIV and its role on the anti-HA antibody response in human NALT tissue, and shown the induction of antigen-specific T\(_{\text{FH}}\) in NALT is critical in LAIV-induced anti-influenza HA antibody response.

RESULTS

**LAIv activates a proliferative T\(_{\text{FH}}\) response in NALT that provides critical help for anti-HA antibody production**

Activation of T\(_{\text{FH}}\) in NALT was examined by LAIV stimulation of adenotonsillar MNC for 3 days followed by enumerating T\(_{\text{FH}}\) numbers using flow cytometry. As shown in Figure 1a+b, LAIV stimulation elicited a significant increase in T\(_{\text{FH}}\) number (CD4\(^+\)CXCR5\(^{\text{hi}}\)ICOS\(^{\text{hi}}\)) compared to unstimulated control (p<0.01). The T\(_{\text{FH}}\) response was further assessed by analysis of T cell proliferation using CFSE cell tracing. As can be seen in Figure 1c+d, stimulation of tonsillar MNC by LAIV elicited a marked T\(_{\text{FH}}\) proliferative response detected at day 5 of cell culture (p<0.001).

Further analysis also demonstrated a marked increase in the number of germinal center B cells (CD19\(^+\)CD38\(^+\)IgD\(^-\)) following LAIV stimulation (Fig 1e+f, p<0.01).
Anti-influenza antibody production was measured in tonsillar MNC culture supernatant following LAIV stimulation for 8 days. As expected, LAIV elicited marked anti-HA antibody production (Fig 1g), and T- B cell co-culture experiment demonstrated B cells co-cultured with purified TFH elicited anti-HA antibody production, whereas no antibody production was shown in B cells co-cultured with non-TFH (CXCR5 CD4+) cells (Fig 1h).

**Induction of antigen-specific TFH by LAIV that correlates with antibody production**

To determine whether LAIV induces TFH differentiation from naive CD4+ T cells in NALT, tonsillar MNC depleted of CD45RO+ T cells (resulting in CD45RO- MNC) were stimulated for 7 days with LAIV. The CD45RO- MNC contained naive T cells but without CD45RO+ cells including CXCR5+ TFH. As shown in Figure 2a+b, LAIV stimulation of CD45RO- MNC induced a marked increase in the number of CD4+ ICOS+CXCR5+ (TFH) cells following 7 days of cell culture. The induced TFH were shown to express the transcription factor BCL6 and cytokine IL21 (Fig 2c+d). The induction of TFH by LAIV was shown in a dose-dependent fashion (Fig 2e, top), which was accompanied by a dose-dependent increase in anti-HA IgG antibody production in the cell culture supernatant detected at day 14 (2e, bottom). All the 3 major antibody isotypes including IgG, IgM and IgA anti-HA antibodies were detected in the culture supernatant at day 14 following LAIV stimulation (Fig 2f).

Having shown the induction of TFH by LAIV, we next examined the specificity of these induced TFH for influenza antigens. As CD154 is considered a reliable functional marker for antigen-activated T cells, i.e. a marker for antigen-specific T cells (5, 19-21), CD154 expression in the CD4+ T cell subsets was analyzed following either an inactivated sH1N1 virus antigen or recombinant HA challenge. A representative dot plot demonstrating the activated TFH (ICOS+CXCR5+, top right quadrant) following the antigen challenge was shown in Fig 3a, and the frequencies of activated TFH (% of CD4+ T cell) following sH1N1 antigen or HA challenge were shown in Fig 3b. Both antigen
stimulations activated a marked increase in the \(T_{FH}\) numbers compared to non-antigen control, and as expected, the sH1N1 virus antigen challenge elicited a higher increase in \(T_{FH}\) frequency than HA (3b). Among the activated \(T_{FH}\) cells following sH1N1 challenge, a large proportion (mean 62.2%) expressed CD154 (3c+d), demonstrating the high frequency of activated influenza antigen–specific T cells in these \(T_{FH}\), substantially higher than the other non-\(T_{FH}\) CD4+ cell populations: 0.45% in ICOS CXCR5\(^{-}\), 3.05% in ICOS CXCR5\(^{+}\), and 20.6% in ICOS\(^{+}\)CXCR5\(^{-}\) populations (p<0.001, p<0.001 and p<0.01 respectively) (Fig 3c+d). A similar proportions of CD154+ CD4+ T cell populations including CD154+ \(T_{FH}\) were shown following the HA antigen challenge (data not shown).

**LAIV-activated induction of \(T_{FH}\) in NALT involves IL21, ICOS, CD40 and BCL6 signalling,**

As LAIV induced \(T_{FH}\) cells expressed a high level of IL21 and ICOS, we determined whether the \(T_{FH}\) induction from naïve T cells involved IL21R and ICOS signalling. Co-incubation of naïve T cell-containing CD45RO\(^{-}\) MNC with either IL21R or ICOS-Ligand blocking antibody led to a marked reduction in \(T_{FH}\) cell induction by LAIV respectively (Fig 3e, p<0.01). Further, co-incubation with CD40-ligand blocking antibody or a BCL6 inhibitor also led to a marked reduction in the \(T_{FH}\) induction (3e). Finally, co-incubation with anti-IL21R, ICOS-L and CD40-L antibodies or the BCL6 blocker respectively inhibited the LAIV-induced anti-HA antibody production in CD45RO\(^{-}\) MNC (3f, p<0.01).

**IL-21 production by LAIV-activated \(T_{FH}\) is critical for anti-HA antibody production**

We next examined the cellular source and production of IL21 in tonsillar MNC following LAIV stimulation, and its effect on \(T_{FH}\) activation and antibody production. Among tonsillar lymphocytes, \(T_{FH}\) were shown as a predominant source of IL21 (4a). Following LAIV stimulation there was an increase of IL21-producing \(T_{FH}\) in tonsillar MNC (Fig 4b), together with a marked increase in IL21 concentration in the MNC culture supernatant (4c). Further, the increase in IL21 concentration was
shown in the co-culture of T<sub>FH</sub> and B cells (4d), but not seen in the co-culture of non-T<sub>FH</sub> with B cells following LAIV stimulation (4c).

IL21 receptor blocking using anti-IL21R antibody abrogated the increase of T<sub>FH</sub> number in tonsillar MNC elicited by LAIV stimulation (4f), followed by a significant reduction in anti-HA antibody production in tonsillar MNC (4g).

**Activation of T<sub>FH</sub>-like cells in PBMC by LAIV**

Recent studies suggest there are T<sub>FH</sub>-like cells in peripheral blood that express CXCR5 and ICOS and have similar B cell-help functions (5, 22-25). To determine whether LAIV activate T<sub>FH</sub>-like cells and antibody production in peripheral blood, freshly isolated PBMC were stimulated by LAIV for up to 14 days followed by flow-cytometry and antibody detection. As shown in figure 5a+b, LAIV stimulation induced an increase of T<sub>FH</sub>-like (CXCR5<sup>+</sup>ICOS<sup>+</sup>) CD4<sup>+</sup> T cells in PBMC (at day 7), followed by the detection of anti-HA IgG and IgM antibodies in the PBMC culture supernatants (Fig 5c). The activation of influenza antigen-specific T<sub>FH</sub>-like cells by LAIV was demonstrated by the finding that a major proportion (mean 45.6%) of these cells expressed CD154 following the H1N1 antigen challenge, markedly higher than the other non-T<sub>FH</sub> cell populations (Fig 5d).

**DISCUSSION**

LAIV is thought to replicate in upper respiratory tract to induce immunity through the local immune tissue NALT, and it was shown to replicate in nasal epithelial cells(26). As part of the mucosal immune system in human nasopharynx, adenotonsillar tissue has a surface reticular epithelial cell layer in which epithelial cells mixed with other cells including a large number of B cells. Many B cells infiltrating the epithelial layer exhibit memory B cell markers and have great antigen-presenting potential(27, 28). In our adenotonsillar MNC culture, the predominant cell populations are lymphocytes of which over 50% are B cells(29). We previously showed a Modified Vaccinia
Virus Ankara (MVA) vectored influenza vaccine predominantly infected tonsillar B cells which were also the major cells presenting vaccine antigens (30). It is likely tonsillar B cells are a major cell population involved in LAIV replication and antigen presentation to T cells, and this B and T cell interaction contributes to the vaccine-induced response in NALT. Our recent pilot data showed a time-dependent increase in HA expression in tonsillar B cells following LAIV stimulation, consistent with virus replication in tonsillar B cells. Fetal bovine serum (10%) was used in our cell culture, and we did not find any evidence suggesting blockade of LAIV replication (data not shown).

In this study, we have demonstrated the activation and induction of antigen-specific T\(_{FH}\) in human nasopharynx immune tissue by LAIV, and show T\(_{FH}\) are critical for LAIV-induced B cell anti-HA antibody response in the immune induction tissue of children and adults.

We showed a marked increase in T\(_{FH}\) number in tonsillar MNC following stimulation by LAIV (Fig 1a+b). With CFSE cell tracing, we also demonstrated T\(_{FH}\) proliferation following the stimulation (Fig 1c+d). The increase in T\(_{FH}\) number was accompanied by the production of anti-HA antibodies in tonsilar MNC (Fig 1g). We further demonstrated in the cell co-culture experiment that purified T\(_{FH}\) from tonsillar MNC helped B cell anti-HA antibody production, whereas non-T\(_{FH}\) cells did not (Fig 1h). These results support that T\(_{FH}\) provide critical help for LAIV-induced B cell anti-HA antibody production in human NALT.

Together with the increase in T\(_{FH}\) and antibody production following LAIV stimulation, a marked increase in GC B cells was also seen in tonsillar MNC (Fig 1e+f). This is consistent with the assumption that LAIV activates T\(_{FH}\) which support GC B cell proliferation and differentiation for antibody production. We reported previously that the number of T\(_{FH}\) correlated with that of GC B cells in NALT (20). These are concordant with previous reports in mouse models that GC B cells correlated with the appearance of T\(_{FH}\) after influenza virus infection (31) and the magnitude of T\(_{FH}\) response was directly correlated with the GC B cell response (32, 33).
We next examined the induction of influenza antigen-specific TFH from naïve T cells by LAIV using TFH-depleted CD45RO+ MNC. 7 days following LAIV stimulation, we have observed a dose-dependent increase in the number of newly differentiated TFH (CXCR5+ICOS+) that co-expressed BCL6 and IL21, which was followed by the detection of anti-HA antibody at day 14 (Fig 2a-e). Both BCL6 and IL21 are known to be essential for TFH differentiation from naïve T cells in animal studies (8, 34, 35). Our results here support TFH induction in human immune tissue also requires BCL6 and IL21. Indeed, further experiment with BCL6 blocker and IL21 blocking antibody demonstrated marked reduction of TFH induction from naïve tonsillar T cells, confirming a critical role of BCL6 and IL21 in TFH induction. We also showed ICOS signalling blocking inhibited ICOS activation and TFH induction, supporting that ICOS activation is required in TFH differentiation. It has been suggested that CD4+ T cells utilize ICOS:ICOSL interactions to upregulate IL21 production through which to contribute to TFH induction (35). Our finding that CD40L blocking antibody abrogated TFH induction is in line with the hypothesis that B and T cell cognate interaction through CD40:CD40L signalling is critical in TFH induction.

One finding we observed was that CD45RO+ cell depletion, which removes memory T cells from tonsillar MNC, markedly reduced anti-HA antibody production analysed at day 8 (for memory response). The fact that anti-HA IgG level could be readily detected at a high level in whole tonsillar MNC following vaccine stimulation at day 8 (Fig 1g), whereas in memory T cell-depleted MNC the antibody production could only be detected at around day 14 at a lower level (Fig 2f) suggests the presence of influenza-specific memory T/B cells in tonsillar MNC. In this study, although tonsillar tissues were from non-vaccinated donors, it is likely some of the donors had experienced an influenza infection previously, and had influenza-specific memory T/B cells. Therefore the presence of the memory T cells including Tfh in tonsillar MNC helped the memory B cell response following LAIV stimulation.
Further to the reduction of $T_{FH}$ induction following BCL6, IL21, ICOS and CD40L signalling blocking, we showed that the blockade of these signalings led to a diminished anti-HA antibody production, supporting the critical involvement of these pathways in $T_{FH}$ induction and $T_{FH}$-mediated B cell antibody production. The induction of influenza antigen-specific $T_{FH}$ by LAIV was demonstrated by the detection of antigen-specific CD4+ T cell activation marker CD154, which was expressed in a large proportion of the induced $T_{FH}$ following influenza antigen challenge (Fig 3). This finding is consistent with the report by Bentebibel et al demonstrating the increase in influenza antigen-specific $T_{FH}$-like cells in peripheral blood following an inactivated vaccine immunization in humans (5).

Studies in animal model demonstrated a critical role of IL21 in $T_{FH}$ activation and $T_{FH}$ were also shown to be the predominant source of IL21(34, 36). We showed here that stimulation of tonsillar MNC with LAIV activated a marked increase in IL21-producing $T_{FH}$ and in IL21 concentration in the cell culture supernatant. These results are consistent with the assumption that $T_{FH}$ are a major cellular source of IL21 in human tonsillar lymphocytes, as we found no significant IL21 production in the absence of $T_{FH}$ in the T-B cell co-culture experiment (Fig 4). We also demonstrated that newly differentiated $T_{FH}$ following LAIV stimulation expressed a high level of IL21 (Fig 2). As tonsillar $T_{FH}$ were also known to express IL21R (35), this co-expression of IL21 and IL21R by tonsillar $T_{FH}$ supports the hypothesis that IL21 acts in an autocrine-loop fashion in the vaccine-induced $T_{FH}$ differentiation and function in human NALT. Indeed, we showed that blocking IL21 signalling by an IL-21R neutralizing antibody inhibited both activation and differentiation of $T_{FH}$ induced by LAIV, and that diminished the anti-HA antibody production. So our results provide direct supporting evidence that IL21 is crucial in vaccine-induced $T_{FH}$ differentiation, and in $T_{FH}$-dependent B cell antibody production in human immune tissue.
Consistent with recent reports that there was an increase in T\textsubscript{FH}-like cells in human peripheral blood following parenteral influenza vaccination which correlated with the anti-HA antibody response (5, 6), we showed LAIV stimulation of PBMC also induced an increase in CXC\textsuperscript{5}\textsuperscript{*} T\textsubscript{FH}-like cells together with the production in anti-HA antibodies in the PBMC (Fig 5). The activation of influenza antigen-specific T\textsubscript{FH} in PBMC by LAIV was demonstrated by the expression of antigen-specific T cell activation marker CD154 upon antigen challenge. These results support the concept that there are T\textsubscript{FH}-like cells in peripheral circulation which are functionally similar to T\textsubscript{FH} found in lymphoid tissue such as NALT, and provide help to B cells for antibody production in an IL21- and ICOS-dependent manner (22).

In conclusion, we demonstrate for the first time the induction of antigen-specific T\textsubscript{FH} in human immune tissue by an intranasal influenza vaccine, and show its critical role in the anti-influenza HA antibody response. Our results suggest promoting antigen-specific T\textsubscript{FH} in human NALT by intranasal vaccines may provide an effective vaccination strategy against respiratory infections in humans.

METHODS

**Patients and samples.** Patients (age 2–30 years) undergoing adenoidectomy and/or tonsillectomy due to upper airway obstruction were recruited, and adenotonsillar tissues obtained following operation. A peripheral blood sample was also obtained before operation. The tonsillar tissues were transported in HBSS medium (Hank’s Balanced salt solution) to the laboratory. Tissue samples exhibiting any signs of gross inflammation were excluded. Patients with any known immunodeficiency were excluded from the study. Subjects who received influenza vaccination previously were also excluded from the study. The Liverpool Paediatric Research Ethics Committee approved the study [08/H1002/92] and written informed consent was obtained in each case.
**LAIV vaccine and influenza antigens.** An intranasal LAIV (FluMist, 2009-10) that included A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B influenza strains was obtained from BEI resources (ATCC, Manassas, VA). 0.2ml of LAIV contains approximately $10^7$ fluorescent focus units (FFU) of each strain, and we used 2µl/ml (~$10^5$FFU/ml) in cell stimulation which was a predetermined optimal concentration for the activation of anti-HA antibody response following dose titration experiments. An inactivated seasonal A/Brisbane/59/2007 H1N1 influenza virus (sH1N1) antigen, which was inactivated by β-propiolactone and partially purified (37) was obtained from the National Institute for Biological Standards and Control (NIBSC, UK). This inactivated sH1N1 antigen contained 83ug/ml of HA. A purified recombinant HA of sH1N1 (ATCC) was used for HA antigen stimulation as well as the coating antigen for anti-HA antibody measurement by ELISA. The recombinant HA contained a C-terminal histidine tag and were produced in High Five insect cells using a baculovirus expression vector system, purified from cell culture supernatant by immobilized-metal affinity chromatography (IMAC) and contain a trimerizing (foldon) domain (38).

**Cell culture and stimulation.** Mononuclear cells (MNC) from adenotonsillar tissues were isolated using Ficoll density centrifugation (39) (40). In some experiments, tonsillar MNC were depleted of effector and memory (CD45RO+) T cells using CD45RO microbeads and magnetic cell sorting (Miltenyi) by passing cells through the depletion column twice as described previously (41, 42). The depletion of CD45RO+ cells from tonsillar MNC removed TFH cells (>98%). Unfractionated whole MNC or CD45RO+ cell-depleted MNC were cultured (4x10⁶/ml) in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), streptomycin (50μg/ml) and penicillin (50U/ml) (Sigma), in the presence the LAIV (2µl/ml unless otherwise stated) for up to 14 days. Cells were collected at pre-defined time points for analysis of TFH cells by flow-cytometry. Cell culture supernatants were collected for measurement of cytokine and antibody production respectively by ELISA.
Flow-cytometry analysis of T<sub>FH</sub> cell proliferation and intracellular cytokine expression. For T<sub>FH</sub> identification, tonsillar MNC were stained with anti-human CD3, CD4, CXCR5 and ICOS antibodies followed by flow cytometry and CD4<sup>+</sup> CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells were identified as T<sub>FH</sub> (43, 44). The tonsillar lymphocytes gated for analysis based on typical FSC/SSC characteristics and singlet selection has a typical viability >98% viability when examined with propidium iodide staining.

Expression of B-cell lymphoma 6 protein (BCL6), a master transcription factor for T<sub>FH</sub> differentiation (8), in newly induced T<sub>FH</sub> cells was analyzed by intracellular staining with anti-human BCL6 antibody after cell fixation/permeabilization following manufacturer’s instructions (eBioscience). Cell proliferation was examined by Carboxyfluorescein succinimidyl ester (CFSE) staining of tonsillar MNC (Molecular Probes), followed by cell stimulation for 5 days and by flow cytometry (41, 42).

Briefly, tonsillar MNC were labelled with CFSE (at 37°C, for 8 min) and resuspended in RPMI before cell stimulation with LAIV (2μl/ml) for 5 days. T<sub>FH</sub> cell proliferation was then examined by analysis of CFSE dilution in T<sub>FH</sub> cells (CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells) by flow cytometry. Intracellular cytokine expression e.g. IL21 was analysed following a standard intracellular staining procedure including cell permeabilization as described previously (40). Flow cytometry data analyzed using FlowJo software.

Germinal center (GC) B cell subset was analyzed by flow-cytometry with a combination of CD19, CD38 and IgD fluorescence-labelled anti-human antibodies and identified as CD19<sup>+</sup>CD38<sup>hi</sup>IgD<sup>-</sup>.

Analysis of antigen-specific T<sub>FH</sub> induction. T<sub>FH</sub> differentiation/induction from naïve tonsillar T cells by LAIV was examined using CD45RO<sup>+</sup> cell-depleted MNC (which resulted in CD45RO<sup>-</sup> MNC) as described earlier. The CD45RO<sup>-</sup> MNC (with T<sub>FH</sub> removed but retained naïve T cell) were stimulated with LAIV (2μl/ml, otherwise as stated) and cultured for 7 days before flow-cytometric analysis for T<sub>FH</sub> cells including CXCR5, ICOS and BCL6 expressions. For the detection of induced influenza antigen-specific T<sub>FH</sub> cells after LAIV stimulation, the cells (at day 7) were washed and incubated for 24 hours in fresh culture medium only, followed by antigen challenge with sH1N1 virus antigen or
recombinant HA (1µg/ml) for 6 hours in the presence of brefeldin A. The cells were then stained for T<sub>FH</sub> including CD4, CXCR5, ICOS, and intracellular CD154 expression after cell fixation/permeabilization which detects antigen-specific T cells by flow cytometry (19-21).

To determine if IL21, ICOS, CD40 and BCL6 signalling pathways are involved in the activation/induction of T<sub>FH</sub>, neutralizing/blocking antibodies to IL21 receptor, ICOS- and CD40-ligand (L) or a BCL6 inhibitor were used to co-incubate with tonsillar MNC before LAIV stimulation. Briefly, recombinant human IL21R-Fc chimera, anti-ICOS-L (R&D systems) and anti-CD40-L antibodies (InvivoGen) or isotype controls (10µg/ml) or BCL6 inhibitor (79-6, Calbiochem)(50 µM) were co-incubated with tonsillar MNC or CD45RO<sup>+</sup> MNC for 1 hour prior to stimulation by LAIV. BCL6 inhibitor 79-6 is a cell-permeable compound that selectively inhibits the transcriptional repression activity of BCL6. The MNC were then cultured for up to 7-14 days before analysis for T<sub>FH</sub> and anti-HA antibody production.

**Measurement of HA-Specific antibodies.** Production of anti-HA IgG, IgM and IgA antibodies to sH1N1 virus in cell culture supernatants was measured as previously described (45, 46). In brief, ELISA plates were coated with recombinant sH1N1 HA overnight. Following blocking, cell culture supernatants were added and incubated for 2 hours. Alkaline phosphatase-conjugated anti-human IgG, IgM or IgA antibody was then added and incubated. Following the addition of pNPP substrate, color development was read at OD405nm at 1 hour and data were analysed using DeltaSoft software. Intravenous immunoglobulin (IVIG, Intratect) containing high titers of anti-sH1N1 HA IgG antibody was used as a reference standard for IgG antibody. Anti-HA IgM and IgA antibody titers were expressed as OD values (read at 30min) as no reference standard was available.

**Cell purification and T<sub>FH</sub>-B cell co-culture.** Tonsillar T<sub>FH</sub> and B cells were purified using magnetic cell sorting as described previously (43). Briefly, B cells were purified by negative selection using B
cell purification kit (EasySep™, Stemcell) which yielded B cell purity >99%. For T<sub>FH</sub> purification, CD4<sup>+</sup>T cells were first isolated by negative selection, followed by positive selection of CXCR5<sup>high</sup> (T<sub>FH</sub>) using biotin anti-CXCR5 antibody. The amount of anti-CXCR5 antibody was optimised to ensure only CXCR5<sup>high</sup>-expressing cells were selected (purity>95%). CXCR5<sup>−</sup>CD4<sup>+</sup>T (non-T<sub>FH</sub>) cells were isolated by negative selection from CD4<sup>+</sup>T cells using an optimised amount of anti-CXCR5 antibody (purity >99%). Purified B cells were co-cultured (1:1 ratio) with either purified T<sub>FH</sub> or non-T<sub>FH</sub> cells at 5x10<sup>5</sup> cells/ml in the presence of LAIV. The cells were cultured for 10 days and cell culture supernatants were collected for anti-HA antibody analysis.

**Statistical Analysis.** Means and standard errors are used unless indicated otherwise. Differences between two groups were analysed using Student’s t test, and paired T test was used for paired samples. Statistical analysis was performed using GraphPad Prism 5 software. P<0.05 was considered statistically significant.

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**REFERENCES**


**Figure 1. LAIV induces T<sub>F</sub>H proliferation that correlates with GC B cell response and antibody production in NALT.** LAIV stimulation induced an increase in T<sub>F</sub>H number (a+b) and T<sub>F</sub>H proliferation (c+d) in tonsillar MNC (b & d, n=15, **p<0.01 vs unstimulated medium controls). (a & c) Representative plots or histogram of T<sub>F</sub>H subset (CXCR5<sup>hi</sup>/ICOS<sup>hi</sup>) in CD4<sup>+</sup> T cells following stimulation (a, day 3), and T<sub>F</sub>H proliferation analysed by CFSE (c, day 5, red line: LAIV, grey shaded: medium control). (e & f) Increase in GC B cell number (CD19<sup>+</sup> CD38<sup>hi</sup>IgD<sup>-</sup>) in tonsillar MNC after LAIV stimulation (n=13, **P < 0.01 vs control). LAIV-induced anti-HA IgG antibody production in tonsillar MNC (g, n=20, **p<0.01 vs control, day 8), and LAIV-induced anti-HA IgG production in B cells co-cultured with T<sub>F</sub>H (red bar) or with non-T<sub>F</sub>H cells (blank bar) (h, n=10, **p<0.01, #p>0.05 vs control). Data in the bar figures are means and SE from a number of different experiments done with tonsils from different donors.

**Figure 2. Induction of T<sub>F</sub>H from naïve tonsillar T cells and antibody production by LAIV.** Representative plots (a) and bar graph (b) show the induction of T<sub>F</sub>H (CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>) from CD45RO<sup>-</sup> MNC by LAIV compared with medium control (n=10, **p<0.01). (c & d) FACS histograms of BCL6 (c) and IL21 expression (d) in LAIV-induced T<sub>F</sub>H as compared to unstimulated medium control (isotype controls: shaded). (e) Dose-dependent induction of T<sub>F</sub>H (day 7, top) and anti-HA IgG antibody production (day 14, bottom) from CD45RO<sup>-</sup> MNC following LAIV stimulation (n=6). (f) LAIV-induced anti-HA IgG, IgM and IgA production in CD45RO<sup>-</sup> MNC (day 14, n=10, **p<0.01).

**Figure 3. Detection of LAIV-induced antigen-specific T<sub>F</sub>H and effect of IL21, ICOS, CD40 and BCL6 signalings on T<sub>F</sub>H and antibody induction.** CD45RO<sup>-</sup> MNC were first stimulated by LAIV for 7 days followed by influenza antigen challenge with sH1N1 or HA antigen. (a) A representative plot showing activated T<sub>F</sub>H (ICOS<sup>+</sup>CXCR5<sup>+</sup>) following sH1N1 antigen challenge, and (b) showing the frequencies of activated T<sub>F</sub>H (% of CD4<sup>+</sup> T cell) after sH1N1 or HA challenge following prior LAIV stimulation (**p<0.01, ***p<0.001 vs LAIV stimulation alone. Medium alone negative
control is also shown). Representative plots (c) and summary frequency (d, n=5) of CD154+ expression in the CD4+ T cell subsets including T<sub>FH</sub> following sH1N1 antigen challenge. (e+f)

Effect of neutralizing antibodies to IL21R, ICOS- and CD40-L or BCL6 blocker on T<sub>FH</sub> induction (e, day 7) and antibody production (f, day 14) in CD45RO<sup>−</sup>ve MNC following LAIV stimulation (**p<0.01 vs LAIV stimulation or isotype control antibodies).

**Figure 4. IL-21 expression in LAIV-activated T<sub>FH</sub> and its effect on anti-HA antibody production.** (a) Representative plots showing T<sub>FH</sub> subset and IL21 expression in tonsillar CD4+ T cells following LAIV stimulation (shaded histogram: isotype control). (b) An increase in IL-21-producing T<sub>FH</sub> (% of CD4+ T cells) of tonsillar MNC following LAIV stimulation (n=10, **p < 0.01 vs control). (c-e) IL21 concentrations following stimulation in the culture supernatants of tonsillar MNC (c, n=22), of B cells co-cultured with T<sub>FH</sub> (d, n=10) or with non-T<sub>FH</sub> cells (e, n=10) (**p < 0.01 vs control, NS: not significant). (f+g) IL-21R blocking by adding anti-IL-21R antibody to tonsillar MNC led to a reduction in T<sub>FH</sub> number (f) and in anti-HA IgG, IgM and IgA antibody production (g) (n=8, **p<0.01).

**Figure 5. Activation of T<sub>FH</sub>-like cells in PBMC.** (a) Representative plots shows the increase of T<sub>FH</sub>-like cells (CD4<sup>+</sup>CXCR5<sup>+</sup>ICOS<sup>+</sup>) in PBMC following stimulation for 3 days by LAIV, as compared to medium control. (b) LAIV-induced increase in T<sub>FH</sub>-like cells in PBMC compared with control (n=10, **p < 0.01). (c) Anti-HA IgG and IgM antibody production in PBMC culture supernatant following LAIV stimulation (n=10, **p < 0.01). (d) Frequency of antigen-specific CD154+ T<sub>FH</sub>-like cells (% of CD4+ T cells, red bar) in PBMC following LAIV stimulation and subsequent sH1N1 antigen challenge, compared to other CD4+ T cell sub-populations as indicated (n=4,**p<0.01, ***p<0.001).