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 Aljurayyan[1], Suttida Puksuriwong[1], Muhammad Ahmed[1], Ravi Sharma[2], Madhan Krishnan[2], Salil Sood[2], Katherine Davies[3], Devika Rajashekar[3]. Sam Leong[4], Paul S McNamara[5], Stephen Gordon[6], Qibo Zhang[1]*

Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool, UK[1] , ENT Department, Alder Hey Children's Hospital, United Kingdom[2], ENT Department, Royal Liverpool and Broadgreen University Hospitals, UK[3], ENT Department, Aintree University Hospital of Liverpool, UK[4], Institute in the Park, Alder Hey Children's Hospital, UK[5], Liverpool School of Tropical Medicine, United Kingdom[6]

Short title: Activation of antigen-specific T_{FH} 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

Word count: Abstract 250. Text: 3440

ABSTRACT

There is increasing interest recently in developing intranasal vaccines against respiratory tract infections. Antibody response is critical in vaccine-induced protection and T_{FH} is considered important in mediating antibody response. Most data supporting the role for T_{FH} in antibody response are from animal studies, and direct evidence from humans is limited, apart from T_{FH}-like cells in blood. We studied activation and induction of T_{FH} and its role on anti-influenza antibody response by live-attenuated influenza vaccine(LAIV) in human nasopharynx-associated lymphoid tissue(NALT). T_{FH} 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_{FH} by LAIV was studied by flow-cytometry for induced T_{FH} and CD154 expression. LAIV induced T_{FH} proliferation which correlated with anti-HA antibody production, and T_{FH} was shown critical for antibody response. Induction of T_{FH} from naïve T cells by LAIV was shown in newly induced T_{FH} expressing BCL6 and CD21, which was followed by the detection of anti-HA antibodies. Antigen specificity of LAIV-induced T_{FH} was demonstrated by the expression of antigen-specific T cell activation marker CD154 upon challenge by H1N1 virus antigen or HA. LAIV-induced T_{FH} 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_{FH} by LAIV in human NALT that provide critical support for anti-influenza antibody response. Promoting antigen-specific T_{FH} in NALT by intranasal vaccines may provide an effective vaccination strategy against respiratory infections in humans.

Journal of Virology JVI Journal of Virology

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_{FH} is important for the antibody response. However, data on whether this T_{FH}-mediated help for antibody production operates in humans is limited, due to the lack of access to human immune tissue containing the T_{FH}. In this study, we demonstrated the induction of T_{FH} 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_{FH} 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_{FH}), 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_{FH}) in vaccine-induced immunity (1, 2) and promoting T_{FH} has been considered a promising vaccination strategy. However, most of the current evidence supporting the importance of T_{FH} in vaccination comes from animal studies, and direct evidence from humans is limited, apart from the detection of T_{FH}-like cells from human peripheral blood samples which are thought as T_{FH} equivalent (3, 4). Whether this T_{FH}–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_{FH} -like" cells in peripheral blood following parenteral influenza vaccination appeared to correlate with an anti-hemagglutinin (HA) antibody response (5, 6).

T_{FH} are a subset of CD4+ 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_{FH} express chemokine receptor CXCR5 and inducible costimulator-ICOS, IL21 and the transcription factor B-cell lymphoma 6 (BCL6) (8). Considering the importance of T_{FH} for B cell antibody response, novel vaccines to induce/activate T_{FH} 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_{FH} by LAIV and its role on the anti-HA antibody response in human NALT tissue, and shown the induction of antigen-specific T_{FH} in NALT is critical in LAIV-induced anti-influenza HA antibody response.

RESULTS

LAIV activates a proliferative T_{FH} response in NALT that provides critical help for anti-HA antibody production

Activation of T_{FH} in NALT was examined by LAIV stimulation of adenotonsillar MNC for 3 days followed by enumerating T_{FH} numbers using flow cytometry. As shown in Figure 1a+b, LAIV stimulation elicited a significant increase in T_{FH} number ($CD4^+CXCR5^{hi}ICOS^{hi}$) compared to unstimulated control ($p<0.01$). The T_{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_{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, $p<0.01$).

117 Anti-influenza antibody production was measured in tonsillar MNC culture supernatant following
118 LAIV stimulation for 8 days. As expected, LAIV elicited marked anti-HA antibody production (Fig
119 1g), and T- B cell co-culture experiment demonstrated B cells co-cultured with purified T_{FH} elicited
120 anti-HA antibody production, whereas no antibody production was shown in B cells co-cultured with
121 non-T_{FH} ($CXCR5^{-}CD4^{+}$) cells (Fig 1h).

122 ***Induction of antigen-specific T_{FH} by LAIV that correlates with antibody production***

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

133 Having shown the induction of T_{FH} by LAIV, we next examined the specificity of these induced T_{FH}
134 for influenza antigens. As CD154 is considered a reliable functional marker for antigen-activated T
135 cells, i.e. a marker for antigen-specific T cells (5, 19-21), CD154 expression in the CD4+ T cell
136 subsets was analyzed following either an inactivated sH1N1 virus antigen or recombinant HA
137 challenge. A representative dot plot demonstrating the activated T_{FH} ($ICOS^{+}CXCR5^{+}$, top right
138 quadrant) following the antigen challenge was shown in Fig 3a, and the frequencies of activated T_{FH}
139 (% 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

163 shown in the co-culture of T_{FH} and B cells (4d), but not seen in the co-culture of non-T_{FH} with B cells
164 following LAIV stimulation (4e).

165 IL21 receptor blocking using anti-IL21R antibody abrogated the increase of T_{FH} number in tonsillar
166 MNC elicited by LAIV stimulation (4f), followed by a significant reduction in anti-HA antibody
167 production in tonsillar MNC (4g).

168 ***Activation of T_{FH}–like cells in PBMC by LAIV***

169 Recent studies suggest there are T_{FH}-like cells in peripheral blood that express CXCR5 and ICOS and
170 have similar B cell-help functions (5, 22-25). To determine whether LAIV activate T_{FH}-like cells and
171 antibody production in peripheral blood, freshly isolated PBMC were stimulated by LAIV for up to
172 14 days followed by flow-cytometry and antibody detection. As shown in figure 5a+b, LAIV
173 stimulation induced an increase of T_{FH}–like *(*$CXCR5^+ICOS^+$) CD4+ T cells in PBMC (at day 7),
174 followed by the detection of anti-HA IgG and IgM antibodies in the PBMC culture supernatants (Fig
175 5c). The activation of influenza antigen-specific T_{FH}–like cells by LAIV was demonstrated by the
176 finding that a major proportion (mean 45.6%) of these cells expressed CD154 following the H1N1
177 antigen challenge, markedly higher than the other non-T_{FH} cell populations (Fig 5d).

178 **DISCUSSION**

179 LAIV is thought to replicate in upper respiratory tract to induce immunity through the local immune
180 tissue NALT, and it was shown to replicate in nasal epithelial cells(26). As part of the mucosal
181 immune system in human nasopharynx, adenotonsillar tissue has a surface reticular epithelial cell
182 layer in which epithelial cells mixed with other cells including a large number of B cells. Many B
183 cells infiltrating the epithelial layer exhibit memory B cell markers and have great antigen-presenting
184 potential(27, 28). In our adenotonsillar MNC culture, the predominant cell populations are
185 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 tonsillar 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 T_{FH} from naïve T cells by LAIV using T_{FH}-depleted CD45RO$^-$ MNC. 7 days following LAIV stimulation, we have observed a dose-dependent increase in the number of newly differentiated T_{FH} (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 T_{FH} differentiation from naïve T cells in animal studies (8, 34, 35). Our results here support T_{FH} induction in human immune tissue also requires BCL6 and IL21. Indeed, further experiment with BCL6 blocker and IL21 blocking antibody demonstrated marked reduction of T_{FH} induction from naïve tonsillar T cells, confirming a critical role of BCL6 and IL21 in T_{FH} induction. We also showed ICOS signalling blocking inhibited ICOS activation and T_{FH} induction, supporting that ICOS activation is required in T_{FH} differentiation. It has been suggested that CD4+ T cells utilize ICOS:ICOSL interactions to upregulate IL21 production through which to contribute to T_{FH} induction (35). Our finding that CD40L blocking antibody abrogated T_{FH} induction is in line with the hypothesis that B and T cell cognate interaction through CD40:CD40L signalling is critical in T_{FH} 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.

234 Further to the reduction of T_{FH} induction following BCL6, IL21, ICOS and CD40L signalling
235 blocking, we showed that the blockade of these signallings led to a diminished anti-HA antibody
236 production, supporting the critical involvement of these pathways in T_{FH} induction and T_{FH}-mediated
237 B cell antibody production. The induction of influenza antigen-specific T_{FH} by LAIV was
238 demonstrated by the detection of antigen-specific CD4+ T cell activation marker CD154, which was
239 expressed in a large proportion of the induced T_{FH} following influenza antigen challenge (Fig 3). This
240 finding is consistent with the report by Bentebibel *et al* demonstrating the increase in influenza
241 antigen-specific T_{FH}-like cells in peripheral blood following an inactivated vaccine immunization in
242 humans (5).
243 Studies in animal model demonstrated a critical role of IL21 in T_{FH} activation and T_{FH} were also
244 shown to be the predominant source of IL21(34, 36). We showed here that stimulation of tonsillar
245 MNC with LAIV activated a marked increase in IL21-producing T_{FH} and in IL21 concentration in
246 the cell culture supernatant. These results are consistent with the assumption that T_{FH} are a major
247 cellular source of IL21 in human tonsillar lymphocytes, as we found no significant IL21 production
248 in the absence of T_{FH} in the T-B cell co-culture experiment (Fig 4). We also demonstrated that newly
249 differentiated T_{FH} following LAIV stimulation expressed a high level of IL21 (Fig 2). As tonsillar
250 T_{FH} were also known to express IL21R (35), this co-expression of IL21 and IL21R by tonsillar T_{FH}
251 supports the hypothesis that IL21 acts in an autocrine-loop fashion in the vaccine-induced T_{FH}
252 differentiation and function in human NALT. Indeed, we showed that blocking IL21 signalling by an
253 IL-21R neutralizing antibody inhibited both activation and differentiation of T_{FH} induced by LAIV,
254 and that diminished the anti-HA antibody production. So our results provide direct supporting
255 evidence that IL21 is crucial in vaccine-induced T_{FH} differentiation, and in T_{FH}-dependent B cell
256 antibody production in human immune tissue.

Consistent with recent reports that there was an increase in T_{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 $CXC5^+$ T_{FH}-like cells together with the production in anti-HA antibodies in the PBMC (Fig 5). The activation of influenza antigen-specific T_{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_{FH}-like cells in peripheral circulation which are functionally similar to T_{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_{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_{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 (~10^5FFU/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 T_{FH} cells (>98%). Unfractionated whole MNC or $CD45RO^+$cell-depleted MNC were cultured (4x10^6/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 T_{FH} cells by flow-cytometry. Cell culture supernatants were collected for measurement of cytokine and antibody production respectively by ELISA.

304 ***Flow-cytometry analysis of T_{FH}, cell proliferation and intracellular cytokine expression***. For T_{FH}
305 identification, tonsillar MNC were stained with anti-human CD3, CD4, CXCR5 and ICOS antibodies
306 followed by flow cytometry and $CD4^+$ $CXCR5^{hi}$ $ICOS^{hi}$ cells were identified as T_{FH} (43, 44). The
307 tonsillar lymphocytes gated for analysis based on typical FSC/SSC characteristics and singlet
308 selection has a typical viability >98% viability when examined with propidium iodide staining.
309 Expression of B-cell lymphoma 6 protein (BCL6), a master transcription factor for T_{FH} differentiation
310 (8), in newly induced T_{FH} cells was analyzed by intracellular staining with anti-human BCL6 antibody
311 after cell fixation/permeabilization following manufacturer' instructions (eBioscience). Cell
312 proliferation was examined by Carboxyfluorescein succinimidyl ester (CFSE) staining of tonsillar
313 MNC (Molecular Probes), followed by cell stimulation for 5 days and by flow cytometry (41, 42).
314 Briefly, tonsillar MNC were labelled with CFSE (at 37°C, for 8 min) and resuspended in RPMI before
315 cell stimulation with LAIV (2µl /ml) for 5 days. T_{FH} cell proliferation was then examined by analysis
316 of CFSE dilution in T_{FH} cells ($CXCR5^{hi}$ $ICOS^{hi}$ cells) by flow cytometry. Intracellular cytokine
317 expression e.g. IL21 was analysed following a standard intracellular staining procedure including cell
318 permeabilization as described previously (40). Flow cytometry data analyzed using FlowJo software.
319 Germinal center (GC) B cell subset was analyzed by flow-cytometry with a combination of CD19,
320 CD38 and IgD fluorescence-labelled anti-human antibodies and identified as $CD19^+CD38^{hi}IgD^-$.

321 ***Analysis of antigen-specific T_{FH} induction***. T_{FH} differentiation/induction from naïve tonsillar T cells
322 by LAIV was examined using $CD45RO^+$cell-depleted MNC (which resulted in $CD45RO^-$ MNC) as
323 described earlier. The $CD45RO^-$ MNC (with T_{FH} removed but retained naïve T cell) were stimulated
324 with LAIV (2µl/ml, otherwise as stated) and cultured for 7 days before flow-cytometric analysis for
325 T_{FH} cells including CXCR5, ICOS and BCL6 expressions. For the detection of induced influenza
326 antigen-specific T_{FH} cells after LAIV stimulation, the cells (at day 7) were washed and incubated for
327 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_{FH} 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_{FH}, 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 $CD45RO^-$ 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_{FH} 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_{FH}-B cell co-culture*.** Tonsillar T_{FH} 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_{FH} purification, $CD4^+$T cells were first isolated by negative selection, followed by positive selection of $CXCR5^{high}$ (T_{FH}) using biotin anti-CXCR5 antibody. The amount of anti-CXCR5 antibody was optimised to ensure only $CXCR5^{high}$-expressing cells were selected (purity>95%). $CXCR5^-CD4^+$T (non-T_{FH}) cells were isolated by negative selection from $CD4^+$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_{FH} or non-T_{FH} cells at $5x10^5$ 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.

ACKNOWLEDGEMENT: We thank the patients who took part in the study and the theatre staff in the Liverpool Children's Hospital and Royal Liverpool and Broadgreen University Hospitals for helping the collection of samples. We also acknowledge funding support from SPARKS Medical Research, UK [15Liv01] and British Medical Association [HC Roscoe Award], and Royal Embassy of Saudi Arabia Culture Bureau [studentship].

REFERENCES

1. **Crotty S.** 2014. T Follicular Helper Cell Differentiation, Function, and Roles in Disease. Immunity **41:**529-542.
2. **Slight SR, Rangel-Moreno J, Gopal R, Lin Y, Fallert Junecko BA, Mehra S, Selman M, Becerril-Villanueva E, Baquera-Heredia J, Pavon L, Kaushal D, Reinhart TA, Randall TD, Khader SA.** 2013. CXCR5(+) T helper cells mediate protective immunity against tuberculosis. The Journal of Clinical Investigation **123:**712-726.

3. **Herati RS, Reuter MA, Dolfi DV, Mansfield KD, Aung H, Badwan OZ, Kurupati RK, Kannan S, Ertl H, Schmader KE, Betts MR, Canaday DH, Wherry EJ.** 2014. Circulating CXCR5(+)PD-1(+) response predicts influenza vaccine antibody responses in young adults but not elderly adults. Journal of immunology (Baltimore, Md : 1950) **193:**3528-3537.
4. **Morita R, Schmitt N, Bentebibel S-E, Ranganathan R, Bourdery L, Zurawski G, Foucat E, Dullaers M, Oh S, Sabzghabaei N, Lavecchio EM, Punaro M, Pascual V, Banchereau J, Ueno H.** 2011. Human Blood CXCR5(+)CD4(+) T Cells Are Counterparts of T Follicular Cells and Contain Specific Subsets that Differentially Support Antibody Secretion. Immunity **34:**108-121.
5. **Bentebibel S-E, Lopez S, Obermoser G, Schmitt N, Mueller C, Harrod C, Flano E, Mejias A, Albrecht RA, Blankenship D, Xu H, Pascual V, Banchereau J, Garcia-Sastre A, Palucka AK, Ramilo O, Ueno H.** 2013. Induction of ICOS+CXCR3+CXCR5+ TH Cells Correlates with Antibody Responses to Influenza Vaccination. Science Translational Medicine **5:**176ra132-176ra132.
6. **Spensieri F, Borgogni E, Zedda L, Bardelli M, Buricchi F, Volpini G, Fragapane E, Tavarini S, Finco O, Rappuoli R, Del Giudice G, Galli G, Castellino F.** 2013. Human circulating influenza-CD4+ ICOS1+IL-21+ T cells expand after vaccination, exert helper function, and predict antibody responses. Proceedings of the National Academy of Sciences **110:**14330-14335.
7. **Choi Youn S, Kageyama R, Eto D, Escobar Tania C, Johnston Robert J, Monticelli L, Lao C, Crotty S.** 2011. ICOS Receptor Instructs T Follicular Helper Cell versus Effector Cell Differentiation via Induction of the Transcriptional Repressor Bcl6. Immunity **34:**932-946.
8. **Nurieva RI, Chung Y, Martinez GJ, Yang XO, Tanaka S, Matskevitch TD, Wang Y-H, Dong C.** 2009. Bcl6 Mediates the Development of T Follicular Helper Cells. Science (New York, NY) **325:**1001-1005.
9. **Barbey-Martin C, Gigant B, Bizebard T, Calder LJ, Wharton SA, Skehel JJ, Knossow M.** 2002. An Antibody That Prevents the Hemagglutinin Low pH Fusogenic Transition. Virology **294:**70-74.
10. **Wu HY, Russell MW.** 1997. Nasal lymphoid tissue, intranasal immunization, and compartmentalization of the common mucosal immune system. Immunol Res **16:**187-201.
11. **Kiyono H, Fukuyama S.** 2004. NALT- versus PEYER'S-patch-mediated mucosal immunity. Nat Rev Immunol **4:**699-710.
12. **Zuercher AW, Coffin SE, Thurnheer MC, Fundova P, Cebra JJ.** 2002. Nasal-Associated Lymphoid Tissue Is a Mucosal Inductive Site for Virus-Specific Humoral and Cellular Immune Responses. The Journal of Immunology **168:**1796-1803.
13. **Tamura SI, Kurata T.** 2004. Defense mechanisms against influenza virus infection in the respiratory tract mucosa. Japanese Journal of Infectious Diseases **57:**236-247.
14. **Belshe RB.** 2004. Current status of live attenuated influenza virus vaccine in the US. Virus Research **103:**177-185.
15. **Moldoveanu Z, Clements ML, Prince SJ, Murphy BR, Mestecky J.** 1995. Human immune responses to influenza virus vaccines administered by systemic or mucosal routes. Vaccine **13:**1006-1012.
16. **Hoft DF, Babusis E, Worku S, Spencer CT, Lottenbach K, Truscott SM, Abate G, Sakala IG, Edwards KM, Creech CB, Gerber MA, Bernstein DI, Newman F, Graham I, Anderson EL, Belshe RB.** 2011. Live and Inactivated Influenza Vaccines Induce Similar Humoral Responses, but Only Live Vaccines Induce Diverse T-Cell Responses in Young Children. The Journal of Infectious Diseases **204:**845-853.
17. **Sridhar S, Brokstad KA, Cox RJ.** 2015. Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines. Vaccines **3:**373-389.

18. **Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, Kemble G, Connor EM.** 2007. Live Attenuated versus Inactivated Influenza Vaccine in Infants and Young Children. New England Journal of Medicine **356:**685-696.

19. **Chattopadhyay PK, Yu J, Roederer M.** 2005. A live-cell assay to detect antigen-specific CD4+ T cells with diverse cytokine profiles. Nat Med **11:**1113-1117.

20. **Frentsch M, Arbach O, Kirchhoff D, Moewes B, Worm M, Rothe M, Scheffold A, Thiel A.** 2005. Direct access to CD4+ T cells specific for defined antigens according to CD154 expression. Nat Med **11:**1118-1124.

21. **Bacher P, Schink C, Teutschbein J, Kniemeyer O, Assenmacher M, Brakhage AA, Scheffold A.** 2013. Antigen-Reactive T Cell Enrichment for Direct, High-Resolution Analysis of the Human Naive and Memory Th Cell Repertoire. The Journal of Immunology **190:**3967-3976.

22. **Morita R, Schmitt N, Bentebibel S-E, Ranganathan R, Bourdery L, Zurawski G, Foucat E, Dullaers M, Oh S, Sabzghabaei N, Lavecchio EM, Punaro M, Pascual V, Banchereau J, Ueno H.** 2011. Human Blood CXCR5+CD4+ T Cells Are Counterparts of T Follicular Cells and Contain Specific Subsets that Differentially Support Antibody Secretion. Immunity **34:**108-121.

23. **Vinuesa CG, Cook MC.** 2011. Blood Relatives of Follicular Helper T Cells. Immunity **34:**10-12.

24. **Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, Manku H, Vyse TJ, Roncador G, Huttley GA, Goodnow CC, Vinuesa CG, Cook MC.** 2010. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. Arthritis & Rheumatism **62:**234-244.

25. **Pallikkuth S, Parmigiani A, Silva SY, George VK, Fischl M, Pahwa R, Pahwa S.** 2012. Impaired peripheral blood T-follicular helper cell function in HIV-infected nonresponders to the 2009 H1N1/09 vaccine. Blood **120:**985-993.

26. **Fischer WA, Brighton M, Jaspers I.** 2014. Live Attenuated Influenza Vaccine Strains Elicit a Greater Innate Immune Response than Antigenically-Matched Seasonal Influenza Viruses during Infection of Human Nasal Epithelial Cell Cultures. Vaccine **32:**1761-1767.

27. **Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C, Liu YJ, Rousset F, Saeland S.** 1994. The CD40 antigen and its ligand. [Review]. Annual Review of Immunology **12:**881-922.

28. **Banchereau J, Brière F, Liu YJ, Rousset F.** 1994. Molecular control of B Lymphocyte growth and differentiation. STEM CELLS **12:**278-288.

29. **Zhang Q, Choo S, Finn A.** 2002. Immune responses to novel pneumococcal proteins (Pneumolysin, PspA, PsaA and CbpA) in adenoidal B cells from children. Infection & Immunity **70:**5363-5369.

30. **Mullin J, Ahmed MS, Sharma R, Upile N, Beer H, Achar P, Puksuriwong S, Ferrara F, Temperton N, McNamara P, Lambe T, Gilbert SC, Zhang Q.** 2016. Activation of cross-reactive mucosal T and B cell responses in human nasopharynx-associated lymphoid tissue in vitro by Modified Vaccinia Ankara-vectored influenza vaccines. Vaccine **34:**1688-1695.

31. **Elsner RA, Ernst DN, Baumgarth N.** 2012. Single and Coexpression of CXCR4 and CXCR5 Identifies CD4 T Helper Cells in Distinct Lymph Node Niches during Influenza Virus Infection. Journal of Virology **86:**7146-7157.

32. **Baumjohann D, Preite S, Reboldi A, Ronchi F, Ansel KM, Lanzavecchia A, Sallusto F.** 2013. Persistent Antigen and Germinal Center B Cells Sustain T Follicular Helper Cell Responses and Phenotype. Immunity **38:**596-605.

33. **Fernandez S, Cisney ED, Hall SI, Ulrich RG.** 2011. Nasal Immunity to Staphylococcal Toxic Shock Is Controlled by the Nasopharynx-Associated Lymphoid Tissue. Clinical and Vaccine Immunology **18:**667-675.
34. **Spolski R, Leonard WJ.** 2010. IL-21 and T follicular helper cells. International Immunology **22:**7-12.
35. **Vogelzang A, McGuire HM, Yu D, Sprent J, Mackay CR, King C.** 2008. A Fundamental Role for Interleukin-21 in the Generation of T Follicular Helper Cells. Immunity **29:**127-137.
36. **Eto D, Lao C, DiToro D, Barnett B, Escobar TC, Kageyama R, Yusuf I, Crotty S.** 2011. IL-21 and IL-6 Are Critical for Different Aspects of B Cell Immunity and Redundantly Induce Optimal Follicular Helper CD4 T Cell (Tfh) Differentiation. PLoS ONE **6:**e17739.
37. **Wood JM, Schild GC, Newman RW, Seagroatt V.** 1977. An improved single-radial-immunodiffusion technique for the assay of influenza haemagglutinin antigen: application for potency determinations of inactivated whole virus and subunit vaccines. J Biol Stand **5:**237-247.
38. **Stevens J, Corper AL, Basler CF, Taubenberger JK, Palese P, Wilson IA.** 2004. Structure of the Uncleaved Human H1 Hemagglutinin from the Extinct 1918 Influenza Virus. Science **303:**1866-1870.
39. **Zhang Q, Bernatoniene J, Bagrade L, Pollard AJ, Mitchell TJ, Paton JC, Finn A.** 2006. Serum and mucosal antibody responses to pneumococcal protein antigens in children: relationships with carriage status. European Journal of Immunology **36:**46-57.
40. **Zhang Q, Leong SC, McNamara PS, Mubarak A, Malley R, Finn A.** 2011. Characterisation of Regulatory T Cells in Nasal Associated Lymphoid Tissue in Children: Relationships with Pneumococcal Colonization. PLoS Pathog **7:**e1002175.
41. **Zhang Q, Bernatoniene J, Bagrade L, Clarke E, Paton JC, Mitchell TJ, Nunez DA, Finn A.** 2007. Low CD4 T cell immunity to pneumolysin is associated with nasopharyngeal carriage of pneumococci in children. Journal of Infectious Diseases **195:**1194-1202.
42. **Gray C, Ahmed MS, Mubarak A, Kasbekar AV, Derbyshire S, McCormick MS, Mughal MK, McNamara PS, Mitchell T, Zhang Q.** 2014. Activation of memory Th17 cells by domain 4 pneumolysin in human nasopharynx-associated lymphoid tissue and its association with pneumococcal carriage. Mucosal Immunol **7:**705-717.
43. **Aljurayyan AN, Sharma R, Upile N, Beer H, Vaughan C, Xie C, Achar P, Ahmed MS, McNamara PS, Gordon SB, Zhang Q.** 2016. A critical role of T follicular helper cells in human mucosal anti-influenza response that can be enhanced by immunological adjuvant CpG-DNA. Antiviral Research **132:**122-130.
44. **Rasheed A-U, Rahn H-P, Sallusto F, Lipp M, Müller G.** 2006. Follicular B helper T cell activity is confined to CXCR5hiICOShi CD4 T cells and is independent of CD57 expression. Eur J Immunol **36:**1892–1903.
45. **Ahmed MS, Jacques LC, Mahallawi W, Ferrara F, Temperton N, Upile N, Vaughan C, Sharma R, Beer H, Hoschler K, McNamara PS, Zhang Q.** 2015. Cross-reactive immunity against influenza viruses in children and adults following 2009 pandemic H1N1 infection. Antiviral Research **114:**106-112.
46. **Mahallawi WH, Kasbekar AV, McCormick MS, Hoschler K, Temperton N, Leong SC, Beer H, Ferrara F, McNamara PS, Zhang Q.** 2013. Infection with 2009 H1N1 influenza virus primes for immunological memory in human nose-associated lymphoid tissue, offering cross-reactive immunity to H1N1 and avian H5N1 viruses. J Virol **87:**5331-5339.

Figure legends

Figure 1. ***LAIV induces T_{FH} proliferation that correlates with GC B cell response and antibody production in NALT***. LAIV stimulation induced an increase in T_{FH} number (a+b) and T_{FH} 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_{FH} subset ($CXCR5^{hi}/ICOS^{hi}$) in CD4+ T cells following stimulation (a, day 3), and T_{FH} proliferation analysed by CFSE (c, day 5, red line: LAIV, grey shaded: medium control). (e & f) Increase in GC B cell number ($CD19^{+}$ $CD38^{hi}$ IgD^{-}) 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_{FH} (red bar) or with non-T_{FH} 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_{FH} from naïve tonsillar T cells and antibody production by LAIV.*** Representative plots (a) and bar graph (b) show the induction of T_{FH} ($CD4^{+}CXCR5^{+}ICOS^{+}$) from $CD45RO^{-ve}$ 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_{FH} as compared to unstimulated medium control) (isotype controls: shaded). (e) Dose-dependent induction of T_{FH} (day 7, top) and anti-HA IgG antibody production (day 14, bottom) from $CD45RO^{-ve}$ MNC following LAIV stimulation (n=6). (f) LAIV-induced anti-HA IgG, IgM and IgA production in $CD45RO^{-ve}$ MNC (day 14, n=10, **p<0.01).

Figure 3. ***Detection of LAIV-induced antigen-specific T_{FH} and effect of IL21, ICOS, CD40 and BCL6 signallings on T_{FH} and antibody induction***. $CD45RO^{-ve}$ 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_{FH} ($ICOS^{+}CXCR5^{+}$) following sH1N1 antigen challenge, and (**b**) showing the frequencies of activated T_{FH} (% of CD4+ 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_{FH} following sH1N1 antigen challenge. (**e+f**) Effect of neutralizing antibodies to IL21R, ICOS-and CD40-L or BCL6 blocker on T_{FH} induction (**e**, day 7) and antibody production (**f,** day 14) in $CD45RO^{-ve}$ MNC following LAIV stimulation (**p<0.01 vs LAIV stimulation or isotype control antibodies).

Figure 4. ***IL-21 expression in LAIV-activated T_{FH} and its effect on anti-HA antibody production.*** (a) Representative plots showing T_{FH} subset and IL21 expression in tonsillar CD4+ T cells following LAIV stimulation (shaded histogram: isotype control). *(b)*An increase in IL-21-producing T_{FH} (% 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_{FH} (d, n=10) or with non-T_{FH} 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_{FH} number (f) and in anti-HA IgG, IgM and IgA antibody production (g) (n=8, **p<0.01).

Figure 5. ***Activation of T_{FH}–like cells in PBMC.*** (a) Representative plots shows the increase of T_{FH}–like cells ($CD4^+CXCR5^+ICOS^+$) in PBMC following stimulation for 3 days by LAIV, as compared to medium control. (b) LAIV-induced increase in T_{FH}–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_{FH}–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).