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ACCEPTED MANUSCRIPT

Title: **A critical role of T follicular helper cells in human mucosal anti-influenza response that can be enhanced by immunological adjuvant CpG-DNA**

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Running title: T_{FH} *in anti-influenza antibody response*

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ABSTRACT

T Follicular helper cells (T_{FH}) are considered critical for B cell antibody response, and recent efforts have focused on promoting T_{FH} in order to enhance vaccine efficacy. We studied the frequency and function of T_{FH} in nasopharynx-associated lymphoid tissues (NALT) from children and adults, and its role in anti-influenza antibody response following stimulation by a live-attenuated influenza vaccine (LAIV) or an inactivated seasonal virus antigen (sH1N1). We further studied whether CpG-DNA promotes T_{FH} and by which enhances anti-influenza response. We showed NALT from children aged 1.5-10 years contained abundant T_{FH}, suggesting efficient priming of T_{FH} during early childhood. Stimulation by LAIV induced a marked increase in T_{FH} that correlated with a strong production of anti-hemagglutinin (HA) IgA/IgG/IgM antibodies in tonsillar cells. Stimulation by the inactivated sH1N1 antigen induced a small increase in T_{FH} which was markedly enhanced by CpG-DNA, accompanied by enhanced anti-HA antibody responses. In B cell co-culture experiment, anti-HA responses were only seen in the presence of $T_{FH,}$ and addition of plasmacytoid dendritic cell to T_{FH}-B cell co-culture enhanced the T_{FH}-mediated antibody production following CpG-DNA and sH1N1 antigen stimulation. Induction of T_{FH} differentiation from naïve T cells was also shown following the stimulation. Our results support a critical role of T_{FH} in human mucosal anti-influenza antibody response. Use of an adjuvant such as CpG-DNA that has the capacity to promote T_{FH} by which to enhance antigen-induced antibody responses in NALT tissue may have important implications for future vaccination strategies against respiratory pathogens.

Keywords: T follicular helper cell (T_{FH}), influenza virus, influenza vaccine, anti-hemagglutinin (HA) antibody response, Nasopharynx-associated lymphoid tissues (NALT), CpG-DNA, children and adults,

1. INTRODUCTION

Follicular helper T cells (T_{FH}) are a specialized T cell subset that provides help to B cells for antibody production(Crotty, 2011). The main effector site of T_{FH} is the germinal center (GC) within the secondary lymphoid organs. The interaction between T_{FH} and B cells leads to GC formation and the development of high affinity antibodies that are central for T cell-dependent antibody response, and therefore T_{FH} are considered critical for infection- or vaccine-induced protective immunity(Crotty, 2014)'(Slight et al., 2013). Most of the evidence supporting a critical role of T_{FH} are derived from studies in mice, whereas direct evidence from humans on T_{FH}'s role in vaccine-induced immunity is lacking due to the difficulty to obtain human secondary lymphoid tissue(Schmitt and Ueno). However, recent studies demonstrated that the response of "T_{FH}-like" cells in peripheral blood following parenteral influenza vaccination correlated well with the anti-hemagglutinin (HA) antibody response, which provide supporting evidence for the importance of T_{FH} in vaccine-induced response in humans (Bentebibel et al., 2013)'(Spensieri et al., 2013). Recently, identification of novel agent/adjuvants that promote T_{FH} number or function thus to enhance antibody response has become an attractive vaccination strategy (Fazilleau et al., 2009; Spensieri et al., 2013).

There is increasing interest recently in developing mucosal vaccines such as intranasally administered vaccines against respiratory tract infections(Lycke, 2012). Nasopharynx-associated lymphoid tissues (NALT) comprising of adenoids and tonsils are secondary lymphoid organs known to be important induction sites for natural immunity against respiratory tract pathogens including influenza virus(Kiyono et al., 2004). Also, they are a major induction site for immunity induced by intranasal vaccines, such as live-attenuated influenza vaccines (LAIV). As immune tolerance is a major feature of the mucosal immune system, it is generally considered that mucosal vaccination needs either a live-attenuated vaccine or an inactivated virus or subunit vaccine antigen together with an adjuvant. LAIV has been demonstrated to be an effective

intranasal vaccine against influenza and been licensed for use in children over 2 years of age. However, LAIV is not licensed for young children < 2 years because of concerns over increased risk of wheezing (Belshe et al., 2007). There is a need for an alternative intranasal vaccine for this age group and that may include inactivated influenza virus antigens with an effective mucosal adjuvant.

Dendritic cells (DC) were shown to be important to initiate T_{FH} cell development(Goenka et al., 2011). Within mucosal lymphoid tissue including human tonsils, plasmacytoid DC (pDC) has been shown to be an important DC population (Polak et al., 2008; Rescigno, 2013). In humans, pDC uniquely express both TLR-7 and TLR-9 which are not found on myeloid DC (Hornung et al., 2002). CpG-DNA, a TLR-9 ligand, has been shown to possess adjuvant activity capable of enhancing antibody responses, including that intranasal administration of CpG-DNA enhanced antibody response to co-administered influenza vaccines in animal models (Klinman, 2006; McCluskie and Davis, 1999; Weeratna et al., 2000) (Moldoveanu et al., 1998). Recent studies in mice suggest CpG-DNA may potentiate T_{FH} response by monocyte-derived DC to modulate antibody production (Chakarov and Fazilleau, 2014). However, it is not known whether CpG-DNA promotes T_{FH} in humans and whether pDC contribute to promoting T_{FH} and by which enhances vaccine antigen-induced response.

A central marker of T_{FH} cells is CXC-chemokine receptor 5 (CXCR5), which is important for T_{FH} positioning in GC(Schaerli et al., 2000). T_{FH} are typically identified by co-expression of CXCR5 together with other markers including ICOS, PD-1, BCL-6 (Breitfeld et al., 2000; Kim et al., 2001) (Choi et al., 2011; Fazilleau et al., 2009; Kerfoot et al., 2011; King, 2009; Laurent et al., 2010). A number of cytokines, particularly IL-21, are produced by T_{FH}, and are considered to have a major role in T_{FH} differentiation and function on B cell antibody response (King, 2009).

In this study, we examined the role of T_{FH} in influenza vaccines- or antigen-induced anti-HA response in human NALT immune cells, and studied whether a candidate adjuvant CpG-DNA

promotes T_{FH} and by which potentiate the inactivated virus antigen-induced anti-influenza antibody responses.

2. METHODS

2.1. Patients and samples. Adenoidal and tonsillar tissues were obtained from patients (age 1.5–36 years) recruited (from 2012 to 2015) for adenoidectomy and/or tonsillectomy due to upper airway obstruction. The tissues were transported in HBSS medium (Hank's Balanced salt solution) in a cold box to the laboratory and proceeded to cell isolation within four hours. Each tissue sample was checked for any signs of gross inflammation and/or necrosis prior to processing and samples that exhibited either of these features were excluded from the study. Patients with known immunodeficiency and those previously vaccinated against influenza 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 all cases.

***2.2. LAIV vaccine, influenza virus antigens and CpG-DNA*.** 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 (Manassas,VA). 0.2ml of LAIV contains about 10^7 fluorescent focus units (FFU) of each strain. An inactivated seasonal A/Brisbane/59/2007 H1N1 influenza virus (sH1N1) antigen, which was inactivated by β-propiolactone and partially purified (Wood et al., 1977) 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 was obtained from BEI Resources and used as the coating antigen for anti-HA antibody measurement by ELISA and ELISpot assays. A type B CpG-DNA (CpG 2006, InvivoGen) (Krieg et al., 1995) was used to study the effect on T_{FH}.

2.3. Cell separation, culture, and stimulation. Mononuclear cells (MNC) from adenotonsillar tissue were isolated using Ficoll density centrifugation (Zhang et al., 2006) (Zhang et al., 2011) and the number of MNC isolated from each patient ranged from $5.0x10^7$-$1.0x10^9$. In some experiments, tonsillar MNC were depleted of effector and memory ($CD45RO^+$) cells using CD45RO microbeads and magnetic cell sorting (Miltenyi) as described previously (Gray et al., 2014; Zhang et al., 2007). The depletion of $CD45RO^+$ cells from tonsillar MNC removed T_{FH} cells (>98%). Unfractionated MNC or CD45RO+cell-depleted MNC were cultured ($4x10^6$/ml) in 96-well flat-bottom culture plates in RPMI-1640 medium with HEPES supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma), in the presence of CpG-DNA, inactivated sH1N1 antigen, or LAIV. Cell culture supernatants were collected and stored at -80°C until analysis for antibody or cytokine production by ELISA.

The effect of IL21 on T_{FH} and T_{FH}-mediated help for antibody production was examined using recombinant human IL21R-Fc chimera (R&D systems) (IL21-Fc). IL21-Fc (or isotype control) (10µg/ml) was incubated with tonsillar MNC or with T_{FH}-B cell co-culture for 1 hour, before the addition of CpG-DNA (0.1µg/ml) and/or sH1N1 antigen (1µg/ml). The cells were then cultured for up to 10 days for analysis of T_{FH} numbers and of antibody production.

2.4. Measurement of HA-Specific antibodies. Production of HA-specific IgG, IgM and IgA antibodies to sH1N1 virus in cell culture supernatants was measured as previously described (Ahmed et al., 2015), (Mahallawi et al., 2013). In brief, ELISA plates were coated with recombinant 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 and data were analysed using DeltaSoft software.

2.5. Analysis of $T_{FH,}$ cell proliferation and intracellular cytokine expression. For T_{FH} identification, tonsillar MNC were stained with anti-human CD3, CD4, CXCR5, ICOS, CCR7, and Bcl-6 antibodies followed by flow cytometry (Rasheed et al., 2006). Cell proliferation was examined by CFSE staining of tonsillar MNC (Molecular Probes, UK), followed by cell stimulation for 5 days and by flow cytometry. Intracellular cytokine staining was performed following overnight cell stimulation as described previously (Zhang et al., 2011). Intracellular staining for Bcl-6 and TLR9 was performed following manufacturer's instructions with anti-human Bcl-6 and TLR-9 antibodies (eBioscience). Flow cytometry was performed using FACS Calibure (BD Biosciences) and data analyzed using WinMDI software (Scripps Institute).

2.6. Purification of T_{FH}, B cells and pDC. T_{FH}, B cells and pDC were purified from tonsillar MNC using magnetic cell sorting (EasySep™, Stemcell). Briefly, tonsillar B cells were purified by negative selection using B cell purification kit which yielded B cell purity >99%. For T_{FH} purification, $CD4^+$T cells were first isolated by negative selection using $CD4^+$ T cell kit, followed by positive selection of $CXCR5^{high}$ (T_{FH}) using biotin anti-human CXCR5 antibody. The amount of anti-CXCR5 antibody was optimised to ensure only $CXCR5^{high}$-expressing cells were selected (purity>95%). $CD4^+CXCR5^-$ (non-T_{FH}) cells were purified by negative selection from $CD4^+$T cells using an optimised amount of anti-CXCR5 antibody to ensure only $CXCR5^-$ cells were obtained (purity >99%). Tonsillar pDC were purified using negative selection with human plasmacytoid DC kit (Stemcell) which yielded a pDC purity >96%. From a total of $5.0x10^7$ MNC, the numbers of isolated T_{FH}, B cell and pDC were ranged $2.0\text{-}4.0x10^6$, $1.0\text{-}1.5x10^7$, and $1.0\text{-}2.0x10^5$ respectively.

2.7. T_{FH}-B cell co-culture. The ability of tonsillar T_{FH} to help B cell antibody production was examined by an autologous B cell- T_{FH} co-culture. 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 a 96-well round bottom plate, in the presence of CpG-DNA and/or sH1N1 virus antigen. The cells were cultured for 10 days and cell

culture supernatant were collected for antibody analysis. In some experiments, purified pDC were added at a concentration of $5x10^4$ cells/ml to the T_{FH}-B cell co-culture.

2.8. Statistical Analysis. Two group comparisons were analysed by student's T test, and paired T test was used for comparison between paired samples. Analysis of variance (ANOVA) was used for multiple group comparisons. Correlation was analysed by Pearson's correlation. Statistical analysis was performed using GraphPad Prism 5 software. $P<0.05$ was considered statistically significant.

3. RESULTS

3.1. Identification and frequency of T_{FH} in human tonsillar tissue and its relationship with age. Adenotonsillar MNC were identified by staining for CD3, CD4, CXCR5 and ICOS expression followed by flow cytometry. Based on CXCR5 and ICOS expression in $CD4^+$T cells, three populations were observed (Figure 1a): $CXCR5^{high}ICOS^{high}$ CD4+T cells (red, designated as T_{FH}), $CXCR5^{int}ICOS^{int}$ (Blue) and $CXCR5^-ICOS^-$ (Green) CD4+T cells. In addition to the high expression of CXCR5 and ICOS, the designated T_{FH} population were also shown to express Bcl-6 and IL-21, but not CCR7 (1b).

To determine whether there is any relationship between the frequencies of T_{FH} and GC B cells, tonsillar B cell subsets were analysed by staining for CD19, CD38 and IgD. Gated for $CD19^+$ B cells, GC B cells were identified as $CD19^+CD38^+IgD^-$ (red circle, 1c). There was a good correlation between the frequencies of GC B cells (% of B cells) and T_{FH} in NALT of children and adults (1d, $r = 0.86$, $P< 0.001$).

When the frequencies of T_{FH} (% of CD4+ T cells) in tonsillar MNC were analyzed in association with age, it was found that the mean frequency in children was significantly higher than in adults (Figure 2a), and there appeared to be an age-associated decrease in the T_{FH} frequency (2b). The mean T_{FH} frequency was shown to be highest in younger children from 1.5 to ~10 years olds (2b),

3.2. T_{FH} mediate LAIV- and inactivated antigen-induced antibody production.

We sought to determine whether T_{FH} play a role in the antibody response induced by current influenza vaccines in an immune induction site eg. NALT. Stimulation by LAIV induced a marked increase in T_{FH} number in tonsillar MNC, which was correlated with a marked production of anti-sH1N1 HA antibodies including IgG, IgM and IgA (Figure 3a). Stimulation by the inactivated sH1N1 antigen induced a modest increase in T_{FH} number which correlated with a modest production of IgG and with little production of IgM and IgA anti-HA antibodies (3b). Further, stimulation by LAIV of co-culture of purified B cells with T_{FH}, but not with non-T_{FH} cells, induced production of IgG, IgM and IgA anti-HA antibodies (3c). Again, stimulation by the inactivated sH1N1 antigen induced a modest IgG anti-HA in the B cell-T_{FH} co-culture (3d), but no IgM and IgA response (data not shown). No antibody response was seen in the co-culture of B cells and non-T_{FH} cells (3c+d).

3.3. CpG-DNA promotes T_{FH} and enhances anti-HA antibody production.

To determine whether CpG-DNA could promote T_{FH} thereby enhancing the B cell anti-HA antibody response, tonsillar MNC were stimulated with CpG-DNA with/without the inactivated sH1N1 virus antigen. As shown in figure 4a, CpG-DNA elicited a dose-dependent increase in T_{FH} frequency in tonsillar MNC. CpG-DNA at a low dose at 0.1 μg/ml, together with the sH1N1 antigen, elicited a marked increase in T_{FH} number (4b) which correlated with a T_{FH} cell proliferative response (4c) in tonsillar MNC, and significantly higher than that elicited by the sH1N1 antigen alone (4b+c, $p<0.01$). In the meantime, CpG-DNA together with sH1N1 antigen elicited a marked increase in IgG, IgM, and IgA anti-HA antibody production, much higher than that elicited by sH1N1 antigen alone (4d).

3.4. CpG-DNA-mediated enhancement of anti-HA antibody production involves T_{FH} and IL21.

To further determine whether T_{FH} contribute to CpG-DNA mediated enhancement of antibody production, purified B cells were co-cultured with T_{FH} or non-T_{FH} cells in the presence of CpG-

DNA and sH1N1 antigen. As shown in Figure 5a, production of anti-HA IgG, IgA and IgM antibodies were seen in the B cell co-culture with T_{FH}, but not with non-T_{FH} cells.

IL21 concentrations in tonsillar MNC and the T_{FH}-B cell co-culture were analyzed. As shown in Figure 5b, CpG-DNA stimulation induced an increase in IL21 concentration in the cultured MNC. In the T_{FH}-B cell co-culture, following stimulation, significant production of IL-21 was only seen in the presence, but not in the absence of T_{FH} (5c). Further, IL21 receptor blocking by the use of IL21R-Fc chimera abrogated the increase in T_{FH} elicited by CpG-DNA and sH1N1 antigen stimulation (5d). In the meantime, the IL-21 receptor blocking reduced the production of anti-HA IgA and IgM antibodies in tonsillar MNC (5e).

3.5. Induction of T_{FH} differentiation by CpG-DNA with influenza antigen.

To determine whether CpG-DNA promotes induction of T_{FH} from naïve T cells, tonsillar MNC depleted of $CD45RO^{+}$ cells (removed effector and memory T cells including T_{FH}) were stimulated with CpG-DNA with the inactivated sH1N1 antigen for 7 days. As shown in Figure 6a, the stimulation induced a marked increase in the number of T_{FH} ($CD4^{+}CXCR5^{high}$) which was significantly higher than the sH1N1 antigen stimulation alone ($p<0.01$).

3.6. Effect of pDC on T_{FH} mediated antibody response.

When we analyzed TLR9 expression using flow cytometry, no significant expression was shown by purified T_{FH} or non-T_{FH} CD4+T cells (data not shown). By contrast, a proportion of B cells and most pDC were shown to express TLR9 (6b). Since there was a prominent number of pDC in tonsillar tissue which expressed a high level of TLR9, we examined the effect of pDC on CpG-DNA and T_{FH} mediated antibody response. Purified pDC were added to the T_{FH}-B cell co-culture, and co-incubated with CpG-DNA with the inactivated sH1N1 antigen. As shown in Figure 6c, the addition of pDC further enhanced the anti-HA IgM and IgA antibody production in the T_{FH}-B cell co-culture.

4. DISCUSSION

NALT is a unique inductive site for B-cell response and plasma cell generation in the upper respiratory tract, which makes the intranasal route of vaccination an attractive strategy against airway infection such as influenza(Brandtzaeg, 2011). As T_{FH} are critical for T cell-dependent antibody response, promoting T_{FH} in NALT may be an effective strategy for intranasal vaccination.

In this study, we first studied the frequency of T_{FH} in NALT tissue of children and adults, and showed that NALT of children aged 1.5-10 years contained abundant T_{FH} (Figure 1+2). The finding that children as young as 1.5 years already developed a prominent number of T_{FH} in NALT suggests the priming of T_{FH} in early childhood is fairly efficient. It may also reflect the period of high level of microbial exposure in the nasopharynx which primes antigen-specific T_{FH} cells during young childhood. The finding that the T_{FH} frequency in children was higher than in adults is consistent with the previous report by Bentebibel et al that also showed differences between children and adults in the T_{FH} subsets including $CXCR5^{high}ICOS^{high}$, $CXCR5^{int}ICOS^{low}$ and $CXCR5^{int}ICOS^{high}$ subsets (Bentebibel et al., 2013).

We then studied the function of T_{FH} in human NALT, and more specifically on whether the T_{FH} play an important role in influenza antigen-induced antibody response. We showed that stimulation by LAIV elicited a marked increase in T_{FH} number in tonsillar MNC that correlated with a strong production of anti-sH1N1 HA IgG, IgM and IgA antibodies (Figure 3a). By comparison, stimulation by an inactivated sH1N1 antigen elicited a small increase in T_{FH} that correlated with a modest anti-HA antibody production which was predominantly IgG (Figure 3b). Further, we demonstrated that the antibody responses induced by both LAIV and the inactivated sH1N1 antigen were dependent on the presence of T_{FH} in the co-culture with B cells (Figure 3c+d). These results support the hypothesis that T_{FH} may be critical in influenza vaccine-induced anti-HA response in humans(Bentebibel et al., 2013; Spensieri et al., 2013).

Information on antibody responses to influenza antigens in human NALT is limited. Our results on antibody responses in the NALT tissue induced by LAIV and the inactivated sH1N1 antigen are in general consistent with previous findings that intranasally administered LAIV induced prominent antibody responses including IgA and IgG in nasal lavage mucosal samples (Moldoveanu et al., 1995), and the inactivated antigen elicits primarily an IgG-predominant memory response (Bentebibel et al., 2013). Given that many subjects would have been exposed previously to infection of various strains of influenza viruses, it is plausible that these previous contact induced memory and would to some degree have impact on the antibody response tested in this study. The relative predominance of IgG antibody production is concordant with the general predominance of IgG immunocytes in adenotonsillar tissue (Boyaka et al., 2000). A live-attenuated vaccine, which resembles more closely to a natural infection, is generally used without an adjuvant and activates a stronger innate and broader immune response than an inactivated vaccine(Siegrist, 2013; Sridhar et al., 2015). For an inactivated virus antigen, an adjuvant is usually needed and some adjuvants have been shown to greatly augment the immune response induced by inactivated influenza vaccines(Nicholson et al., 2001).

We further studied whether an adjuvant could be used to promote T_{FH} and by which enhances the antibody response induced by the inactivated influenza antigen. CpG-DNA, as a TLR9 ligand, has been studied as a candidate adjuvant. We found that CpG-DNA stimulation of tonsillar MNC promoted the T_{FH} number in a dose-dependent manner. Also, a low dose of CpG-DNA (0.1ug/ml) with the inactivated sH1N1 antigen markedly increased T_{FH} number, and correlated with an enhanced anti-HA response including IgG, IgM and IgA antibodies in tonsillar MNC (Figure 4), a pattern similar to that induced by LAIV. Furthermore, the enhanced anti-HA antibody response was observed only in the co-culture of B cells with T_{FH} but not with non-T_{FH} cells following stimulation by the antigen and CpG-DNA (Figure 5a). These suggest that with the inactivated influenza virus, CpG-DNA may promote influenza-specific T_{FH}, and thereby enhances the T_{FH}

mediated antibody response, including both primary and memory anti-influenza responses. These results are concordant with the recent reports in mouse models that CpG-DNA could increase T_{FH} and B cell responses (Chakarov and Fazilleau, 2014; Mastelic et al., 2012; Rookhuizen and DeFranco, 2014).

IL-21 has been suggested to play an important role in T_{FH} differentiation(Rodríguez-Bayona et al., 2012). We showed that stimulation with CpG-DNA enhanced IL-21 production that was correlated with the expansion of T_{FH} in tonsillar MNC. Further we showed that it was mainly the T_{FH} but not non-T_{FH} CD4+T cells produced IL-21. IL-21 receptor blocking inhibited the increase in T_{FH} number and anti-HA antibody production induced by CpG-DNA with sH1N1 antigen (Figure 5). These findings support an important role of IL-21 in CpG-DNA mediated expansion of T_{FH} and in the enhanced B cell antibody production.

Whereas no significant expression of TLR9 was shown in T_{FH} cells, both B cell and pDC in NALT were shown to express TLR-9 (Figure 6). It is possible that CpG-DNA promotes T_{FH} number and function in tonsillar MNC through both B cell and pDC. We found that the addition of pDC to T_{FH}-B cell co-culture enhanced the anti-HA antibody production following stimulation by CpG-DNA and sH1N1 antigen. This suggests that pDC contributes to T_{FH} function in mediating B cell antibody response. The addition of pDC enhanced mainly IgM and IgA but not IgG response, which may suggest that pDC contributes mainly to T_{FH}-mediated primary antibody response (mainly IgM and IgA). It has been reported that the T_{FH} equivalent $CXCR5^{+}CD4^{+}$ T cells in circulation were efficient to help memory B cells for memory antibody response (predominantly IgG), but were unable to help naïve B cells(Bentebibel et al., 2013). By activation of pDC, CpG-DNA may enhance T_{FH}-mediated primary B cell anti-HA response induced by an inactivated virus antigen. An optimal primary response is likely to be critical for effective immunization in young children or that against a new avian influenza virus infection in humans. CpG-DNA has a strong immunostimulatory effects on pDC (Krug et al., 2001a; Krug et al.,

2001b; Rothenfusser et al., 2002) and pDC has been shown to be important in anti-influenza and anti–rotavirus antibody responses upon virus infection/stimulation (Deal et al., 2013; Jego et al., 2003). Considering that tonsillar tissue contains a prominent number of pDC (Polak et al., 2008; Rescigno, 2013) (Summers et al., 2001), this contribution by pDC to T_{FH}-mediated antibody response in NALT tissue may be explored in future vaccination strategy against respiratory infection.

In conclusion, abundant T_{FH} exist in NALT of young children which suggests efficient T_{FH} priming and it will be possible to prime T_{FH} effectively in NALT through immunization during early childhood. T_{FH} cells are critical in human mucosal anti-influenza antibody responses in NALT tissue. Use of an immunological adjuvant such as CpG-DNA that has the capacity to promote T_{FH} and thereby to enhance influenza antigen-induced antibody response in NALT may have important implications for novel vaccination strategies, such as mucosal vaccines against respiratory infections such as influenza.

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Figure legends

Figure 1. Identification of T_{FH} in tonsillar tissues of children and adults, and relationship with GC B cells. Adenotonsillar MNC were stained with anti-human CD3, CD4, CXCR5 and ICOS antibodies followed by flowcytometry (a). Within CD4+ Tcells (R2), T_{FH} were identified as $CXCR5^{high}$ $ICOS^{high}$ CD4+ T cells (red, R3). In addition to CXCR5 and ICOS, the designated T_{FH} population were also shown to express Bcl-6 and IL-21, but not CCR7 (b). GC B cell was identified as $CD38^{++}$ IgD^{-} $CD19^{+}$ (c, red circle), and the relationship between the frequencies of T_{FH} and GC B cells (% of B cells) in tonsillar MNC is shown (d, r = 0.86, n=16, P< 0.001).

Figure 2. Frequencies of T_{FH} in children and adults. Tonsillar T_{FH} frequencies (expressed as the % of T_{FH} in CD4+ T cells) were compared between children (n=80) and adults (n=30) (a), and the relationship between the T_{FH} frequencies and age is shown (b, r=-0.62, n=110, p<0.001).

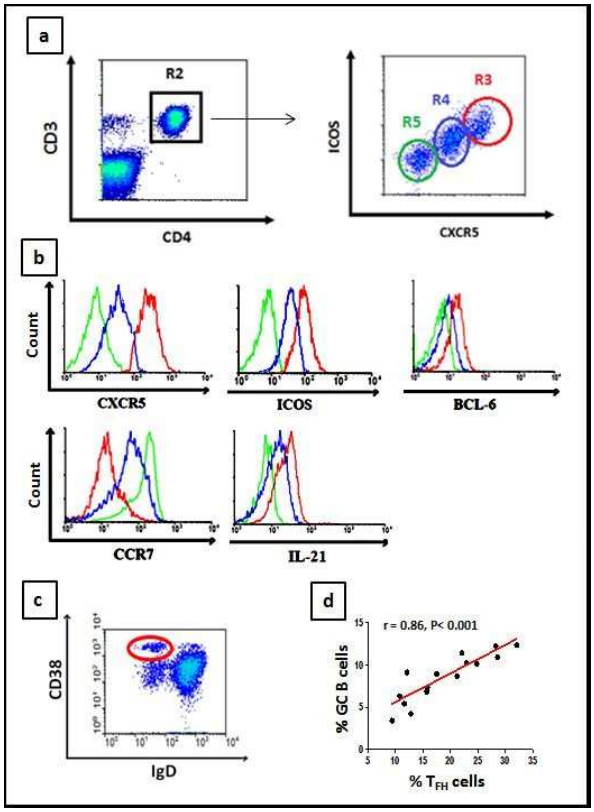
Figure 3. T_{FH} mediate LAIV and inactivated virus antigen-induced antibody production in NALT. Tonsillar MNC were stimulated by LAIV (2ul/ml, approx. 10^5FFU/ml) (a) and the inactivated seasonal virus (sH1N1) antigen (1ug/ml) (b) followed by analysis of T_{FH} number and anti-HA IgG, IgA and IgM antibody responses using flow cytometry and ELISA respectively (**p<0.01, n=20, aged 2-20 years). Co-culture of purified B cells with T_{FH} ($CD4^{+}$ $CXCR5^{high}$) or with non-T_{FH} CD4+T cells ($CD4^{+}$ $CXCR5^{-}$) were stimulated with LAIV (c) or the sH1N1 antigen (d), and anti-HA antibody responses were seen in the presence, but not in the absence of T_{FH} (c+d, **p<0.01, n=10, aged 2-20 years).

Figure 4. **CpG-DNA promotes T_{FH} and enhances anti-HA antibody production**. Tonsillar MNC were stimulated by CpG-DNA with or without the inactivated sH1N1 antigen, followed by analysis of T_{FH} frequency (a+b), proliferation index (c) and antibody production (d). CpG-DNA stimulation induced a dose-dependent increase in T_{FH} frequency (a, *p<0.05,**p<0.01, n=10). T_{FH} number (b) and proliferation index (c) in tonsillar MNC were shown following stimulation

with CpG-DNA (0.1 μg/ml) with or without sH1N1 antigen (1 μg/ml) (*p<0.05, **p<0.01, p=10). CpG-DNA together with sH1N1 antigen elicited a marked production of anti-sH1N1 HA IgG, IgM, and IgA antibodies (d, *p<0.05, **p<0.01, n=10, aged 2-20 years).

Figure 5. CpG-DNA-mediated enhancement of anti-HA antibody production involves T_{FH} and IL-21. Co-culture of purified B cells with T_{FH} ($CD4^+$ $CXCR5^{high}$) or non-T_{FH} ($CD4^+$ $CXCR5^-$) cells were stimulated with CpG-DNA (0.1μg/ml) and sH1N1 antigen (1μg/ml), followed by analysis of anti-HA IgG, IgA and IgM antibody production (a, **p<0.01, n=20, aged 2-20 years). IL-21 concentrations in tonsillar MNC (b, **p<0.01, n=20) and in the co-culture of B cells with T_{FH} or non-T_{FH} (c, **p<0.01, n=15) were analyzed following stimulation by CpG-DNA (0.1μg/ml). IL-21R-Fc chimera or isotype control was co-incubated with tonsillar MNC in the presence of CpG-DNA and sH1N1 antigen, followed by analysis of T_{FH} frequency (d, *<0.05) and IgA and IgM antibody production (e, **p<0.01), n=15, aged 2-20 years). IgG was not shown because of the cross-reactivity to the Fc portion of IL-21R-Fc.

Figure 6. Induction of T_{FH} from naive $CD4^+$ T cells by CpG-DNA and antigen, and effect of pDC on T_{FH}-mediated antibody response. Tonsillar MNC depleted of $CD45RO^+$ T cells were stimulated with CpG-DNA (0.1μg/ml) and sH1N1 antigen for 7 days. CD45RO+ cell depletion removed effector and memory T cells including T_{FH} from tonsillar MNC but retained naïve T cells. CpG-DNA with sH1N1 antigen induced a significant number of T_{FH} differentiation from naïve T cells in tonsillar MNC (a, *p<0.05, **p<0.01, ***p<0.001, n=16, aged 2-20 years). A representative figure shows expression of TLR9 in tonsillar B cells and pDC (b). Purified pDC was added to the co-culture of purified T_{FH} and B cells followed by stimulation by CpG-DNA and sH1N1 antigen. The addition of pDC was shown to enhance HA-specific IgA and IgM, but not IgG antibody production induced by CpG-DNA and sH1N1 antigen (c, *p<0.05, n=10, aged 2-20 years).



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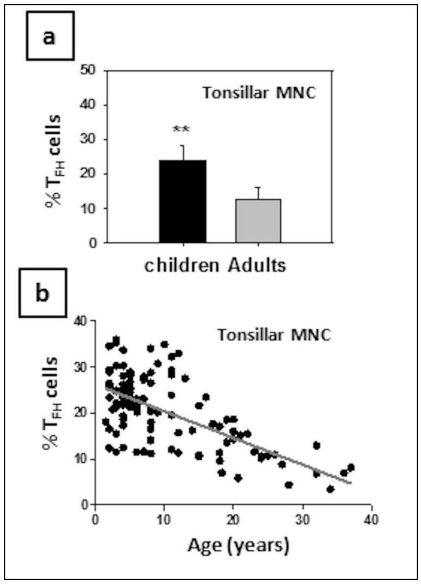

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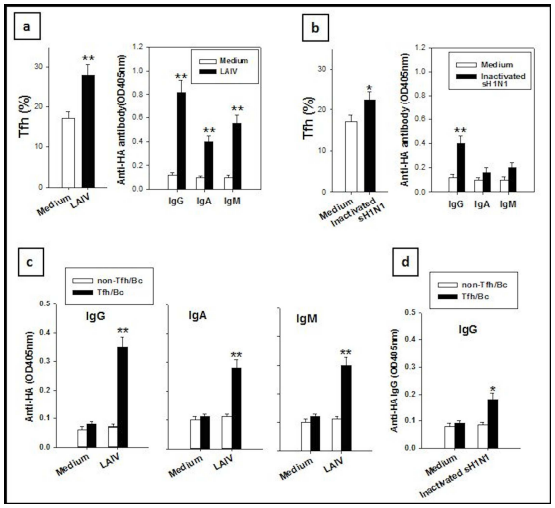

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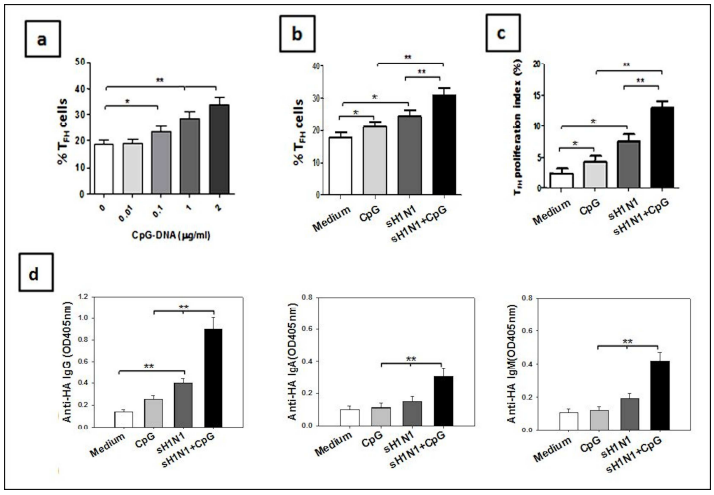

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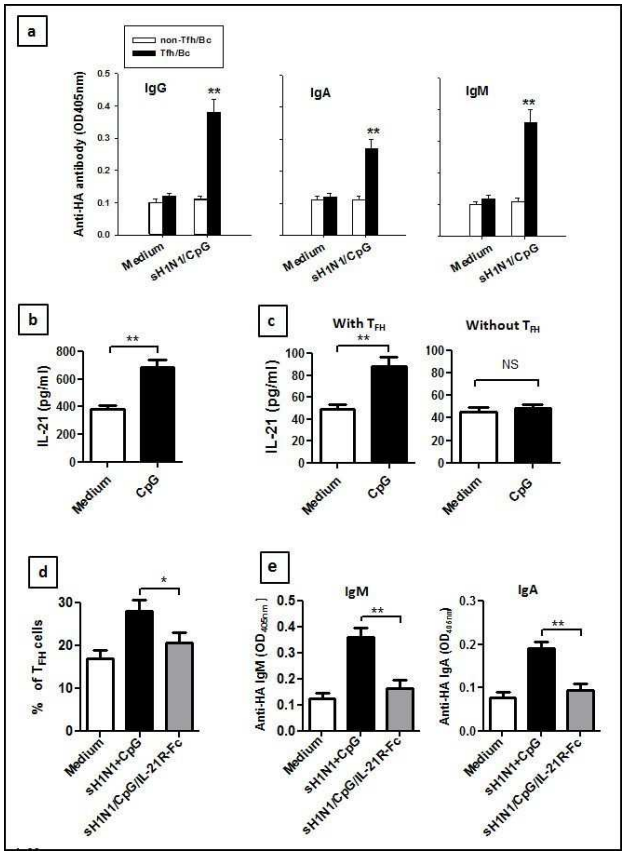

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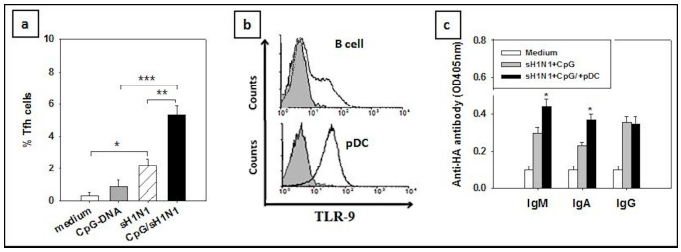

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Highlights

- We analysed the role of T_{FH} cells in anti-influenza response in human immune tissue.
- T_{FH} are critical for anti-influenza antibody response following vaccine stimulation.
- CpG-DNA can promote T_{FH} and by which enhances antigen-specific antibody response.

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