



**Institute of Infection and Global Health**

**Department of Clinical Infection, Microbiology and Immunology**

**Characterisation of T Follicular Helper Cell  
(T<sub>FH</sub>) in Nasopharynx-Associated Lymphoid  
Tissue and Its Effect on Regulation of Immune  
Response to Influenza Virus**

Thesis submitted in accordance with  
the requirements of the University of Liverpool  
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**by**

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## Abstract

T<sub>FH</sub> cells have been identified as a new T helper subset specialized to regulate the development of effector and memory B cells and long-lived plasma cells. The interaction between T<sub>FH</sub> and B cells leads to the activation of B cells and germinal centers (GC) formation. Considering the importance of T<sub>FH</sub> for B cell antibody response, novel vaccine adjuvants and intranasal vaccines to boost T<sub>FH</sub> number or function may be an attractive vaccination strategy to enhance vaccine efficacy in humans. Adenotonsillar tissues are major parts of nasopharyngeal associated lymphoid tissues (NALT) and they are important in response to upper respiratory tract pathogens and intranasal vaccination. This PhD project investigated the frequencies of T<sub>FH</sub> in human NALT and PBMC in children and adults. The effects of CpG-DNA and live attenuated influenza vaccine (LAIV) on T<sub>FH</sub> in human NALT and the T<sub>FH</sub>-mediated B cell immunity to influenza virus were studied. The importance of the cytokine IL-21 and plasmacytoid dendritic cells (pDC) in T<sub>FH</sub> cell-mediated B cell antibody production was also investigated.

Adenotonsillar MNC and PBMC were isolated from adenotonsillar tissues and peripheral blood respectively. T<sub>FH</sub> (CD4<sup>+</sup> CXCR5<sup>high</sup> ICOS<sup>high</sup>) numbers and function were analysed by flowcytometry and intracellular cytokine staining. Purified T<sub>FH</sub> (CD4<sup>+</sup> CXCR5<sup>hi</sup>) and non-T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>-</sup>) were co-cultured with B cells in the presence of influenza virus antigen and CpG-DNA or of LAIV. Purified pDC were added to the T<sub>FH</sub>-B cell co-culture to study their importance in T<sub>FH</sub> -mediated B cell antibody production. Haemagglutinin (HA)-specific antibody production was analysed by ELISA and ELISpot assay. IL-21 receptor blocking by neutralization was used to study the importance of IL-21 in T<sub>FH</sub>-mediated B cell antibody production.

A prominent number of T<sub>FH</sub> were found in human NALT which were considerably higher than in PBMC. There was an age-associated difference in T<sub>FH</sub> numbers in NALT and PBMC, i.e. the mean T<sub>FH</sub> number was higher in children than in adults. T<sub>FH</sub> in NALT were shown to express high levels of IL-4, IL-10 and IL-21 and that were important for B cell antibody production. A good correlation between the numbers of GC B cell and T<sub>FH</sub> in NALT was seen. Co-culture of purified T<sub>FH</sub> but not non-T<sub>FH</sub> with B cells promoted antibody production. Stimulation of adenotonsillar MNC by CpG-DNA significantly increased T<sub>FH</sub> number and that was correlated with HA-specific antibody production following influenza antigen stimulation. Co-incubation of T<sub>FH</sub>-B cell with pDC enhanced the CpG-DNA-mediated antibody production. We also found that stimulation with LAIV significantly increased T<sub>FH</sub> number and that was correlated with HA-specific antibody production. Blocking the IL-21R significantly reduced the number of T<sub>FH</sub> that was correlated with a significant reduction of HA-specific antibody production.

Enhancing vaccine immunogenicity through modulation of T<sub>FH</sub> numbers or function in human NALT using immunological adjuvants such as CpG-DNA and through intranasal vaccination may be an effective vaccination strategy against respiratory pathogens.

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## ***Dedication***

*All my thanks to Allah for helping me bring this work to a successful completion. I dedicate this work to my parents and family for supporting me constantly by their unlimited care and prayers. A dedication also goes to my beloved wife (Hissah) for her patience, support and inspiration through the good and bad times in the past four years.*

## **Declaration**

No part of the work referred to in this thesis has been submitted in support of an application for another degree or qualification at this or any other university, or other institution of learning. All laboratory experiments described here have been carried out by the author, in the Department of Clinical Infection, Microbiology and Immunology, Institute of Infection and Global Health, University of Liverpool.

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## **List of Abbreviations**

AID	Activation-Induced Cytidine Deaminase
ANOVA	Analysis of variance
Anti-HA	Anti-haemagglutinin
AP	Alkaline Phosphatase
APC	Antigen Presenting Cell
ASC	Antibody Secreting Cell
BALT	Bronchus-Associated Lymphoid Tissue
BCL-6	B Cell Lymphoma 6
BCR	B Cell Receptor
BDCA	Human blood dendritic cell antigen
BSA	Bovine Serum Albumin
BTLA	B and T lymphocyte attenuator
CCL19	Chemokine (C-C motif) ligand 19
CCL21	Chemokine (C-C motif) ligand 21
CCR7	C-C chemokine receptor type 7
CD	Cluster of Differentiation
CDC	Centres for Disease Control and Prevention
CFSE	5-(6) Carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalovirus
CO <sub>2</sub>	Carbon dioxide
CpG-DNA	CpG oligodeoxynucleotides
CT	Cholera Toxin
CVID	Common Variable Immunodeficiency
CXCL13	C-X-C motif chemokine 13
CXCR5	C-X-C chemokine receptor type 5
DC	Dendritic Cell
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetate
ELISA	Enzyme linked immunosorbent assay

ELISpot	Enzyme linked immunosorbent spot
FACS	Fluorescence activated cell sorting
FBS	Foetal Bovine Serum
FDC	Follicular dendritic cell
Foxp3	Forkhead box P3
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
g	Centrifugal force
GALT	Gut Associated Lymphoid Tissues
GATA-3	Trans-acting T-cell-specific transcription factor
GC	Germinal Centre
HA	Haemagglutinin protein
HAI	Haemagglutination Inhibition
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
H <sub>2</sub> SO <sub>4</sub>	Sulphuric acid
HRP	Horseradish peroxidase
IFA	incomplete Freund's adjuvant
IFN- $\alpha$	Interferon alpha
IFN- $\gamma$	Interferon gamma
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IL-21R	Interleukin-21 Receptor
ILT7	Immunoglobulin like transcript 7
KLH	Keyhole Limpet Hemocyanin
l	Litre
LAIV	Live Attenuated Influenza Vaccine
LN	Lymph Node
LPS	Lipopolysaccharide
M	Molar

MALT	Mucosal-associated lymphoid tissue
mg	Milligram
MHC	Major Histocompatibility Complex
min	Minute
ml	Millilitre
mM	Millimolar
MNC	Mononuclear cell
mRNA	Messenger Ribonucleic acid
NALT	Nasal Associated Lymphoid Tissue
NA	Neuraminidase protein
NIBSC	National Institute for Biological Standards and Control
NF- $\kappa$ B	Nuclear factor-kappa B
NK	Natural killer cell
NS	Not Significant
OD	Optical density
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed Death 1
PD-L	Programmed Death 1 Ligand
pDC	Plasmacytoid Dendritic Cell
PE	Phycoerythrin
PGNs	Peptidoglycans
pT <sub>FH</sub>	Peripheral T <sub>FH</sub> cells
PNPP	P-Nitrophenyl Phosphate
PRR	Pathogen Recognition Receptor
RNA	Ribonucleic acid
ROR $\gamma$ t	Retinoid related Orphan Receptor
rpm	Revolutions per minute
SAP	Signalling lymphocytic Activation molecule-associated Protein
SEB	Staphylococcus aureus Enterotoxin
SEM	Standard error of the mean

S-IgA	Secretory Immunoglobulin A
sH1N1	seasonal H1N1 influenza
SSC	Side scatter
T-bet	T-box transcription factor
TCR	T-cell receptor
T <sub>FH</sub>	T Follicular helper cells
T <sub>H</sub>	T helper
T <sub>H1</sub>	T helper 1 cell
T <sub>H17</sub>	T helper 17 cell
T <sub>H2</sub>	T helper 2 cell
TIV	Intramuscular Trivalent Inactivated Influenza Vaccine
TLR	Toll like receptor
TNF- $\alpha$	Tumour necrosis factor alpha
T <sub>reg</sub>	T regulatory cells
U	Units
URT	Upper Respiratory Tract
URTI	Upper Respiratory Tract Infection
WHO	World Health Organization
$\alpha$ -IL-21R	Human anti-IL-21R Fc blocking antibody
$\mu$ g	Microgram
$\mu$ l	Microlitre
$\mu$ M	Micromolar



# **Chapter 1**

## **General Introduction**

## **1. Introduction**

It is known that T cell help to B cells is an essential process for T cell-dependent antibody responses. Recently, a new T helper subset has been identified, named as T follicular helper cells ( $T_{FH}$ ). Within secondary lymphoid organs,  $T_{FH}$  cells are specialized to regulate the development of effector and memory B cells and long-lived plasma cells. The interaction between  $T_{FH}$  cells and B cells leads to the development of B cells and germinal centre (GC) formation. Considering the importance of  $T_{FH}$  cells for B cell antibody response, novel vaccine adjuvants and intranasal vaccines to boost  $T_{FH}$  number or function may be an attractive vaccination strategy to enhance vaccine efficacy in young children.

### **1.1 Vaccines**

After Edward Jenner's discovery of the smallpox vaccine in 1798, a great interest in vaccine development has occurred in vaccinology. Vaccines are one of the most successful and cost-effective public health and medical achievements. Worldwide, it is estimated that over 3 million lives are saved each year because of successful vaccination programs (Shaw & Feinberg 2008). The global eradication of smallpox is a great example of how vaccinations are very effective. Similarly, efforts to eliminate other diseases including poliomyelitis, measles and others have made tremendous progress due to vaccination programs (Mak & Saunders 2006). Progress in the development of new vaccines significantly increased in the past 10 years by developing new vaccines against emerging diseases as well improving existing vaccines for better efficiency. Still, the need for new and improved vaccines is constantly present.

The major types of vaccines presently licensed and under development include: (i) live attenuated vaccines, (ii) killed or inactivated whole cell vaccines, (iii) subunit vaccines consisting of purified components of an organism, and (iv) DNA and vector vaccines (Mak & Saunders 2006).

### **1.1.1 Live attenuated vaccines**

The main concept of live attenuated vaccines is to mimic the natural infection for an effective host immune response. A live attenuated vaccine includes live, whole viral particles or bacterial cells that are handled in a way that they lost their virulence within the host but maintain their ability to initiate an immune response (He et al. 2006). This type of vaccine has been used to develop vaccines against number of viral and bacterial infections including measles, mumps, rubella, varicella-zoster, yellow fever, polio, influenza and tuberculosis vaccine (Shaw & Feinberg 2008). This type of vaccine has proven to be highly effective and protection is often long lasting. Live attenuated vaccines can induce  $CD4^+$  and  $CD8^+$  T cell responses, as well as specific neutralizing antibodies and memory B cell responses. However, there is always a risk that the attenuated pathogen gains its virulence, as has been reported with the oral polio vaccine (Plotkin 2008).

### **1.1.2 Killed or inactivated whole cell vaccines**

In a killed vaccine, a whole organism is involved but remains harmless due to assault by physical or chemical methods to kill it or otherwise inactivate the pathogenic organism. In most cases, the organism is treated with chemical agents such as  $\beta$ -propiolactone and formaldehyde to eliminate the pathogen infectivity. This only works with organisms that do not contain toxic substances. Killed bacterial vaccines

including, *Yersinia pestis* (plague) and *Vibrio. cholerae* (cholera) and killed viral vaccines for polio, rabies and influenza are successfully in use (Girard et al. 2005). Killed or inactivated vaccines have been shown to generate memory B cells and pathogen-neutralizing antibodies, likely in concert with memory CD4<sup>+</sup> T cell; however, they do not typically induce CD8<sup>+</sup> T cell responses (Plotkin 2008). In addition, killed vaccines are generally less immunogenic than live attenuated vaccines, thus, they are commonly administered with an adjuvant (Mak & Saunders 2006; Shaw & Feinberg 2008).

### **1.1.3 Subunit vaccines**

Using a purified subunit antigen from a pathogen in designing a vaccine is a method to avoid unwanted side effects and problems of reversion associated with using whole bacterial cells or viral particles. The principle is to use a component that is able to induce an immune response that protect against the whole pathogen. A lot of interest in vaccine design is now using purified subunit antigens, including, toxoids, recombinant proteins, and purified lipopolysaccharides (LPS) (Shaw & Feinberg 2008). Examples of subunit vaccines are the acellular pertussis vaccine, toxoid vaccines against diphtheria and tetanus, carbohydrate vaccine against pneumococcus (Takahashi et al. 2009). These subunit antigens benefit from being safe and specific, but they usually have a low immunogenicity. Consequently, most subunit vaccines contain immune enhancing agents such as adjuvants and may also require specific delivery systems (McKee et al. 2010).

#### **1.1.4 DNA vaccines**

The recombinant DNA technologies provided great advances in vaccine development. No antigens are found in vaccines based on DNA, instead they provide the genetic material for antigen production by the host cell. These types of vaccines mimic a viral infection by inducing antigen presenting cells (APC) to ensure optimal MHC class I presentation and CD8<sup>+</sup> T cells response. DNA vaccines have also been reported to induce antibody response against expressed antigens (Donnelly, Wahren & Liu 2005). In order to improve delivery to target cells and recruitment of APC, these types of vaccines have been mixed with different components such as, lipid complexes, microparticulates and adjuvants (Ferraro et al. 2011).

#### **1.2 Mucosal vaccinations**

The first interaction between pathogens and the human host commonly occurs within the mucosal surfaces, specifically, the nasal, oropharyngeal, respiratory, gastrointestinal and genitourinary mucosa. In the past few years, major developments in the production of mucosal vaccines have occurred. Examples for licensed mucosal vaccines includes, the intranasal influenza vaccine (FluMist) (Belshe 2004), the Cholera vaccine (Dukoral) (Jelinek & Kollaritsch 2008), Rotavirus vaccine (RotaTeq) (Plosker 2010) and the oral Polio vaccine (OPV) (WHO 2014). Mucosal vaccines offer a number of potential advantages over systemic vaccines including, 1) promoting both mucosal and systemic immunity, 2) increased stability, 3) increased shelf life, and 4) no need for needles or trained healthcare specialists to administer vaccines. A major limiting factor for the development of mucosal vaccines is the availability of a safe and an effective adjuvant that functions in the mucosa (Freytag & Clements 2005). Mucosal immunizations are characterized by inducing antigen

specific secretory IgA (S-IgA) antibodies directed against specific pathogens of mucosal surfaces. These S-IgA may block the attachment of pathogens by neutralizing their toxins, and they also inactivate invading viruses inside epithelial cells (Eriksson & Holmgren 2002). The immune responses induced by mucosal immunization are initiated in specific mucosal-associated lymphoid tissue (MALT) structures such as the tonsils and adenoids in the nasal cavity in response to intranasal vaccination (Lycke 2012).

### **1.3 Vaccine Adjuvants**

The word ‘adjuvant’ (derived from the Latin *adjuvare*, to help) refers to any element added to a vaccine formula that improves both the quality and durability of the immunological response to an antigen (Shaw & Feinberg 2008). Until recently, the exact mechanisms of how these adjuvants improve immune responses were unknown. Recent advances in understanding the mechanisms of how dendritic cells (DC) recognizes foreign antigens, have led to massive improvements in adjuvant discovery and optimization. These advances in understanding adjuvants mechanisms helped to shape a better quantity, quality, and durability of host cellular and humoral immune responses. A better quality, and durability response is meant by a more specific antibody response that lasts for a longer period. Several substances have been identified as potent adjuvants including, Alum, Oil-in-water emulsions (like MF59) (Ott, Barchfeld & Van Nest 1995) and TLR agonists (Weeratna et al. 2005). Alum is the most commonly used adjuvant in vaccine formulations worldwide (Clements & Griffiths 2002; Kool, Fierens & Lambrecht 2012).

### **1.3.1 Toll like receptor (TLR) agonists as adjuvants**

TLR ligands/agonists activate innate immune response through TLR and have been studied as immunological adjuvants. Currently, many efforts for developing novel adjuvants are focused on the activation of TLRs that are expressed on innate immune cells and they are crucial for the initiation of innate and adaptive immune responses. A number of ligands for TLR have been identified including, double-stranded RNA (recognized by TLR-3), lipopolysaccharide (LPS) from bacterial cell walls (recognized by TLR-4), flagellin (recognized by TLR-5), single-stranded RNA (recognized by TLR-7) and CpG-DNA (recognized by TLR-9). Of the defined TLR agonists being explored as vaccine adjuvants, CpG-DNA, which activate TLR-9, are being evaluated in clinical studies (Halperin et al. 2003; Klinman 2006; McCluskie & Davis 1999).

### **1.3.2 Mucosal adjuvants**

The adjuvant to be selected for a mucosal vaccine is perhaps as important as the selection of the vaccine antigen. The initiation of an immune response following a mucosal immunization usually relies on the co-administration of an adjuvant which induces and supports the transition from innate to adaptive immunity. The most studied mucosal adjuvants include, the ADP-ribosylating enterotoxins, cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) (Freitag & Clements 2005; Lycke 2012). However, unwanted side effects of these mucosal adjuvants have been reported (Fujihashi et al. 2002; Mutsch et al. 2004). Among all adjuvants, TLR agonists constitute a major category of candidate mucosal adjuvants. For example, CpG-DNA, a TLR-9 ligand, have been tested in experimental vaccine studies and in clinical trials (Blaas et al. 2009; McCluskie & Davis 1999). Other TLR ligands

including monophosphoryl lipid A (MPL) (Childers et al. 2000) and flagellin (Bates et al. 2008) (TLR-4 and 5 ligands respectively) have also been tested as mucosal adjuvants. CpG-DNA as an immunological adjuvant will be discussed in further detail later in this chapter.

## **1.4 The Immune System**

Every day, people are challenged by millions of pathogenic agents such as microorganisms that try to invade our bodies. The main function of our immune system is to eliminate and protect us from these invading pathogens including influenza virus. Importantly, the distinction between self and non-self is an essential component of immune activation (Chi & Flavell 2008). Cells specialised in immunosurveillance monitor the body for pathogens, and transport fragments of any foreign material they encounter back to lymphoid organs. This function needs highly complex mechanisms which can be done by both the innate immune system (non-specific) and the adaptive immune system (specific).

Innate immunity represents the first line of our immunological defence against invading pathogens including influenza virus through its ability to distinguish between what is non-self and self. This function by the innate immune system requires special tools such as phagocytes, complements and pattern recognition receptors (PRRs) that have the ability to recognize conserved pathogen associated molecular patterns (PAMPs) which are able to distinguish self/non-self cells (Khalturin & Bosch 2007; Tosi 2005).

Unlike innate responses, adaptive immunity are slower to respond and require the recognition of a specific antigen. The adaptive immunity requires the involvement of

T and B lymphocytes and is characterized by immunological memory and antibody responses. Pathogens taken up by APC including DC are fragmented then presented as antigens on MHC molecules. Priming of naïve T cells takes place in the T cell zone of lymphoid organs, in close proximity to the B cell follicles. Activated CD8<sup>+</sup> and some CD4<sup>+</sup> T cells exit the lymphoid organs into the peripheral blood. Activated CD4<sup>+</sup> T cells that remain in the lymph node (LN) may become T<sub>FH</sub> cells that migrate towards the B cell follicle, where they help B cells in forming GC (Victora & Nussenzweig 2012).

### **1.5 The Mucosal Immune System in the Upper Respiratory Tract**

Most viral and bacterial infections gain entry via mucosal surfaces (respiratory tract, gastrointestinal tract and genital epithelium). The mucosal immune system, known as mucosa-associated lymphoid tissue (MALT), is composed of lymphoid tissues inhabiting the internal lining of our bodies. This mucosal immune system includes: the upper respiratory tract immune system named as nasopharyngeal-associated lymphoid tissues (NALTs), the lower respiratory tract immune system known as the bronchus-associated lymphoid tissue (BALT) and the gastrointestinal immune system named as gut-associated lymphoid tissues (GALTs) (van Kempen, Rijkers & Van Cauwenberge 2000).

The mucosal respiratory tract (epithelial-cell surfaces) has a large interface with the external environment ranging from the oral and nasal cavities to the alveoli in the lungs, with an estimated surface area of 70 m<sup>2</sup> in human adults (Holt et al. 2008). The upper respiratory tract mucosal immunity is a vital component of the body functioning as a first line of physical and immunological defence against invading pathogens such as bacteria, viruses, fungi, etc., dietary proteins and other

environmental molecules. This immune system has a highly complicated function of distinguishing harmful (invading pathogens) from non-harmful (commensal microorganisms) using its unique innate and acquired immune mechanisms (Nochi & Kiyono 2006).

The Waldeyer's ring of lymphoid tissues in the oropharynx including tonsils and adenoids that form NALT, and Peyer's patches, one of the well-characterized GALT elements, are two of the main components of MALT. These tissues (NALT and GALT) have been shown to be important inductive sites for the generation of antigen-specific immune responses including IgA isotype switching, generation of IgA<sup>+</sup> memory B cells and the induction of Ag-specific cytotoxic T lymphocytes (CTL), in diffuse mucosal effector sites (such as the lamina propria of the intestinal and respiratory tracts, and glandular tissues) (Fujihashi & Kiyono 2009; Kiyono & Fukuyama 2004).

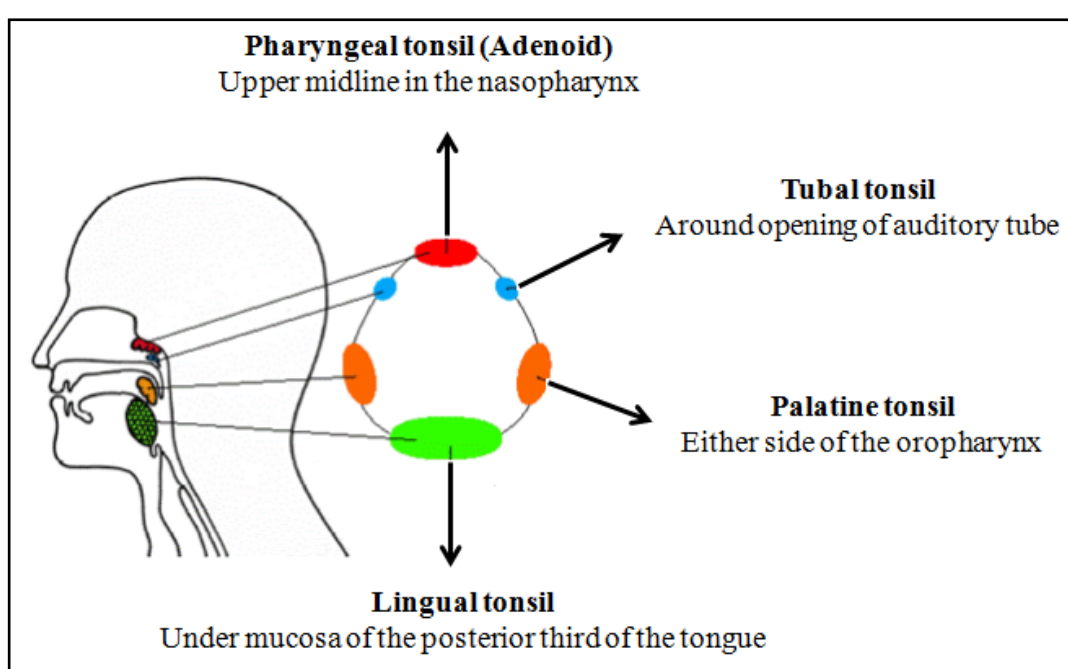
In general, the MALT can be functionally classified in two main types of tissues:

- i. Inductive sites: where naive B and T cells are activated and clonally selected and expanded upon antigens contact.
- ii. Effector sites: where activated B and T cells relocate after antigen priming in inductive sites to express their effector functions (Zuercher et al. 2002).

### **1.5.1 Nasopharyngeal Associated Lymphoid Tissues (NALT)**

NALT is a major front line of immune defence against inhaled pathogens. The Waldeyer's ring is considered as the inductive site of NALT to generate antigen specific immune responses. The Waldeyer's ring anatomically is especially arranged around the wall of the throat and consists of the palatine tonsil, adenoid

(nasopharyngeal tonsil) and lingual tonsils as major parts of the Waldeyer's ring. The lateral pharyngeal bands and tubal tonsils are less prominent components of the Waldeyer's ring (Hellings, Jorissen & Ceuppens 2000; van Kempen, Rijkers & Van Cauwenberge 2000) (Figure 1.1). Reticular crypt epithelium, the extrafollicular area, the mantle zones of lymphoid follicles and the follicular GC are all special lymphoid elements that have major roles in the immune responses in NALTs, particularly in tonsils and adenoids (Brandtzaeg 2003).



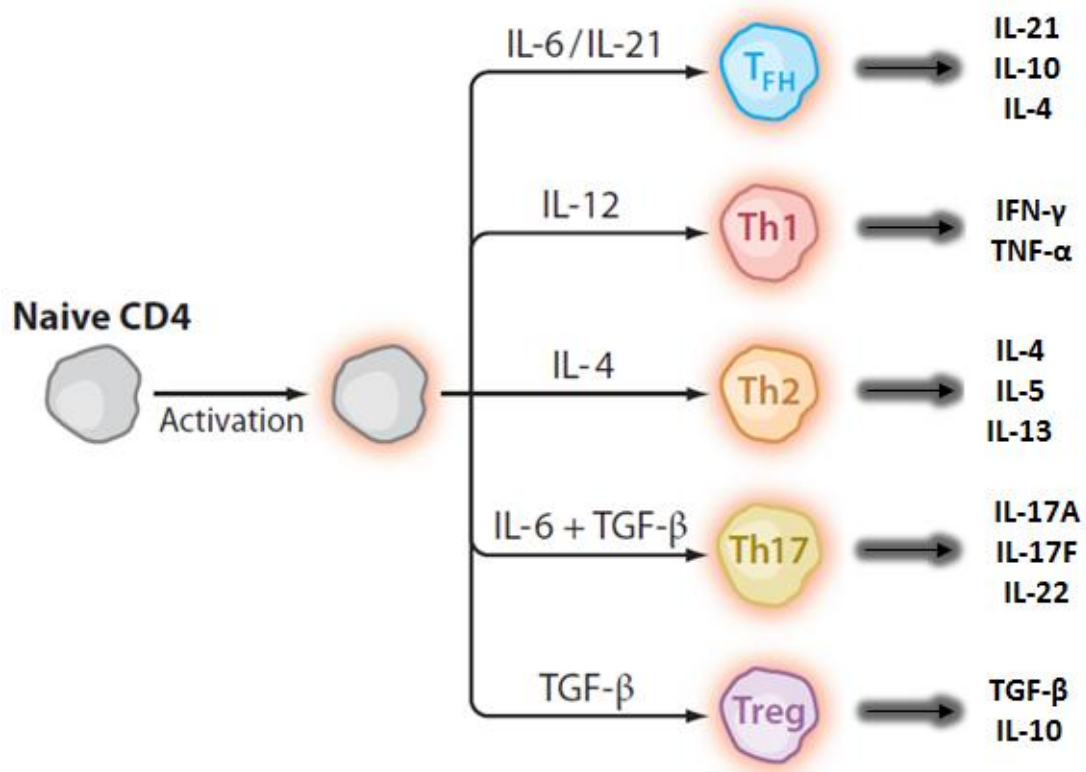
**Figure 1.1. The Waldeyer's ring**

The Waldeyer's ring is formed by the adenoid, tubal tonsils, palatine tonsils and lingual tonsil. Adapted from (Perry & Whyte 1998).

### 1.6 CD4<sup>+</sup> T cell subsets

Upon activation by APC, naïve CD4<sup>+</sup> T cells (T<sub>H</sub>0) can develop into one of many functionally distinct effector CD4<sup>+</sup> T subsets. Some of the major types of CD4<sup>+</sup> T cell subsets include T helper 1 (T<sub>H</sub>1), T helper 2 (T<sub>H</sub>2), T helper 17 (T<sub>H</sub>17), T

regulatory ( $T_{reg}$ ) cells, and the more recently discovered T follicular helper cells ( $T_{FH}$ ) (Figure 1.2).  $CD4^+$  T cell subsets are classified based on cytokines produced, transcription factors and cell surface markers expressed. The differentiation of a naïve T cell into a specific subset depends on the type of cytokines found in the microenvironment at the time of T cell activation (Chaplin 2010; Zhu, Yamane & Paul 2010).



**Figure 1.2.  $CD4^+$  T helper subsets**

$CD4^+$  T cells can differentiate into different subsets depending on the cytokine milieu present during T cell activation. Adapted from (Crotty 2011)

### 1.6.1 T helper 1 cells ( $T_H1$ )

$T_H1$  are characterized by the expression of T-box transcription factor (T-bet) and production of pro-inflammatory cytokines, particularly, interferon gamma (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ) and IL-2 (Szabo et al. 2000). IL-12 and IFN- $\gamma$

induce the  $T_H1$  differentiation by up regulating the expression of STAT-1 and STAT-4 leading to the expression of T-bet (Anne & Arai 2000).  $T_H1$  functions during intracellular bacterial and viral infections and their cytokines are important for macrophage activation by enhancing the phagocyte-mediated defence (Chaplin 2010; Li, Zhang & Sun 2011).

### **1.6.2 T helper 2 cells ( $T_H2$ )**

$T_H2$  are regulated by the Trans-acting T-cell-specific transcription factor (GATA-3) and secrete a range of cytokines, predominantly, IL-4, IL-5 and IL-13. IL-4 induces the  $T_H2$  cell differentiation by up regulating the expression of the transcription factor STAT-6 leading to the expression of GATA-3 (Anne & Arai 2000; Li, Zhang & Sun 2011).  $T_H2$  cells are important in the protection against helminth and parasite infections (Pearce et al. 2004) and allergens (Maezawa et al. 2003). In allergy, a dysfunctional  $T_H2$  regulation results in an IgE and eosinophil/mast cell mediated immune responses and a release of histamine in the affected tissue (Licona-Limon et al. 2013).

$T_H2$  cells have long been viewed as the main T helper subset supporting B cells for antibody production. Recently, a new T helper subset named as  $T_{FH}$  cells have been identified as a subset distinct from  $T_H1$  or  $T_H2$  cells (Chtanova et al. 2004).  $T_{FH}$  cells are discussed in further detail later in this chapter.

### **1.6.3 T helper 17 cells ( $T_H17$ )**

A third T helper cell termed  $T_H17$  cells are characterised by secreting large amounts of IL-17 (A and F) and IL-22.  $T_H17$  cells have also been reported to secrete IL-21 in an autocrine manner for  $T_H17$  cells development (Wei et al. 2007). These cells

express high levels of the transcription factors retinoid-related orphan receptor  $\gamma$ t (ROR $\gamma$ t). T<sub>H</sub>17 cells differentiate in response to transforming growth factor- $\beta$  (TGF- $\beta$ ) together with IL-6 and IL-1, while IL-23 may also be involved in their generation and or maintenance (Korn et al. 2009; Stockinger & Veldhoen 2007). T<sub>H</sub>17 cells have major roles in the recruitment, activation and migration of neutrophils. T<sub>H</sub>17 cells are important in immune responses to extracellular bacteria and fungi and are also potent inducers of autoimmunity and tissue inflammation (Korn et al. 2009; Kurts 2008).

#### **1.6.4 T regulatory cells (T<sub>reg</sub>)**

T<sub>regs</sub> are CD4<sup>+</sup> T cells that highly express CD25 (also known as IL-2R $\alpha$ ), the transcription factor forkhead box protein P3 (FoxP3) and TGF- $\beta$ . Two types of this subset have been described, the naturally occurring T<sub>regs</sub> population that develops in the thymus, and IL-10 producing regulatory T cells known as the inducible subtype (iT<sub>regs</sub>) (Ito et al. 2008). Unlike natural T<sub>regs</sub>, iT<sub>regs</sub> differentiate from naïve CD4<sup>+</sup> T cells in the periphery in a TGF- $\beta$  dependent manner. T<sub>regs</sub> has a critical role in controlling the immune system against inflammation, autoimmunity and allergy via suppressing effector T cells (Zheng & Rudensky 2007).

#### **1.7 T Follicular Helper cells (T<sub>FH</sub> cells)**

In the last decade, T Follicular helper CD4 (T<sub>FH</sub>) cells in humans have been identified as a new T helper subset. Within secondary lymphoid organs, T<sub>FH</sub> cells are specialized to regulate the development of effector and memory B cells and long-lived plasma cells. These cells were first described by several research groups which reported a unique CD4 T cell phenotype expressing high levels of C-X-C chemokine receptor type 5 (CXCR5) in tonsils (Breitfeld et al. 2000; Kim et al. 2001). Loss of

the chemokine receptor CCR7 and high expression of CXCR5 is a major player to drive T<sub>FH</sub> cells migration into B cells follicles in secondary lymphoid tissue, using a CXCL13-dependent manner (Choi et al. 2011; Fazilleau et al. 2009).

It is generally considered that T cell help to B cells is an essential process for adaptive immunity. The first interaction between T<sub>FH</sub> cells and B cells happens at the T cell-B cell border leading to B cells differentiation and GC formation within the follicle (Choi et al. 2011). In secondary lymphoid tissue, high expression of the costimulatory molecules including: CD40L, OX40 and inducible T-cell co-stimulator (ICOS), as well as the inhibitory molecule Programmed Death 1 (PD-1) are major players in modifying antigen specific contact of T<sub>FH</sub> cells to B cells expressing peptide major histo-compatibility complex class II (pMHC-II), CD40, OX40L, ICOSL and PDL-1 (Fazilleau et al. 2009; Kassiotis & O'Garra 2009).

### **1.7.1 T<sub>FH</sub> cell characterization**

T<sub>FH</sub> cells have been established as a distinct T helper subset based on characteristic cell surface markers expressed and cytokine profile. In addition to CD4 and CXCR5 (CD185), T<sub>FH</sub> cells are identified by expressing high levels of ICOS (CD278), PD-1 (CD279), the transcriptional repressor B cell lymphoma 6 (BCL-6), IL-21R, OX40 (CD134), CD40L (CD154) and GL7 (Kerfoot et al. 2011; King 2009; Laurent, Fazilleau & Brousset 2010). Elevated expression of signalling lymphocytic activation molecule-associated protein (SAP), B and T lymphocyte attenuator (BTLA) and CD200 have been reported (Crotty 2011). Other markers have also been reported in humans including, CD25 (IL-2R $\alpha$ ), CD69, CD95 and CD57 (Laurent, Fazilleau & Brousset 2010).

### **1.7.2 T<sub>FH</sub> Cell Differentiation**

Where and when a naïve T cell becomes a T<sub>FH</sub> cell is controversial and remains to be defined. At present, four models for T<sub>FH</sub> cell differentiation have been suggested: Model 1: direct T<sub>FH</sub> differentiation via cytokine (IL-6 or IL-21); Model 2: B cell-dependent direct T<sub>FH</sub> differentiation; Model 3: a non-distinct subset differentiated from T<sub>H</sub>1/T<sub>H</sub>2/T<sub>H</sub>17 cells (secondary program); Model 4: T<sub>FH</sub> differentiation is initially induced by DC and maintained by cognate interaction with B cells (Crotty 2011; Deenick et al. 2011).

In support of the first model, studies have shown the development of T<sub>FH</sub> cells as a distinct and independent subset after exposing purified CD4 T cells (T<sub>H</sub>0) to IL-6 or IL-21 (Dienz et al. 2009; Nurieva et al. 2008; Nurieva et al. 2009; Suto et al. 2008). A second model of T<sub>FH</sub> development has suggested that T<sub>FH</sub> cells are a distinct CD4 T cell subset and they are directly differentiated in a B cell-dependent interaction. It was shown that in the absence of B cells, T<sub>FH</sub> cells were not observed after viral infection (Johnston et al. 2009), and protein immunization (Haynes et al. 2007). In support of the third model (secondary program), a study has shown T<sub>FH</sub> cells to develop from another T<sub>H</sub> subset under certain conditions (Zaretsky et al. 2009). The fourth model of T<sub>FH</sub> differentiation is based on more recent findings which suggest prolonged antigen presentation by DC is critical in the generation of T<sub>FH</sub> cells (Chakarov & Fazilleau 2014; Deenick et al. 2011; Goenka et al. 2011).

### **1.7.3 Cell markers of T<sub>FH</sub> cells**

In addition to CD4 and CXCR5, several other molecules have been associated with T<sub>FH</sub> cells. These markers include, ICOS, PD-1, BCL-6 and IL-21.

### 1.7.3.1 CXCR5 and CCR7

CXCR5 was the first (and most widely used) cell surface marker to identify T<sub>FH</sub> cells discovered in 2000 (Breitfeld, Ohl et al. 2000). CXCR5 is a G protein-coupled seven transmembrane chemokine receptor, which specifically allows migration of cells towards the follicular chemokine ligand CXCL13 (Forster et al. 1994). In the activation process of naïve T<sub>H</sub> cells when interacted with APC, CXCR5 expression is upregulated which then binds to its receptor CXCL13, a chemokine responsible for T<sub>FH</sub> and B cells entry into follicles produced mainly by follicular dendritic cells (FDC) (Laurent, Fazilleau & Brousset 2010). However, the CXCR5 upregulation is not applied on all activated T cells and only a part of CXCR5<sup>+</sup> CCR7<sup>low</sup> cells migrates towards the follicle following an antigen exposure by APC (Yu & Vinuesa 2010a).

By down-regulation of CCR7, these activated T<sub>H</sub> cells become less reactive to both CCL19 (also known as Epstein-Barr virus-induced receptor ligand chemokine (ELC)) and CCL21 (also known as secondary lymphoid tissue chemokine (SLC)) which are main T-zone chemokines (Haynes 2008). Together, the CXCR5-CXCL13 interaction and down-regulation of CCR7 expression levels are responsible for T<sub>FH</sub> cells follicular homing. This leads to regulation of antigen-specific B-cell responses using co-stimulatory molecules including ICOS, CD28 and OX40 (Gómez-Martín et al. 2010; Laurent, Fazilleau & Brousset 2010).

It has been reported that a subset of human peripheral blood memory CD4<sup>+</sup> T cells, but not naïve CD4<sup>+</sup> T cells, T<sub>H</sub>1 cells and T<sub>H</sub>2 cells, are CXCR5<sup>+</sup> (Morita et al. 2011). A small proportion (<2%) of blood memory cytotoxic CD8<sup>+</sup> T cells has also been reported to express CXCR5 (Moser, Schaerli & Loetscher 2002). CD25<sup>+</sup>

Foxp3<sup>+</sup> cells that localize in the follicles has also been shown to express high levels of CXCR5 in addition to ICOS and PD-1 and they have recently been termed as follicular T<sub>reg</sub> (Wollenberg et al. 2011).

In a murine study, Haynes and Allen et al. showed that overexpression of CXCR5 is not enough for T cells access to follicles unless the CCR7 expression is down regulated. The absence of CXCR5 in T cells resulted in impairment of T cells entry into B cells follicles leading to a reduction of 2-fold in GC response (Haynes et al. 2007). Arnold and Campbell et al. reported that GC B cells response is significantly impaired in CXCR5 deficient T cells in mice (Arnold et al. 2007).

#### **1.7.3.2 Inducible co-stimulator molecule (ICOS)**

Inducible co-stimulator molecule (ICOS, also called CD278) is a homodimeric protein of relative molecular mass 55,000-60,000 (*Mr* 55K-60K). ICOS has been identified as a member of the CD28 receptor family of co-stimulatory molecules that regulates T cell activation and function expressed primarily on activated human T cells. As a member of CD28 family, ICOS induces T cell proliferation, differentiation, cytokine secretion (such as IL-2, IL-4 and especially IL-10 and IL-21), upregulates cell-cell interaction molecules and offers effective help to B cells for antibody production (Hutloff et al. 1999; Simpson, Quezada & Allison 2010).

In addition to CXCR5, ICOS has commonly been used to identify T<sub>FH</sub> cells in humans (Yu & Vinuesa 2010a). Highest expression of ICOS is found on T<sub>FH</sub> cells. Recent studies show that following T cell activation, ICOS is upregulated leading to T cell proliferation and promotion of either T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17 or T<sub>FH</sub> cells response depending on the type of inflammation. These activation effects are controlled by the

interaction of ICOS molecules with its specific ligand, ICOS-L (also named as: LICOS, B7RP-1, B7h, B7-H2 and GL50) and expressed on B cells as well as antigen presenting cells including DC and macrophages (Haynes 2008; Simpson, Quezada & Allison 2010). Studies have shown that ICOS are expressed in the apical light zone of GC in human tonsillar tissues (Shilling, Bandukwala & Sperling 2006).

Both murine and human studies have shown the importance of ICOS in the development and function of T<sub>FH</sub> cells. It has recently been reported that ICOS or ICOS-L deficiency is correlated with fewer T<sub>FH</sub> cells and a decrease in GC formation suggesting a major role of ICOS in T<sub>FH</sub> cell differentiation (Laurent, Fazilleau & Brousset 2010). Bauquet et al reported that ICOS is a major player in T<sub>FH</sub> cells development by enhancing the transcription factor c-MAF expression on T<sub>FH</sub> cells. On the other hand, ICOS-deficient mice had significantly less c-MAF expression resulting in fewer T<sub>FH</sub> cells. c-MAF also controls IL-21 production which consequently regulates T<sub>FH</sub> cell development (Bauquet et al. 2009).

Reports show that ICOS deficiency in humans is related with different immunodeficiency syndromes including late-onset common variable immunodeficiency (CVID) resulting in impaired B cells memory and major defects in immunoglobulin class switching (Grimbacher et al. 2003; Haynes 2008). These ICOS or ICOS-L deficiencies resulted in reduction of serum IgG production in particular IgG1 levels in a T-dependent pathway; whereas, the antibody levels were normal against T-independent antigens (Akiba et al. 2005). Studies suggested a crucial role of ICOS/ICOS-L interaction in regulating the formation of GC and development of effector and memory B cells and long-lived plasma cells, although the mechanism controlling this interaction remains unclear (Akiba et al. 2005). Akiba et al reported an essential role of ICOS/B7RP-1 interaction for the up-

regulation of CXCR5 expression on CD4<sup>+</sup> cells in LN and spleen for the development of CXCR5<sup>+</sup> T<sub>FH</sub> cell in vivo (Akiba et al. 2005).

### **1.7.3.3 Programmed cell death 1 (PD-1)**

The receptor ‘programmed cell death 1’ (PD-1; also called CD279), a member of the CD28 family and a potent inhibitory receptor, is an important molecule for T cell tolerance. PD-1 is induced on activated CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, natural killer T cells, B cells, myeloid cells and activated monocytes (Iwai et al. 2002; Sharpe et al. 2007). PD-1 is highly expressed on T<sub>FH</sub> cells and has been used as an identification marker for T<sub>FH</sub> cells (Haynes et al. 2007; Xu et al. 2014). PD-1 is suggested to contribute to GC B cell survival and the formation and affinity of long-lived plasma cells through interaction with its ligands PD-L1 (also called CD274 or B7-H1) and PD-L2 (also called CD273 or B7-DC) which are members of the B7 (CD28 ligand) family. These PD-L1 and PD-L2 are highly expressed on GC B cells (Good-Jacobson et al. 2010).

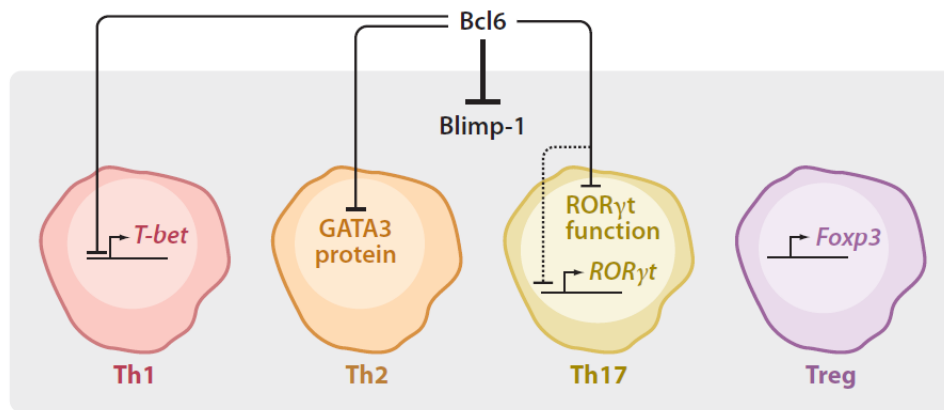
PD-1 provide an inhibitory signal to GC T<sub>FH</sub> cells to prevent excess proliferation of CD4 T cells in GCs (Crotty 2011). The extended TCR signalling from B cells that occur in the GC induces the PD-1 expression on T<sub>FH</sub> cells (Crotty 2011). Haynes and colleagues have shown that within CXCR5<sup>high</sup> CCR7<sup>low</sup> population the PD-1 expression levels and the abundance of IL-4 transcripts were the highest (Haynes et al. 2007). A study with human tonsillar tissue by immunohistochemistry has shown that PD-1<sup>+</sup> T cells are highly enriched within the GC light zone (Iwai et al. 2002). PD-1 has been reported to increase during viral infections to prevent activated T cells of becoming autoreactive or causing immunopathology (Sang-Jun et al. 2008; Sharpe et al. 2007). Recent studies have reported alterations in T<sub>FH</sub> subsets including

reduced IL-4 and IL-21 production in the absence of PD-1 (Good-Jacobson et al. 2010).

#### **1.7.4 BCL-6 as a Regulator of T<sub>FH</sub> Differentiation**

Master regulatory transcription factors control CD4 T cell differentiation. T<sub>H</sub>1 cells express T-bet (Szabo et al. 2000), T<sub>H</sub>2 cells express GATA-3 (Zheng & Flavell 1997), T<sub>H</sub>17 cells express ROR $\gamma$ t (Kurts 2008) and T<sub>reg</sub> express Foxp3 (Zheng & Rudensky 2007).

Most recently, BCL-6 has been identified as the master regulatory transcription factor which controls T<sub>FH</sub> cell differentiation program (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009b). Up-regulation process of BCL-6 by T<sub>FH</sub> cells leads to down-regulation and blocking for T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 cells proliferation by suppressing their transcription factors (See Figure 1.3) (Laurent, Fazilleau & Brousset 2010; Yu et al. 2009b). Over-expression of BCL-6 in activated T helper cells has been reported to stimulate IL-6 receptor and IL-21 receptor expression and they are both necessary for T<sub>FH</sub> cells differentiation (Laurent, Fazilleau & Brousset 2010). However, the expression of BCL-6 by T<sub>FH</sub> cells is heterogeneous and around 10-15% of T<sub>FH</sub> cells in GC have detectable BCL-6 protein (Vinuesa et al. 2005). Thanks to gene expression profiling, T<sub>FH</sub> cells has been distinguished from T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17 and iT<sub>reg</sub> gene expression profiles (Choi et al. 2011). Recent studies have shown that T<sub>FH</sub> differentiation does not occur in vivo in the absence of BCL-6 (*Bcl6*<sup>-/-</sup>). However, the differentiation of other CD4 T cell subsets is relatively unaffected by the lack of BCL-6 (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009b).



**Figure 1.3. BCL-6 inhibition of other CD4 T cell differentiation pathways**

BCL-6 can cause a generalized inhibition of other CD4 T cell differentiation pathways by blocking Blimp-1 expression. Taken from (Crotty 2011).

BCL-6 is an antagonist of Blimp-1 and Blimp-1 is an antagonist of BCL-6. BCL-6 is highly expressed on T<sub>FH</sub> cells and Blimp-1 is highly expressed on non-T<sub>FH</sub> cells, i.e., T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>17, or other subsets (Crotty, Johnston & Schoenberger 2010; Johnston et al. 2009). BCL-6 can limit all non-T<sub>FH</sub> differentiation by repressing Blimp-1. Recent studies have reported that BCL-6 can inhibit transcription factors important for T<sub>H</sub>1 cells (Nurieva et al. 2009; Yu et al. 2009b), T<sub>H</sub>2 cells (Kusam et al. 2003; Nurieva et al. 2009), or T<sub>H</sub>17 cells (Mondal, Sawant & Dent 2010) differentiation. Other transcription factors such as c-Maf, Batf, and BCL-6-binding partners also play essential roles in the development and function of T<sub>FH</sub> cells (Crotty 2011). However, more studies are needed to fully understand the mechanisms by how BCL-6 controls T<sub>FH</sub> cell differentiation.

### 1.7.5 Cytokine profile for T<sub>FH</sub> cells

It is clear that T<sub>FH</sub> cells can produce many cytokines in biologically relevant quantities in particular, IL-21, IL-10 and IL-4 which are all important for B cell

development and proliferation (Eto et al. 2011; Leddon & Sant 2012; Luthje et al. 2012; Reinhardt, Liang & Locksley 2009; Yusuf et al. 2010). Interferon- $\gamma$  (INF- $\gamma$ ), IL-4 and IL-17 which are usually related to T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 cells, respectively, has also been reported to be produced by T<sub>FH</sub> cells (Yu et al. 2009a).

#### **1.7.5.1 IL-21**

IL-21 influences the function of T cells, NK cells, and B cells. In humans, IL-21 is mainly produced by T<sub>FH</sub> cells (Eto et al. 2011; Nurieva & Dong 2009; Reinhardt, Liang & Locksley 2009) although not exclusively expressed by T<sub>FH</sub> cells (Suto et al. 2008; Wei et al. 2007). IL-21 has been reported to play a role in T<sub>FH</sub> cell formation and it is crucial in stimulating the differentiation of human B cells (Ettinger et al. 2005; Linterman et al. 2010). Major defects in IgG1 production following antigen priming, has been reported in IL-21R-deficient mice (Ozaki et al. 2002). Recent studies have also shown that IL-21 is important for optimal GC B cell proliferation. Impaired affinity maturation and reduction in the proportion of IgG1<sup>+</sup> GC B cells was seen in IL-21 deficiency. However, the formation of early memory B cells was not affected by the lack of IL-21. A reduction of BCL-6 expression on GC B cells has been reported in absence of IL-21 (Linterman et al. 2010; Zotos et al. 2010).

#### **1.7.5.2 IL-10**

IL-10 is known to act as a survival, proliferation, and differentiation factor for B cells and is more efficiently provided by T<sub>FH</sub> cells than other T cells (Kim et al. 2001). Chevalier et al. reported that CXCR5<sup>+</sup> T cells expressed significantly higher levels of IL-10 as compared with CXCR5<sup>-</sup> T cells in human blood. This study also showed that plasma cell differentiation and immunoglobulin secretion were

significantly increased when B cells were cultured with CXCR5<sup>+</sup> T cells compared with CXCR5<sup>-</sup> T cells (Chevalier et al. 2011). Using an IL-21 reporter mouse Luthje and co-workers reported that the expression of IL-10 mRNA was restricted to IL-21<sup>+</sup> T<sub>FH</sub> cells (Luthje et al. 2012). Kim et al. reported in human tonsillar tissues that GC-T<sub>FH</sub> cells (CXCR5<sup>+</sup> CD57<sup>+</sup>) are more efficient in production of IL-4 and IL-10 as evidenced by intracellular staining and ELISA compared to CXCR5<sup>+</sup> CD57<sup>-</sup> cells (non- GC-T<sub>FH</sub> cells), naive or CXCR5<sup>-</sup> T cells (Kim et al. 2001).

### **1.7.5.3 IL-4**

IL-4 is widely recognized as the main marker for T<sub>H</sub>2 polarized CD4<sup>+</sup> T cells and as a B cell survival and differentiation factor. T<sub>FH</sub> cells have frequently been reported to produce IL-4 (Fazilleau et al. 2009; King & Mohrs 2009; Reinhardt, Liang & Locksley 2009). T<sub>FH</sub> cells are the major producers of IL-4 in secondary lymphoid tissues where humoral immune responses develop. Production of IL-4 by GC T<sub>FH</sub> cells is required for optimal B cell help (Vijayanand et al. 2012). Using a IL-4 GFP reporter mice, Yusuf et al. demonstrated that IL-4 production was observed by GC-T<sub>FH</sub> cells (GL7<sup>+</sup> CXCR5<sup>+</sup> CD4 T cells) but not CXCR5<sup>-/low</sup> virus-specific CD4 T cells. The majority of the IL-4 was produced by the highest CXCR5 expressing cells, even within the T<sub>FH</sub> population and no IL-4 was made by non-T<sub>FH</sub> CD4 T cells. The IL-4 expression by the GC T<sub>FH</sub> population was independent of T<sub>H</sub>2 differentiation, because the expression of GATA3 was not increased above basal levels in any virus-specific CD4 T cells (Yusuf et al. 2010).

### 1.7.6 Peripheral T<sub>FH</sub> cells (pT<sub>FH</sub>)

Recent studies in humans have also shown a circulating CD4<sup>+</sup> T cell population characterized by high CXCR5 expression in peripheral blood (Chevalier et al. 2011; Ma & Deenick 2014; Morita et al. 2011; Simpson et al. 2010). These circulating cells, termed “peripheral T<sub>FH</sub>” (pT<sub>FH</sub>) share similar functional characteristics to T<sub>FH</sub> cells found in human secondary lymphoid tissues by expressing higher levels of PD-1 and IL-21 compared to CD4<sup>+</sup> CXCR5<sup>-</sup> T cells (Ma & Deenick 2014). However, a main difference between T<sub>FH</sub> cells found in secondary lymphoid organs and pT<sub>FH</sub> cells is the lack of expression of the transcription factor BCL-6 and much lower expression of ICOS by pT<sub>FH</sub> cells (Chevalier et al. 2011; Morita et al. 2011; Simpson et al. 2010).

Humans who show severely impaired GC formation through deficiency of CD40-ligand or ICOS show significantly less circulating CD4<sup>+</sup> CXCR5<sup>+</sup> T cells (Bossaller et al. 2006). A study by Morita et al. has shown the ability of these CXCR5<sup>+</sup> pT<sub>FH</sub> cells in providing better help to B cells to differentiate and proliferate compared to their CD4<sup>+</sup> CXCR5<sup>-</sup> counterparts. This study also showed that CD4<sup>+</sup> CXCR5<sup>-</sup> express higher Blimp-1 mRNA than their CD4<sup>+</sup> CXCR5<sup>+</sup> cell (pT<sub>FH</sub>) counterparts (Morita et al. 2011). These pT<sub>FH</sub> cells have been suggested to represent the memory counterparts of T<sub>FH</sub> cells outside the lymphoid organs, although it remains unknown whether there is a direct relationship between pT<sub>FH</sub> cells and T<sub>FH</sub> cells found in secondary lymphoid tissue (Morita et al. 2011; Simpson et al. 2010; Vinuesa & Cook 2011).

In concordance with T<sub>FH</sub> cells found in secondary lymphoid tissue, blood CD4<sup>+</sup> CXCR5<sup>+</sup> T cells (pT<sub>FH</sub>) was found to support naïve and memory B cells to become

antibody-producing cells via IL-21, IL-10, ICOS, and secrete CXCL13. In contrast with  $T_{FH}$  cells found in NALT,  $pT_{FH}$  cells express less CD69, ICOS, and PD-1 suggesting that they are in a resting state and express CCR7 and CD62L, suggesting their capacity to migrate into secondary lymphoid organs (Kim et al. 2001; Ma et al. 2009; Simpson et al. 2010). Whether  $pT_{FH}$  originate from cells that migrated out of GCs,  $T_{FH}$  precursors, is an important question, although it remains unclear in humans.

### **1.8 Germinal Centre B cells**

B cells are found circulating in the peripheral blood. However, within special follicles in secondary lymphoid tissues including tonsils and adenoids, B cells are most abundant. B cells activation needs either a soluble antigen or an antigen delivered on DC, macrophages or FDC through BCR stimulation. B cells can be activated by either a T cell-independent or a T cell-dependent manner (Jeurissen & Bossuyt 2004). In a T cell-independent response, the crosslinking in BCRs by repeated antigenic or multiple polyclonal epitopes can provide enough stimuli for B cells to be activated. Neutralizing antibodies can be generated within 2-3 days in T cell-independent responses, although without affinity maturation or memory B cell responses (Shih, Roederer & Nussenzweig 2002). In T cell-dependent responses, the initiation of the interaction between B and T cells requires antigen presentation by B cells via MHC class II, the binding of antigen specific T cells, and the formation of an immunological synapse between the two cells (Parker 1993).

The up-regulation of CCR7 and down-regulation of CXCR5, initiates the B cell migration towards the boundary of the T/B cell zones. The subsequent  $T_{FH}$  cell interaction initiates proliferation and formation of GCs (Cyster 2010; Victora &

Nussenzweig 2012). After interaction with T<sub>FH</sub> cells, B cells can either be programmed to become memory B cells, or plasma cells producing high affinity antibodies.

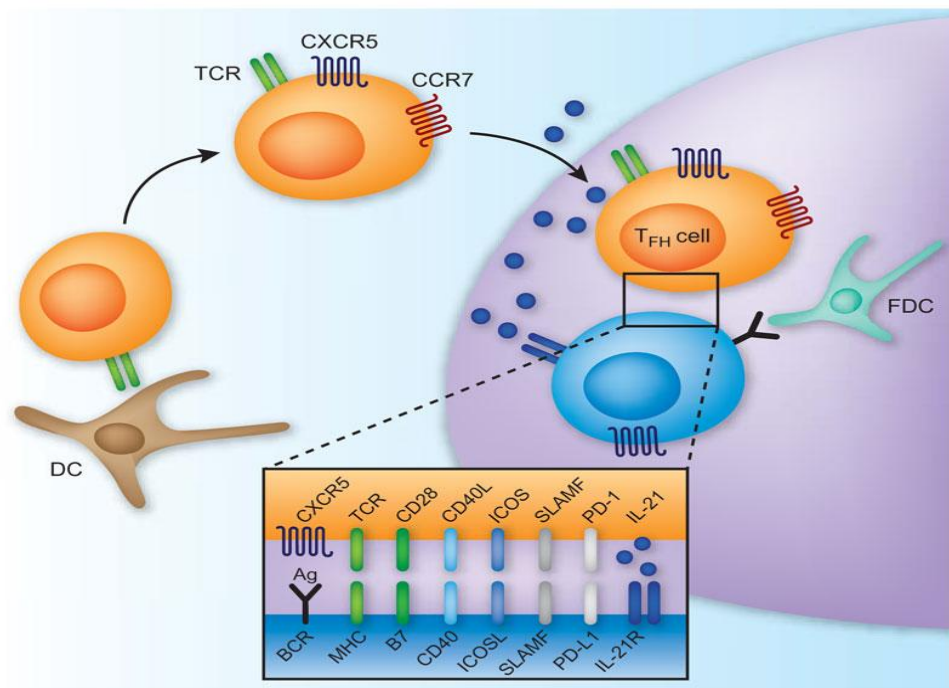
In addition to its importance for T<sub>FH</sub> cell differentiation, BCL-6 is also an important regulator for GC B cells. BCL-6 is able to block Blimp-1, the transcription factor regulating differentiation of plasma cells, to keep B cells in the GC response (Shaffer et al. 2000). Up-regulation of the transcription factor Blimp-1, which is accompanied by the down-regulation of BCL-6, drives GC B cells to become either short lived plasma cells that reside in secondary lymphoid tissue or long-lived plasma cells found in the bone marrow (Shapiro-Shelef & Calame 2005). CXCL13 is mainly expressed by FDCs, retaining B cells and attracting T<sub>FH</sub> cells expressing high levels of CXCR5 to the GC (Laurent, Fazilleau & Brousset 2010). These processes leads to the formation of memory B cells and plasma cells producing high affinity antibodies, which is the goal of most vaccinations, although the exact signals that determine GC B cells differentiation into memory cells or plasma cells are unknown (Klein & Dalla-Favera 2008).

### **1.9 T<sub>FH</sub> cell – B cell Interactions in the GC**

Specific antigen activated T cells which were primed on DC in the T cell zone, upregulate the expression of CXCR5, ICOS and PD-1, and downregulate CCR7 allowing them to migrate to the B cell follicles (Yu & Vinuesa 2010b). These activated T cells mature into T<sub>FH</sub> cells after interacting with follicular B cells. Antigen activated follicular B cells migrate towards the T cell zone boundary. Some of these B cells differentiate into either extrafollicular plasmablasts or become early

memory B cells, however, most of them return to the follicle and initiate massive proliferations to form a GC (Nutt & Tarlinton 2010). (See Figure.1.4)

Antigen activated follicular B cells require a cognate interaction with  $T_{FH}$  cells which takes place at the B-T cell boundary, to initiate the B cell differentiation process towards forming GCs. Before these interactions happen, activated follicular B cells and T cells adjust their chemokine receptor expression allowing them to relocate. Activated B cells upregulate CCR7 expression and migrate to the T cell zone (Reif et al. 2002), while activated T cells upregulate CXCR5 expression allowing them to migrate towards the follicle (Fillatreau & Gray 2003).



**Figure 1.4. Important interactions between  $T_{FH}$  and B cells in the GC**

In the GC,  $T_{FH}$  cells interact with GC B cells through an array of molecular pairings leading to the secretion of cytokines by  $T_{FH}$  cells, particularly IL-4, IL-10 and IL-21, which are received by B cells for optimal GC function. Taken from (Nutt & Tarlinton 2010).

T<sub>FH</sub> and GC B cell interactions are crucial in GC response. In the GC, T<sub>FH</sub> cells interact with GC B cells via a range of molecular pairings, including those between T cell antigen receptor and CD4 with major histocompatibility complex class II (TCR-MHC-II); CD28-B7 family members; ICOS and its ligand ICOSL (ICOS-ICOS-L); CD40 and its ligand CD40L (CD40-CD40L); PD-1 and its ligand PDL-1 (PD1-PDL-1); IL-21 and its receptor IL-21R (IL-21-IL-21R) and signaling lymphocytic-activation molecule family members (SLAMF) on both cell types. These interactions leads to the secretion of IL-4, IL-10 and IL-21 by T<sub>FH</sub> which are received by GC B cells for optimal GC function by forming high affinity memory B cells and long-lived plasma cells (Nutt & Tarlinton 2011). (See Figure 1.4)

These interactions, which occur in the GC, have been intensively examined. Major defects in the immune response that developed in the absence of one of these specific molecules from either T<sub>FH</sub> cells or B cells have been documented. For example, a significant reduction in long-lived plasma cells was seen in the absence of PD-1 or its ligands (Good-Jacobson et al. 2010). ICOS or ICOS-L deficiency was correlated with fewer T<sub>FH</sub> cell numbers and major defects in GC formation (Laurent, Fazilleau & Brousset 2010). Significant reduction in both GC B cells and antibody levels was also reported in the absence of IL-21 or its receptor (Linterman et al. 2010; Ozaki et al. 2002; Zotos et al. 2010).

### **1.10 The Germinal Centre Response**

The GC is a distinct microanatomical region of secondary lymphoid organs. GCs have a dynamic microenvironment where antigen activated B cells go through various essential modification including, clonal expansion, somatic hypermutation,

selection of high-affinity B cells and class-switch recombination (Klein & Dalla-Favera 2008). During T-cell-dependent antibody responses to exogenous antigen, GCs have been shown to initiate after around 6 days of a primary immunization, when foci of rapidly proliferating B cells begin to appear within the B cell follicles of secondary lymphoid organs including tonsils. The size of these foci increase rapidly and within a few days of their formation they differentiate into a mature GC reaction (Victora & Nussenzweig 2012).

The GC contains two major zones known as the light zone, proximal to the LN capsule or the spleen marginal zone, and the dark zone, proximal to the T cell zone. The dark zone is consists almost entirely with rapidly proliferating GC B cells with a high nucleus-to-cytoplasm ratio, consequently appearing “dark” by light microscopy. In the light zone GC B cells are intermixed with a network of FDCs which give this zone a “light” appearance (van Nierop & de Groot 2002). The light zone also contains a large number of T<sub>FH</sub> cells (Crotty 2011; Nutt & Tarlinton 2011) as well as a small number of DCs (Grouard et al. 1996). Tingible body macrophages, a specialized group of phagocytes that engulf dying B cells that develop during GC selection, are found throughout the GC (Smith et al. 1998; Victora & Nussenzweig 2012). These two areas have separate functions. The dark zone is where GC B cells proliferate and go through the process of somatic hypermutation and class switch recombination. Whereas the light zone is the site for clonal selection through recognition of antigen-bearing FDCs and interactions with T<sub>FH</sub> cells (Victora & Nussenzweig 2012).

To increase antibody diversity and affinity, activated B cells undergo somatic hypermutation and then alter antibody affinity and undergo class-switch recombination.

Class-switch recombination changes the constant region of the Ig heavy chain from IgD or IgM to IgG, IgA, or IgE which is an essential stage in humoral immunity (isotype-switching) (Xu et al. 2012). Somatic hyper mutation and class-switch recombination require the presence of the enzyme activation-induced cytidine deaminase (AID) which initiates DNA strand-breaks in the V(D)J section of the Ig variable regions and is expressed by proliferating B cells (Klein & Dalla-Favera 2008; Vinuesa, Sanz & Cook 2009).

### **1.11 Dendritic Cells**

DCs are considered to be the most effective APCs and provide an important link between the innate and adaptive immunity (Stockwin et al. 2000). DCs are usually found where pathogens are most likely to first be encountered, for example, dermal tissues, mucosal surfaces of the nasal cavity, lungs and intestine (Stockwin et al. 2000). Upon antigen encounter, DCs migrate to the draining LNs via lymphatic vessels where they interact with T cells. Following degrading of antigens by DCs, immuno-peptides (epitopes) are presented by MHC class I or II molecules. The presentation of MHC class I by DC is recognized by specific CD8<sup>+</sup> cytotoxic T cells (CTL), and MHC class II is recognized by CD4<sup>+</sup> T helper cells. Driving the differentiation of naïve T cells into a certain T helper subset can be the difference between inflammation and tolerance responses (Merad et al. 2013). The common property of several agents with adjuvant activity, including, CpG-DNA, is that they stimulate immune responses by inducing the maturation of DCs (Ramírez-Pineda et al. 2004).

Currently, many subtypes of DCs have been identified including conventional DCs (cDC), plasmacytoid DCs (pDC), monocyte-derived DCs and the epidermal

Langerhans cells. DCs are derived from blood-circulating precursors, originating from the bone marrow, and differentiate in peripheral tissues including lymphoid organs (Geissmann et al. 2010). Immature DC are characterized by low surface MHC and co-stimulatory molecules expression, however, they are highly phagocytic and have a large range of PRRs (Banchereau & Steinman 1998).

The initiation of the maturation process starts after the encountered antigens are engulfed by endocytosis. These internalized antigens are then degraded in lysosomes to be loaded on MHC molecules and transported to the cell surface to be recognized by T cells (Banchereau & Steinman 1998). Another feature of DC maturation is the expression of co-stimulatory molecules CD80 and CD86. Mature DC also express CCR7 to enable a gradient-dependent migration to the T cell zone of lymphoid tissues, which are rich in the chemokines CCL21 and CCL19 (Weber et al. 2013).

Recently, it has been reported that antigen presentation by DC is necessary to initiate T<sub>FH</sub> cell development (Choi et al. 2011; Goenka et al. 2011), despite the fact that in most cases, antigen presentation by B cells is ultimately responsible for promoting the full differentiation program of T<sub>FH</sub> cells (Ballesteros-Tato & Randall 2014; Johnston et al. 2009; Nurieva et al. 2009).

#### **1.11.1 Plasmacytoid DC (pDC)**

Plasmacytoid DC differs from classical DC morphologically as pDC are more spherically formed and classical DC have extending dendrites. pDC represents a small subset of DC and can be found in the bone marrow, blood and all lymphoid organs (including tonsils) (Polak et al. 2008; Rescigno 2013) and enter the LN via the blood circulation (Merad et al. 2013). pDC have been previously described in the

T-cell area of human mucosa, especially in tonsils (Polak et al. 2008; Rescigno 2013)

They are characterized with rapid production of type I interferons (IFN- $\alpha/\beta$ ) in response to viral infections. They also can act as APC and control T cell responses (Geissmann et al. 2010). Several relatively pDC specific surface markers have been established for pDC identification, including, ILT7 (immunoglobulin like transcript 7) (also known as CD85g), human blood dendritic cell antigen (BDCA)-2, BDCA-4 and CD123 (known as human IL-3R $\alpha$ ) (Reizis 2010; Reizis et al. 2011). At the same time, pDC lack the expression of CD11b and only express low levels of CD11c. In humans, pDC uniquely express both TLR-7 and TLR-9 which are not found on other DC types (Fuchsberger, Hochrein & O'Keeffe 2005; Hornung et al. 2002).

### **1.12 Pattern Recognition Receptors (PRRs)**

The innate immune system has the ability to recognize different microorganisms and distinguish them from self cells by several phylogenetically germ line encoded Pattern Recognition Receptors or PRRs. These PRRs are considered to act as a key component of the innate immunity and are expressed in cells of innate system, including mononuclear phagocytes, such as circulating monocytes and tissue macrophages, DCs, mucosal epithelial cells, and lymphocytes (Akira, Uematsu & Takeuchi 2006; Tosi 2005). PRRs can identify specific microbial structures known as pathogen associated molecular patterns (PAMPs), which are important microbial molecules for pathogens to survive and they are unique to each microorganism that are not found in the host body.

In addition, it's unlikely for these infectious agents to change these molecules as any major changes will lead to disadvantages to the invader (Chi & Flavell 2008). Several bacterial components are included such as, lipopolysaccharide (LPS) and peptidoglycans (PGNs), bacterial DNA and viral RNA in case of a virus infection (Martinon & Tschopp 2005). Although PAMPs are important to trigger an innate immune response, there are other elements that can be detected to initiate the innate immunity such as abnormal self cells or danger signals like DNA, RNA or even uric acid that normally should not be outside cells or at certain areas inside the cell (Martinon & Tschopp 2005).

Additionally, there are several types of PRRs in the innate immune system including members of the Toll-like receptors (TLRs) family which are found to be expressed at the cell surface or monitor endosomal compartments (Lotz, Ménard & Hornef 2007). Furthermore, each of these PRRs acts in response to specific PAMPs that will activate specific signaling pathways resulting in distinct anti-pathogen responses (Akira, Uematsu & Takeuchi 2006).

#### **1.12.1 The role of Toll-like receptors (TLRs)**

The first human TLR was identified by Medzhitov et al. (Medzhitov, Preston-Hurlburt & Janeway 1997). However, the Toll gene was originally discovered in the fruit fly *Drosophila* long time earlier. TLRs are conserved transmembrane signaling receptors of the innate immune system characterized by an extracellular domain including leucine-rich repeats (Abrahams & Mor 2005; Takeda & Akira 2005) and an intracellular domain which shows homology with the interleukin-1 receptor (IL-1R) that forms the TOLL/IL-1R (TIR) family. In addition, TLRs are considered the

first line of defence against bacteria, fungi and some viruses (Janeway & Medzhitov 2002; Papadimitraki, Bertsias & Boumpas 2007).

Furthermore, several types of TLRs have been identified in humans and each one of them has the ability to recognize some unique PAMPs (Medzhitov & Janeway 2002). For example, TLR-4 has the ability to recognize LPS of Gram-negative bacteria and the F protein of the respiratory syncytial virus (RSV). Whereas TLR-2 has the ability to recognize several groups of components derived from pathogens such as lipopeptides from Gram-positive and Gram-negative bacteria, mycobacteria and some parasites, while TLR-3, -7, -8 and -9 are able to recognize nucleic acids of some pathogens (Suzuki, Chow & Downey 2008; Takeda & Akira 2005). Bacterial DNA is highly rich in unmethylated CpG motifs and they are detected by TLR-9. Exposure to unmethylated CpG-DNA released during an infection provides a “danger signal” to the innate immune system, triggering a protective immune response that improves the host’s capacity to eliminate the pathogen. Synthetic ODNs expressing CpG motifs similar to those found in bacterial DNA stimulate a similar response (Klinman 2006).

### **1.13 CpG-DNA a TLR-9 Ligand**

CpG oligodeoxynucleotides (CpG-DNA) are short single-stranded synthetic oligonucleotides containing unmethylated Cytosine-Guanine (CpG, where the p indicates the phosphodiester bond) dinucleotides known as CpG motifs. CpG-DNA is recognized as PAMPs due to their abundance in bacterial DNA compared to mammalian DNA. CpG-DNA mimic the immunostimulatory activity of bacterial DNA and are recognized by the Toll-like receptor 9 (TLR-9) leading to strong immunostimulatory effects. Three types of stimulatory CpG-DNA have been

suggested based on their immunostimulatory activities named as CpG-DNA type A, B and C (Klinman 2006; McCluskie & Davis 1999).

### **1.13.1 CpG-DNA classes**

Three major classes of immune stimulatory CpG-DNA has been described, with distinct structural and biological characteristics; CpG-DNA class A (also known as type D), CpG-DNA class B (also known as type K) and CpG-DNA class C. One of the first class A CpG-DNA was described in 2001 by Krug et al. (Krug et al. 2001a). This class is characterized by its strong stimulatory effect on pDC to produce high quantities of IFN- $\alpha$ , induces APC maturation and through IFN- $\alpha$  (indirectly) activates natural killer (NK) cells. In addition, CpG-DNA class A have a weak effect on B cell activation compared to CpG-DNA type B (Krug et al. 2001a; Vollmer et al. 2004).

On the other hand, CpG-DNA type B is the strongest B cell stimulatory class. CpG-DNA class B was first identified in 1995 by Krieg and colleges (Krieg et al. 1995). Members of this class of CpG-DNA mainly induce B cell proliferation, differentiation and immunoglobulin and cytokine secretion. They induce pDC expression of costimulatory molecules, including CD86, CD54, MHC II and CD40. Class B CpG-DNA was also reported to activate NK cells but stimulate little IFN- $\alpha$  secretion (Hartmann & Krieg 2000; Krieg & Yi 2000; Krieg et al. 1995).

Class C CpG-DNA, which was firstly identified by Marshall et al., combines the properties of both class A and B CpG-DNA by being potent stimulators of pDC IFN- $\alpha$  production, APC activation and maturation, indirect NK cell activation, and direct

B cell stimulation and proliferation, but to a lower extent than class A or B alone (Marshall et al. 2003; Vollmer et al. 2004).

Recently a new CpG-DNA was identified, called the P-class CpG-DNA. This new class combines the preferred properties of the other CpG-DNA classes. It is able to induce the strongest type I IFN secretion by pDC, compared to the other classes, and induces superior stimulation of cytokine production *in vivo* (Samulowicz et al. 2010).

### **1.13.2 CpG-DNA clinical applications**

In recent years, novel adjuvants and vaccine delivery systems aimed to enhance vaccine immunogenicity have been under study (Spensieri et al. 2013). CpG-DNA is a TLR-9 ligand and has several potential therapeutic uses such as treatment for allergic disorders (Kitagaki, Businga & Kline 2006), immunotherapy for cancer (Mason et al. 2005; Mason et al. 2006) and as immune stimulants to improve host resistance to infection (Klinman 2006). CpG-DNA were also suggested previously as a candidate vaccine adjuvant capable of enhancing antibody responses when combined to antigens (Klinman 2006; McCluskie & Davis 1999; Weeratna et al. 2000). However, it remains unclear whether such effect was mediated by the induction of higher numbers of T<sub>FH</sub> or through direct effect on B cells via TLR activation (Mastelic et al. 2012).

CpG-DNA has strong TLR-9 mediated immunomodulatory effects developing antigen specific humoral and cellular responses and is currently undergoing clinical trials (Halperin et al. 2003; Krieg et al. 1995; Sagara et al. 2009). Several mechanisms are involved in the B cell stimulatory effects of CpG-DNA, including the cross-linking between TLR-9 and B cell antigen receptor (BCR) signaling,

rescuing B cells from apoptosis (Krieg & Yi 2000). CpG-DNA was also reported to enhance the T cell-mediated help through DC maturation and differentiation (Krieg 2002). Therefore, CpG-DNA may have a dual effect on T<sub>FH</sub> and B cell activation allowing GC B cells to provide a feedback loop signal to T<sub>FH</sub> cells. Subsequently, the T<sub>FH</sub> will help and enhance GC B cell responses. (Mastelic et al. 2012).

Accordingly, for working with T<sub>FH</sub> cells and B cells, CpG-DNA class B was chosen to be examined as a candidate adjuvant. The member of this class that has been used in this project is CpG 2006 (5'- TCG TCG TTT TGT CGT TTT GTC GTT -3' (24 mer)) which was first identified by Hartmann and Krieg (Hartmann & Krieg 2000). Since then, this member of B-class CpG-DNA has become the most widely studied B-class member.

#### **1.14 The Upper Respiratory Tract Infections**

According to WHO, acute respiratory tract infections are shown to be the leading cause of the global burden of disease worldwide particularly in low-income countries. It is estimated that more than 4 million deaths yearly; 50% of them children under five years, are due to respiratory tract infections (WHO 2004). Large numbers of infectious agents gain entry into the human host through the upper respiratory tract (URT).

Respiratory tract infections can be classified into two main groups: the upper respiratory tract infection (URTI), and the lower respiratory tract infection (LRTI). The URTI includes sinusitis, pharyngitis, tonsillitis, middle ear infections and rhinitis (Elwood & Bailey 2005; Heikkinen & Ruuskanen 2006). On the other hand, pneumonia and bronchiolitis are the main LRTIs (Chang et al. 2009). The highest

rates for developing a fatal respiratory infection can be seen in young children <5 years, elderly people >65 years and in immune-compromised patients. The main pathogens responsible for respiratory tract infections include viruses such as influenza virus, RSV (Nair et al. 2010) and parainfluenza virus type 3 (PIV-3) (Hsieh et al. 2010). In addition, several bacteria have also been known to cause acute respiratory tract infections including *Streptococcus pneumoniae* (Barnett & Klein 2011; Klugman & Feldman 2011) and *Haemophilus influenza* (Nicholson et al. 2002).

#### **1.14.1 Influenza virus**

Influenza virus is a major cause of respiratory tract infection and responsible for 3–5 million clinical infections and 250,000–500,000 fatal cases annually worldwide (Stöhr 2002). Influenza virus infection induces host immune responses that help to reduce virus replication and prevent further spread. Protection against influenza virus is mediated by neutralizing antibodies, and their induction at high and sustained titres is essential for successful vaccination (Spensieri et al. 2013). Immunological memory induced by an infection may offer protection against subsequent influenza virus infection of the same subtype, but may offer limited protection against other subtypes (Kreijtz, Fouchier & Rimmelzwaan 2011).

There are three types of influenza viruses, type A, B and C, and all have many biological properties in common (Taubenberger & Morens 2008). A key difference between these types is their host range, influenza A viruses have been isolated from many animal species, including humans, pigs, horses, marine mammals, and a wide range of domestic and wild birds (Taubenberger & Kash 2010), whereas influenza viruses of types B and C are predominantly human pathogens although have

sporadically been isolated from seals and pigs (Osterhaus et al. 2000).

Influenza virus is highly contagious and it is transmitted through airborne droplets and via the nasal mucosa. The virus infects by binding their surface glycoprotein haemagglutinin (HA) to sialic acid receptors on the host epithelia cell surface (Barbey-Martin et al. 2002). Intranasal vaccination has been proposed for a more effective and biologically relevant way of immunization against influenza. Intranasal vaccination essentially depends on the local mucosal immune tissue. Mucosal immunity in the upper respiratory tract is considered the first line of defence against a number of pathogens of both bacterial and viral origin. Human adenoids and tonsils are major components of the NALT and known to be main induction sites for both mucosal and systemic immunity against upper respiratory tract pathogens including influenza infection (Kiyono & Fukuyama 2004; Tamura & Kurata 2004; Wu & Russell 1997; Zuercher et al. 2002).

In the 1940s, inactivated influenza vaccines were first presented and they are still considered the main formulation of influenza vaccine. The aim of influenza vaccination is to offer an immunological memory protection against influenza infection. Protection following natural infection with influenza is primarily mediated by anti-HA specific antibodies in serum and mucosa, and T-cell responses associated with reduced disease severity (Brokstad et al. 2001). However, influenza vaccination is the main method of preventing influenza infection and its associated complications (Brokstad et al. 2001; Cox & Subbarao 1999).

The surface HA glycoprotein of influenza virus is a major target for antiviral activity as immune response to HA offer neutralizing antibodies following vaccination or natural infection (Wilks et al. 2012). As HA is a major virulence factor and essential

for influenza virus - host cells binding process, HA-specific neutralizing antibodies are important to prevent the virus attachment to host cell thus prevent infection (Wang et al. 2011).

## **1.15 Influenza vaccine types**

### **1.15.1 Trivalent Inactivated Influenza Vaccine (TIV; intramuscular)**

The current inactivated influenza vaccine contains three strains of influenza viruses: one influenza A (H1N1) virus, one influenza A virus (H3N2), and one influenza B virus (WHO). It is given as an injection subcutaneously or intramuscularly. In primed individuals it will induce a quick systemic humoral immune response in the blood. In serum, the influenza specific IgG antibody response is predominant after vaccination (Belshe et al. 2004; Cox & Subbarao 1999). Wrammert et al. reported that the majority of antibody secreting cells (ASC) produced in blood are IgG positive, with minor IgA and IgM positive cells in vaccinated individuals (Wrammert et al. 2008).

### **1.15.2 Live Attenuated Influenza Vaccine (LAIV; intranasal)**

Intranasal Live Attenuated Influenza Vaccine (LAIV) is a live, trivalent intranasal spray vaccine that produces the HA and NA surface antigens from a number of influenza viruses including seasonal strains such as A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B influenza strains. Studies have reported using LAIV vaccines delivered intranasally was able to induce strong immune response including induction of mucosal IgA and IgG, and serum IgG and enhanced local cytokine responses, which may provide better protection. LAIV vaccines also shown to stimulate stronger cellular immune response by inducing influenza specific

memory T cells and B cells (De Filette et al. 2006; Vajdy et al. 2007). Intranasal vaccination may offer certain advantages over intramuscular vaccination by inducing a protective local immunity preventing local spread and transmission of the virus. Intranasal vaccination with LAIV has been used successfully in several countries including USA and Canada (under the commercial name FluMist<sup>®</sup>) with good efficacy (Belshe 2004). Most recently, LAIV Intranasal vaccination has also been licensed in Europe including the UK (under the commercial name Fluenz<sup>™</sup>).

Both live attenuated vaccine and inactivated vaccines are currently in use. At present, seasonal influenza vaccination are recommended to be taken every year using TIV for all individuals in particular, children aged 6 months or older, those with a diversity of chronic illnesses and health care workers. In addition to TIV, LAIV is also recommended for healthy non-pregnant people aged 2-49 years (Osterholm et al. 2012).

Although it has been shown to be safe and effective in humans, not much research has been done to evaluate the local mucosal immunity induced by these LAIV intranasal vaccines. As these vaccines contain live attenuated viruses and administered through the nasal mucosa mimicking natural infection, it may induce an immune response resembling natural immunity. NALT components, including adenotonsillar tissues are local mucosal immune organs in the upper respiratory tract, therefore, intranasal vaccines are likely to depend on these immune tissues to induce specific immune responses.

### **1.16 Aims of the Thesis**

The aims of this PhD project include:

1. To characterise T<sub>FH</sub> cells in human NALT in children and adults,
2. To investigate the effect of CpG-DNA on T<sub>FH</sub> cells and the adjuvant effect on B cell antibody response to influenza antigen,
3. To determine the effect of T<sub>FH</sub> cells in LAIV-induced immune response to influenza virus antigen,
4. To study the effects of IL-21 and plasmacytoid DC on T<sub>FH</sub> cell mediated B cell antibody production.

# **Chapter 2**

## **Materials and Methods**

## **2.1 Subjects and Samples**

### **2.1.1 Subjects**

Samples used in this study were collected from children and adult patients undergoing adenoidectomy and/or tonsillectomy due to upper airway obstruction or tonsillitis, attending Alder Hey Children's Hospital and Royal Liverpool and Broadgreen University Hospitals. Patients previously vaccinated against influenza or who had any known immunodeficiency were excluded from the study. The study was approved by the local ethics committee (Liverpool Paediatric Research Ethics Committee) and written informed consent was obtained from each patient or their legal guardians as appropriate.

### **2.1.2 Patient Samples**

#### **2.1.2.1 Adenotonsillar Tissues**

In sterile conditions, adenoids and tonsils were kept in a 25ml universal tube, containing 5ml HANK'S balanced salt solution (Sigma Aldrich, UK) supplemented with 1% L-glutamine, streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma-Aldrich). The adenotonsillar tissues were then transported to the laboratory immediately.

#### **2.1.2.2 Peripheral Blood**

Peripheral blood samples (obtained from the patients by venous puncture) were collected into a 25ml universal tube containing Heparin as an anti-coagulant (Heparin 100µl, LEO Pharma, UK). These blood samples were then transported to the laboratory immediately.

## **2.2 Mononuclear Cell Isolation**

### **2.2.1 Adenotonsillar Mononuclear Cells (MNC)**

All cell separation procedures were processed within a class-II Bio-safety cabinet and under a negative pressure situation to avoid any probable contamination. Mononuclear cells were freshly isolated from adenoids and/or tonsils. To remove any blood, adenotonsillar tissues were rinsed with 10 ml HANK'S medium before being placed into a sterile Petri-dish. Using a sterile forceps, the tissues were minced by a scalpel to release cells into the medium. Then the cell suspension was filtered through a 70µm cell strainer (BD biosciences, USA) using a sterile Pasteur pipette. The cells were then carefully layered onto 15 ml Ficoll-Paque™ PREMIUM (GE Healthcare Life Sciences, UK) and spun at 400g for 30 min at room temperature with no brake. After centrifugation, the cloudy layer of mononuclear cells formed at the sample/Ficoll-Paque™ interface were carefully harvested using a Pasteur pipette. Then MNC were washed twice with sterile PBS solution (Sigma, UK), at 400g for 10 min with the brake. The pellet was re-suspended in 5 ml RPMI-1640 culture medium with HEPES (Sigma-Aldrich, UK) supplemented with 10% heat inactivated Foetal Bovine Serum (FBS) (Sigma), 1% L-glutamine (Sigma), streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma-Aldrich); and counted in an automated cell counter (BioRad, UK). After counting, each cell suspension was adjusted to contain  $4 \times 10^6$  cells/ml concentrations.

### **2.2.2 Peripheral Blood Mononuclear Cells (PBMC)**

Peripheral blood mononuclear cells (PBMC) were isolated from sample blood by density gradient centrifugation using Ficoll-Paque™ PREMIUM (GE Healthcare

Life Sciences, UK). Peripheral blood was carefully layered onto 10 ml Ficoll-Paque™ PREMIUM (GE Healthcare Life Sciences, UK) and spun at 400g for 30 min at room temperature with no brake. After centrifugation, the cloudy layer of mononuclear cells formed at the sample/Ficoll-Paque™ interface were carefully harvested using a Pasteur pipette. Then PBMC were washed twice with sterile PBS solution (Sigma, UK), at 400g for 10 min with the brake. The pellet was re-suspended in 1 ml RPMI-1640 culture medium with HEPES (Sigma-Aldrich, UK) supplemented with 10% heat inactivated FBS (Sigma), 1% L-glutamine (Sigma), streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma-Aldrich); and counted in an automated cell counter (BioRad, UK). Then further re suspended in culture medium and adjusted to  $4 \times 10^6$  cells/ml concentrations.

## **2.3 Influenza virus antigens and proteins**

### **2.3.1 Seasonal H1N1 (sH1N1) virus antigen**

The sH1N1 virus antigen used in this study for cell stimulation experiments was derived from a seasonal H1N1 (A/Brisbane/59/2007) strain. This influenza antigen was  $\beta$ -propiolactone inactivated, partially purified A/Brisbane/59/2007 virus antigen containing HA (83µg/ml) from National Institute for Biological Standards and Control (NIBSC, UK).

### **2.3.2 Intranasal Live Attenuated Influenza Vaccine (LAIV)**

Intranasal LAIV (FluMist formula 2009-10) is a live, trivalent intranasal spray vaccine that produces the Hemagglutinin (HA) and Neuraminidase (NA) surface antigens from a number of influenza viruses including A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B influenza strains. The vaccine viruses are

characterized being temperature sensitive, cold adapted and attenuated viruses (FluMist, BEI resources ATCC).

### **2.3.3 Recombinant HA**

The recombinant HA from a seasonal H1N1 influenza virus (A/Brisbane/59/2007) (sH1N1), contains an N-terminal part of HA with histidine Tag and produced in Sf9 insect cells using a baculovirus expression vector system. This HA was purified from cell culture supernatant by conventional chromatographic techniques (Biodefense and Emerging Infections Research Resources Repository, BEI Resources, ATCC, Manassas VA, USA).

### **2.3.4 CpG-DNA**

CpG oligodeoxynucleotides (CpG-DNA) are short single-stranded synthetic oligonucleotides containing unmethylated Cytosine-Guanine (CpG, where the p indicates the phosphodiester bond) dinucleotides also known as CpG motifs. CpG-DNA is recognized as PAMPs due to their abundance in bacterial DNA compared to mammalian DNA. CpG-DNA are recognized by TLR-9 leading to immunostimulatory effects. Three types of stimulatory CpG-DNA have been suggested based on their immunostimulatory activities named as CpG-DNA type A, B and C (Klinman 2006; McCluskie & Davis 1999). In this study type B CpG-DNA (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3' (24 mer) (CpG 2006, InvivoGen, USA) was chosen to be examined as a candidate adjuvant. CpG-DNA class B was first identified in 1995 by Krieg and colleges (Krieg et al. 1995).

## **2.4 Cell culture and stimulation by LAIV, influenza virus antigen and CpG-**

### **DNA for antibody and IL-21 production**

Adenotonsillar MNC were isolated from adenotonsillar tissues and PBMC were isolated from blood as described earlier. The cell suspension was adjusted to contain  $4 \times 10^6$  cells/ml concentrations in RPMI-1640 culture medium with HEPES (Sigma-Aldrich, UK) supplemented with 10% heat inactivated FBS (Sigma), 1% L-glutamine (Sigma), streptomycin (50 µg/ml) and penicillin (50 U/ml) (Sigma-Aldrich). 500 µl/well of cells were cultured in 48-well flat bottom culture plates (Corning Inc., USA) in the presence or absence of different stimulants.

These cells were stimulated with (1 µg/ml) sH1N1 antigen alone or in combination with (0.1 µg/ml) CpG-DNA (InvivoGen, USA) or CpG-DNA alone. Samples were also stimulated with (2 µl/ml) of LAIV alone. An unstimulated (RPMI culture medium) control was included in each experiment. To prevent fungal contamination, all cultures and medium used for antibody production throughout was treated with 2.5 µl/ml amphotericin B (Sigma). Cells then were cultured for 24 hours for cytokine production and for 14 days for antibody production in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub>. After that, cell culture supernatants were collected from each well and stored at -80°C until further analysis by ELISA.

## **2.5 Enzyme-Linked Immunosorbent Assay (ELISA)**

### **2.5.1 Measurement of HA specific antibodies from cell culture supernatants**

ELISA assay in this study was designed and developed to detect specific HA of sH1N1 IgG, IgM and IgA antibodies production in cell culture supernatants using the following protocol. Coating solution (2 µg/ml) of HA/H1N1 recombinant purified

proteins (ATTC) was prepared by adding 20µl of stock HA antigen into 10ml of PBS. The HA recombinant purified proteins were tested and optimized to reach optimal coating concentration.

#### **2.5.1.1 Measurement of HA-specific IgG antibody**

Using a 96-well flat-bottom, high binding Costar EIA plate (Corning Inc., USA) 100µl of coating solution was added per well and covered by adhesive seal then incubated overnight in 4°C. The following day, plates were washed 5 times with PBS containing 0.05% Tween-20 (Sigma-Aldrich) then plates were blotted on a paper towel. The plates were then blocked with 150µl/well blocking buffer (10% FBS in PBS) for 1 hour at room temperature. In the meantime, samples were prepared by diluting them (1:5) in blocking buffer. Diluted samples (50 µl/well) were then added in duplicate to the plate and incubated for 2 hours in room temperature.

Plates were then washed 5 times with PBS containing 0.05% Tween-20 and blotted on a paper towel. Followed by the addition of 50µl/well of alkaline phosphatase-conjugated mouse anti-human IgG (1:2000 diluted in blocking buffer) (Sigma). After 2 hours, plates were washed 5 times with PBS containing 0.05% Tween-20 and then blotted on a paper towel. By the end of the last washing cycle, the substrate was prepared by dissolving one PNitrophenyl Phosphate (PNPP) (5mg) tablet into 5ml of substrate buffer and then 50µl substrate were added to each well. Then the plates were incubated at room temperature and in dark protecting it from direct light for 30 min. Finally, the Optical Density (OD) at 405 nm was measured using a microtiter plate reader (Opsys MR, Thermo labsystems, UK) and data were analysed using DeltaSoft software (BioMetallics Inc, NJ).

### **2.5.1.2 Measurement of HA-specific IgM antibody**

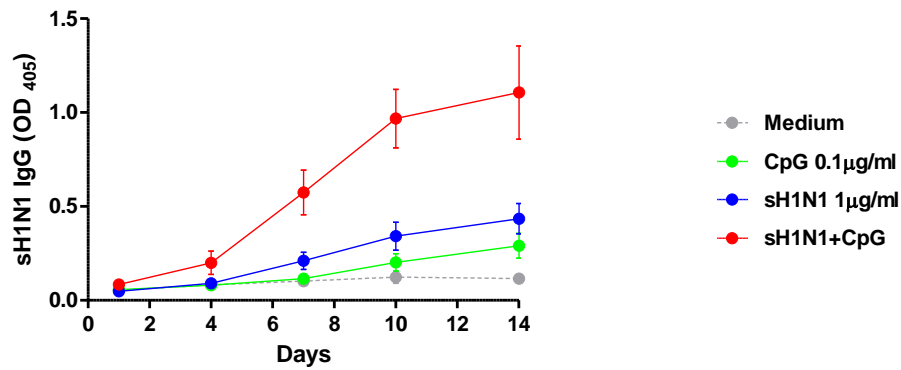
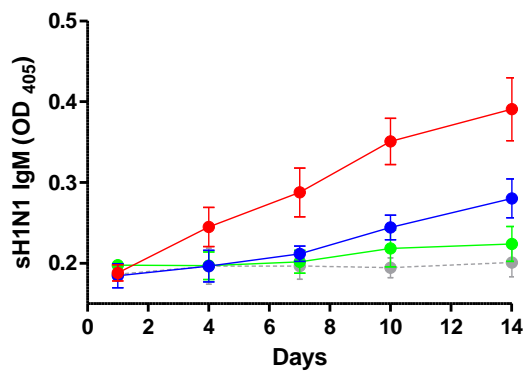
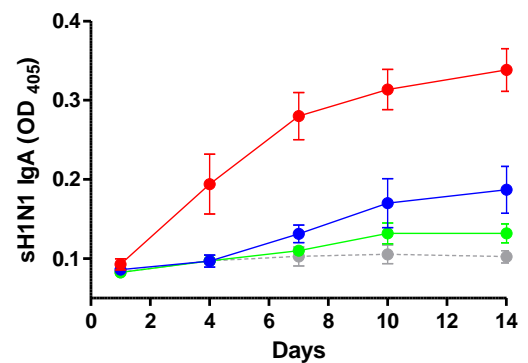
Specific HA of H1N1 IgM in cell culture supernatants were also measured by ELISA following a procedure similar to specific HA of H1N1 IgG measurement, except alkaline phosphatase-conjugated mouse anti-human IgM (1:1000 dilution) (Sigma) was used as conjugate.

### **2.5.1.3 Measurement of HA-specific IgA antibody**

Specific HA of H1N1 IgA in cell culture supernatants were also measured by ELISA following a procedure similar to specific HA of H1N1 IgG measurement, except alkaline phosphatase-conjugated mouse anti-human IgA (1:1000 dilution) (Sigma) was used as conjugate.

## **2.5.2 Time-course of HA-specific antibody production**

Time-course of antibody production for HA-specific antibodies to sH1N1 in adenotonsillar MNC following sH1N1 virus antigen and CpG-DNA stimulation was determined. MNC culture supernatants were collected at different time points (1, 4, 7, 10 and 14 days) following stimulation with sH1N1 virus antigen with or without CpG-DNA in 5 patients. Production of HA-specific sH1N1 IgG, IgM and IgA was seen to rise 4 days after stimulation with sH1N1 antigen combined with CpG-DNA. The time-course of antibody production was shown in (Figure 2.1). The highest level of HA-specific sH1N1 IgG, IgM and IgA was observed at day 14 samples. In subsequent experiments, cell culture supernatants were collected at day 14 of stimulation for measurement of specific HA-specific sH1N1 antibody production by ELISA.

**A****B****C**

**Figure 2.1. Time course of HA-specific sH1N1 IgG, IgM and IgA production**

Adenotonsillar MNC were stimulated with sH1N1 virus antigen with or without CpG-DNA and CpG-DNA alone. Cell culture supernatants were collected at 1, 4, 7, 10 and 14 days and analysed for anti-sH1N1 HA IgG (A), IgM (B) and IgA (C) antibody production using ELISA. The value represents the mean OD read at 405 nm  $\pm$  SEM of 5 patient samples.

## **2.6 Enzyme Linked Immuno Spot Assay (ELISpot)**

### **2.6.1 Principle of the test**

ELISpot assay is a sensitive immunoassay which measures the frequency of Antibody Secreting Cells (ASC) at a single-cell level. In this assay, adenotonsillar MNC cells were cultured on a solid surface lined with a polyvinylidene difluoride (PVDF) and pre-coated with a specific antigen of interest. Secreted antibodies which are produced by B lymphocytes will be captured by the specific antigen on the surface. These secreted antibodies are detected using a similar procedure like ELISA by using a secondary antibody-enzyme conjugate and its reaction with the substrate, resulting in the development of visible spots on the surface where each spot represents an ASC. In this study, ELISpot assay was used to measure the frequencies of antibody-producing cells specific to HA of sH1N1 (A/Brisbane/59/2007).

### **2.6.2 Culture of adenotonsillar MNC**

Adenotonsillar MNC cells were resuspended in RPMI-1640 medium ( $4 \times 10^6/\text{ml}$ ) and stimulated with ( $1 \mu\text{g}/\text{ml}$ ) sH1N1 (A/Brisbane/59/2007) (NIBSC, UK) antigen alone or in combination with ( $0.1 \mu\text{g}/\text{ml}$ ) CpG-DNA (InvivoGen, USA) and CpG-DNA alone. Samples were also stimulated with ( $2 \mu\text{l}/\text{ml}$ ) of LAIV alone. An unstimulated control (RPMI culture medium) was included in each experiment. The cells were incubated in a 24-well cell culture plate (Corning Inc., USA) at  $37^\circ\text{C}$ , in 5%  $\text{CO}_2$  for 5 days. Thereafter, the cells were harvested and washed with sterile PBS with 1% BSA followed by centrifugation at  $400 \times g$  for 10 min. The pellets were then resuspended in RPMI-1640 medium to a final cell concentration of  $4 \times 10^6/\text{ml}$ .

### **2.6.3 Antigen coating of ELISpot plate**

A filtered 96-well ELISpot plate (Millipore, UK) was coated on the day before cell harvesting. Immediately before coating, the wells were pre-wetted with 15µl of 35% ethanol for 1 min, then washed 3 times with 150µl of PBS containing 0.05% Tween-20. For detecting sH1N1-HA specific memory B cells, the ELISpot plate was coated with (2µg/ml) of HA/sH1N1 (A/Brisbane/59/2007) recombinant purified proteins (ATTC, Manassas VA, USA) and incubated overnight at 4°C. For enumeration of total IgG spots as positive controls, some wells were also coated with a Fab specific anti-human IgG (Sigma) antibody in 1:1000 dilutions.

### **2.6.4 Incubation of cells in the ELISpot plate**

On the following morning, the plate was washed 3 times with PBS containing 0.05% Tween-20. Then the plate was blocked with 150µl of RPMI-1640 media supplemented with 10% FBS for an hour at room temperature. In the meantime, adenotonsillar MNC were harvested from the incubation as described earlier. 100µl of cell suspension was added into each of the designated wells in triplicate. Cell concentration used for different antigens were:  $4 \times 10^6$ /ml for antigen and  $4 \times 10^5$ /ml for total IgG. RPMI-1640 media with no cells was added into the wells designated as negative controls (at least one well for each antigen). The plate was then incubated overnight at 37°C in 5% CO<sub>2</sub> incubator.

### **2.6.5 ELISpot assay procedure**

The following day, the plate was washed 3 times with PBS containing 0.05% Tween-20 and 50µl of goat anti-human IgG (H+L) biotin (1:2,000 dilutions) (Invitrogen, USA) was added to the appropriate wells. After 30 min incubation in

room temperature, the plate was washed 3 times with PBS containing 0.05% Tween-20, followed by the addition of 50µl of Horseradish peroxidase Avidin D conjugate (1:10,000 dilutions) (Vector laboratories Inc., USA). After 30 min incubation in room temperature, the plate was washed 3 times with PBS containing 0.05% Tween-20. During the last washing cycle, the substrate was prepared by adding 0.5 ml 3-amino-9 ethylcarbazole into 9.5 ml acetate buffer and addition of 25µl H<sub>2</sub>O<sub>2</sub> immediately before use (Sigma-Aldrich). After adding freshly prepared AEC substrate (50µl/well), the plate was incubated in dark for 15-20 min at room temperature. The reaction was stopped by washing the plate with running cold tap water. Finally, the plate was blotted gently with adsorbent paper to remove the excess water and allowed to dry for 2-3 hours in room temperature.

#### **2.6.6 Counting of ELISpots**

The spots were imaged by Chemi-doc XRS system, and analysed with NIST's Integrated Colony Enumerator software (Version 1.2.1, National Institute of Standards and Technology, USA). The result was expressed as number of ASC/million of lymphocytes. For antigen-specific HA/sH1N1 ASC, the average spot counts were multiplied by 2.5 to get this result. This calculation was obtained by dividing  $1 \times 10^6$  (one million) by  $4 \times 10^5$  that was the number of cells in 100µl of ( $4 \times 10^6$ ) cell suspension, which was put into each well. For total IgG, the result was obtained by multiplying the spot counts with 50.

#### **2.7 Measurement of IL-21 in cell culture supernatants**

*In vitro* IL-21 production in adenotonsillar MNC and PBMC cultures was measured with human IL-21 ELISA Ready-Set-Go® set (eBioscience, UK), following

manufacturer's instructions. 96-well flat-bottom, high binding Costar EIA plate (Corning Inc., USA) were coated with the capture antibody which was prepared by adding 48µl of purified anti-human IL-21 into 12ml coating buffer (1:250 dilutions). 100µl of the capture antibody solution was added into each well of the plates and incubated overnight at 4°C. In the following day, the plate was washed 5 times with PBS containing 0.05% Tween-20 (Sigma-Aldrich) then blotted on a paper towel.

The plate was then blocked with 1x assay diluent (200µl/well) for 1 hour at room temperature. Then the plate was washed 5 times with PBS containing 0.05% Tween-20. In the meantime, standards and samples were prepared by diluting them in 1x assay diluent. For standards, the top concentration (1000pg/ml) was prepared by adding 10µl of human IL-21 recombinant protein into 10ml of 1x assay diluent. The samples from cell culture supernatants were prepared by diluting them 1:10 in 1x assay diluent. Then, 100µl/well of standards and samples were added to the appropriate wells. A total of 8 standards were prepared by 2-fold serial dilutions from the top standard (1000pg/ml). For maximal sensitivity, the plates were then incubated overnight at 4°C.

In the following morning, the plate was washed 5 times with PBS containing 0.05% Tween-20. In the meantime, detection antibody was prepared by adding 48µl of purified anti-human IL-21 Biotin into 12ml 1x assay diluent (1:250 dilutions). After that, 100µl of detection antibody was added to each well. Following 1 hour incubation in room temperature, the plate was washed 5 times with PBS containing 0.05% Tween-20. Thereafter, 100µl of Avidin-HRP (diluted 1:250) was added to each well; and incubated at for 30 min at room temperature. After washing 7 times with PBS containing 0.05% Tween-20, 100µl of TMB substrate solution was added

to each well, and incubated for 15 min at room temperature in dark. The reaction was then stopped by adding 50µl of stop solution (1M H<sub>2</sub>SO<sub>4</sub>) to each well. Finally, the plate was read at 450nm, and the concentration (pg/ml) of each sample was calculated against the standard curve, with the help of DeltaSoft software (BioMetallics Inc, NJ).

## **2.8 Flow Cytometric Analysis of Cells**

### **2.8.1 Principle of the test**

Flow cytometry is widely used as an important tool in both diagnostic and research laboratories. This device is able to analyse the physical and optical features of the fluorochrome-labelled cells passing through a focused laser beam. In this process, cells passing through the laser beam will scatter light; resulting in what is known as forward scatter (FSC) and side scatter (SSC). FSC is usually used as an indicator of the cell size allowing the differentiation of different cell types such as erythrocytes from lymphocytes and monocytes. The FSC is also been used to distinguish dead cells with low FSC distribution from the viable cells.

On the other hand, SSC is an indicator of cells density and internal complexity or granularity, distributing the more granular cells to be more scattered. A combination of FSC and SSC characteristically distribute different subsets of cells in sample containing a mixed population such as blood, bone marrow, spleen, adenotonsillar, and LN single cell preparations.

In this study, a BD FACS Calibur (BD Biosciences) has been used to carry out flow cytometry analyses. Different lymphocyte subsets were defined and characterized using multiple parameters, including FSC and SSC based gating. Fluorescence

emissions of different fluorochromes were measured at different wavelengths; FL1 was used for FITC and Alexafluor488 at 519nm, FL2 for PE at 578nm, FL3 for PerCPCy5.5, PECy5 and PECy7 at 695nm and FL4 for APC at 660nm. The data were acquired using Cell Quest software (BD Biosciences) and analysed with WinMDI 2.9 software.

## **2.8.2 Cell culture and stimulation by influenza virus antigen, LAIV and CpG-DNA for FACS analysis**

Adenotonsillar MNC were isolated from adenotonsillar tissues and PBMC were isolated from blood as described earlier. The cell suspension was adjusted to contain  $4 \times 10^6$  cells/ml concentrations in RPMI-1640 culture medium with HEPES (Sigma-Aldrich, UK) supplemented with 10% heat inactivated FBS (Sigma), 1% L-glutamine (Sigma), streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma-Aldrich). 500 µl/well of cells were cultured in 48-well flat bottom culture plates (Corning Inc., USA) in the presence or absence of different stimulants. These cells were stimulated with (1µg/ml) sH1N1 antigen alone or in combination with (0.1µg/ml) CpG-DNA (InvivoGen, USA) and CpG-DNA alone. Samples were also stimulated with (2µl/ml) of LAIV alone. An unstimulated (RPMI culture medium) control was included in each experiment. Unfractionated adenotonsillar MNC were cultured for 3 days and CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC were cultured for 7 days in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub>. After that, cells were harvested for staining.

### 2.8.3 Cell staining for surface phenotyping for FACS analysis

Adenotonsillar MNC and PBMC were harvested and washed twice with 0.02% BSA-PBS buffer (Sigma-Aldrich), by centrifuging at 400xg for 5 min. The pellets were resuspended in 50µl of 0.02% BSA-PBS and fluorochrome-labelled anti-human antibodies were added depending on the phenotype. Anti-human antibodies used for flow cytometric analysis are described in the table below (**Table 2.1**). For T<sub>FH</sub> cell identification, adenotonsillar MNC and PBMC were stained with anti-human-CD3, -CD4, -CXCR5 and -ICOS antibodies. In some samples, PD-1 and CCR7 were also stained to confirm the T<sub>FH</sub> cell phenotype. For GC B cells identification, adenotonsillar MNC were stained with anti-human-CD19, -CD38 and -IgD antibodies. For pDC staining, purified pDC were stained with anti-human-CD123 and -CD85g (ILT7), a unique marker for pDC and not found on other DC types (Cho et al. 2008). All cell staining was performed in Falcon 5ml round bottom tubes (BD bioscience). Cells were kept at room temperature for 30 minutes in the dark then washed twice with 2ml of 0.02% BSA-PBS (500xg for 8 min) to remove unbound antibodies. Finally, the pellets were resuspended in 300µl of 0.02% BSA-PBS; and immediately acquired/analysed in the BD FACS Calibur (BD bioscience) or fixed and permeabilized if needed for intracellular staining.

**Table 2.1. Antibodies used for flow cytometry**

Antibody	Fluorochrome	Clone	Species and Isotype	Company
<b>CD3</b>	APC	HIT3a	Mouse IgG2a	BD Biosciences
<b>CD4</b>	PE	RPA-T4	Mouse IgG1	BD Bioscience
<b>CD4</b>	PE-Cy5	RPA-T4	Mouse IgG1	BD Bioscience
<b>CD4</b>	PE-Cy7	SK3	Mouse IgG1	BD Bioscience
<b>CD19</b>	PE	H1B19	Mouse IgG1	BD Bioscience
<b>CD19</b>	FITC	H1B19	Mouse IgG1	BD Bioscience
<b>CD38</b>	PE	H1T2	Mouse IgG1	BD Bioscience
<b>CD45RO</b>	FITC	UCHL1	Mouse IgG2a	BD Bioscience
<b>CD45RA</b>	PE	HI100	Mouse IgG2b	BD Bioscience
<b>CD85g (ILT7)</b>	PE	17G10.2	Mouse IgG1	eBioscience
<b>CD123</b>	PE-Cy5	9F5	Mouse IgG1	BD Bioscience
<b>CXCR5</b>	Alexa Fluor® 488	RF8B2	Rat IgG2b	BD Bioscience
<b>CXCR5</b>	PE	MU5UBEE	Mouse IgG2a	eBioscience
<b>CXCR5</b>	Biotin	RF8B2	Rat IgG2b	BD Bioscience
<b>CXCR5</b>	FITC	51505	Mouse IgG2b	R&D Systems
<b>PD1</b>	PE	MIH4	Mouse IgG1	BD Bioscience
<b>ICOS</b>	APC	ISA-3	Mouse IgG1	eBioscience
<b>ICOS</b>	PE	DX29	Mouse IgG1	BD Bioscience
<b>BCL6</b>	PE	K112-91	Mouse IgG1	BD Bioscience
<b>IL-4</b>	PE	8D4-8	Mouse IgG1	BD Bioscience
<b>IL-10</b>	PE	JES3-9D7	Rat IgG1	eBioscience
<b>IL-21</b>	APC	3A3-N2	Mouse IgG1	Biolegend
<b>IL-21</b>	PE	3A3-N2	Mouse IgG1	eBioscience
<b>IgD</b>	PE-Cy7	IA6-2	Mouse IgG2a	BD Bioscience
<b>TLR9</b>	PE	Eb72-1665	Rat IgG2a	eBioscience
<b>CCR7</b>	FITC	3D12	Rat IgG2a	BD Biosciences

## **2.9 T cell Proliferation Assay**

### **2.9.1 Labelling of adenotonsillar MNC with CFSE**

Adenotonsillar MNC were washed with PBS and pelleted at 400xg for 10 min and the cell pellet was dissolved in 3ml sterile PBS. Just before use, a working concentration of CFSE was prepared by adding 5µl of CFSE stock solution (5µM, stored at -20°C) to 10ml of sterile PBS. Next, 3 ml of this CFSE solution (50µM) was added to the cell suspension and mixed by vortexing thoroughly. After incubating for 8 min at 37°C, 15ml of ice-cold RPMI-1640 media was added. Then, cells were spun at 400xg for 10 min; and the pellet was dissolved in RPMI-1640 culture medium with HEPES (Sigma-Aldrich, UK) supplemented with 10% heat inactivated FBS (Sigma), 1% L-glutamine (Sigma), streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma-Aldrich).

Cells were then adjusted to  $4 \times 10^6$  cells/ml suspensions. 500µl of these cell suspensions was stimulated with (1µg/ml) sH1N1 antigen alone or in combination with (0.1µg/ml) CpG-DNA (InvivoGen, USA) and CpG-DNA alone. Samples were also stimulated with (2µl/ml) of LAIV alone. An unstimulated (RPMI culture medium) control was included in each experiment and a positive control stimulated with 0.1µg/ml Staphylococcal enterotoxin B (SEB) (Sigma). The cells were then added into a 96-well cell culture plate (Corning); and incubated at 37°C in 5% CO<sub>2</sub> for 5 days. After incubation, cells were harvested for T cell proliferation assay and analysed by flow cytometry using a BD FACS Calibur (BD bioscience).

### **2.9.2 Staining of the CFSE labelled cells for FACS analysis**

At the end of incubation, the cells were harvested and washed with 0.02% BSA-PBS buffer (Sigma-Aldrich), by centrifuging at 500xg for 8 min. The pellets were resuspended in 50µl of 0.02% BSA-PBS and anti-Human CD4-PE-Cy5 (BD bioscience), anti-Human CXCR5-PE (eBioscience) and anti-Human ICOS-APC (eBioscience) were added to stain CD4<sup>+</sup> CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells (T<sub>FH</sub> cells). After incubated in dark at 4°C for 30 min, the tubes were washed (500xg for 8 min) with 1ml of 0.02% BSA-PBS. The pellets were resuspended in 300µl of 0.02% BSA-PBS; and immediately acquired/analysed in the BD FACS Calibur (BD bioscience). T<sub>FH</sub> cell proliferation index was examined by gating from T<sub>FH</sub> cells (CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells).

## **2.10 Intracellular staining for BCL-6, TLR-9 and cytokines**

### **2.10.1 Cell stimulation**

Cell suspension (4x10<sup>6</sup>/ml) were cultured in 48-well flat bottom culture plates (500 µl/well) (Corning Inc., USA) in the presence or absence of different stimulants. These cells were stimulated with (1µg/ml) sH1N1 antigen alone or in combination with (0.1µg/ml) CpG-DNA (InvivoGen, USA) and CpG-DNA alone. Samples were also stimulated with (2µl/ml) of LAIV alone. For cytokine analysis, stimulated cells were incubated for overnight; thereafter, 10µg/ml brefeldin A (Sigma) was added to each well and further incubated for 4 hours at 37°C in 5% CO<sub>2</sub>. Cells in one well was also stimulated with Ionomycin (1µg/ml) and PMA (20ng/ml) for the last 4 hours as a positive control. After that, cells were harvested for staining and cell culture supernatants were collected from each well and stored at -80°C until further analysis by ELISA.

### **2.10.2 Intracellular cytokine and BCL-6 staining**

For BCL-6 analysis, either freshly isolated cells or stimulated cells incubated for 3 days (unfractionated) and 7 days (for CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC) were used for staining. For cytokine analysis, either freshly isolated cells or stimulated cells incubated for overnight were used for staining. Cells were harvested and washed twice with FACS staining buffer (0.02% PBS-BSA) (500xg for 5 min). Firstly, cells were stained for surface markers such as CD4, CXCR5 and ICOS in a same procedure described earlier. Thereafter, cells were washed (500xg for 5 min) and the pellets were resuspended and fixed with 100µl intracellular fixation buffer (eBioscience). After incubation in dark at room temperature for 20 min, cells were washed twice with 2 ml 1x permeabilization buffer (eBioscience) (500xg for 5 min); and the pellets were resuspended with 100µl of 1x permeabilization buffer (eBioscience). Then the proper amount of fluorochrome-labeled antibody (see Table 2.1) was added to each samples; and incubated in the dark at room temperature for 30 min. After that, 2ml of 1x permeabilization buffer was added to each tube. After washing twice (500xg for 5 min), the stained cells were resuspended in 400µl of FACS staining buffer; and immediately acquired/analysed by flow cytometry.

### **2.10.3 Intracellular TLR-9 staining**

Freshly purified T<sub>FH</sub> cells, non-T<sub>FH</sub> cells, B cells and pDC were harvested and washed twice with FACS staining buffer (0.02% PBS-BSA) by centrifugation (500xg for 5 min). Cells were then stained for surface staining markers in a same procedure described earlier. Thereafter, the cells were washed (500xg for 5 min) and the pellets were resuspended and fixed with 100µl intracellular fixation buffer (eBioscience). After incubation in dark at room temperature for 20 min, cells were

washed twice with 2 ml 1x permeabilization buffer (eBioscience) (500xg for 5 min); and the pellets were resuspended with 100µl of 1x permeabilization buffer (eBioscience). Then 2µg/ml of anti-human-TLR9-PE (eBioscience) was added to each sample; and incubated in the dark at room temperature for 30 min. After that, 2ml of 1x permeabilization buffer was added to each tube. After washing twice (500xg for 5 min), the stained cells were resuspended in 400µl of FACS staining buffer; and immediately analysed by flow cytometry.

## **2.11 Anti-IL-21R blocking experiments**

Adenotonsillar MNC were isolated from adenotonsillar tissues as described earlier. The cell suspension was adjusted to contain  $4 \times 10^5$  cells/ml concentrations supplemented with 10% heat inactivated FBS (Sigma), 1% L-glutamine (Sigma), streptomycin (50µg/ml) and penicillin (50U/ml). Cells were cultured (250 µl/well) in 96-well round bottom plate (Corning Inc., USA) in the presence or absence of different stimulants with or without the recombinant human anti-IL-21R Fc blocking antibody ( $\alpha$ -IL-21R) (R&D systems, UK). Firstly, 10µg/ml  $\alpha$ -IL-21R was added to the allocated wells and incubated for 1 hour at 37°C in a 5% CO<sub>2</sub> incubator. These cells were then stimulated with (0.5µg/ml) sH1N1 antigen in combination with (0.05µg/ml) CpG-DNA (InvivoGen, USA). In some experiments, cells were also stimulated with (0.5µl/ml) of LAIV. An unstimulated medium control was included in each experiment. Cells were then cultured at 37°C, 5% CO<sub>2</sub> for 3 days for T<sub>FH</sub> cell measurement by FACS and 10 days for antibody production. After 3 days, cells were harvested and washed twice with FACS staining buffer (0.02% PBS-BSA) by centrifugation (500xg for 5 min). Next, cells were stained for surface staining markers such as CD4, CXCR5 and ICOS in a same procedure described earlier.

Furthermore, after 10 days incubation, cell culture supernatants were collected from each well and stored at -80°C until further analysis by ELISA to measure the HA-specific IgM and IgA antibodies to sH1N1 virus.

## **2.12 CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC**

To evaluate the proliferative and cytokine production response of CD45RO<sup>-</sup> naïve T cells, effector and memory (CD45RO<sup>+</sup>) T cells were depleted from the adenotonsillar MNC with the help of magnetic beads (Miltenyi Biotech, Germany), magnetic column (Miltenyi Biotech), and a MACS magnet (Miltenyi Biotech).

### **2.12.1 Principle of the MACS separation**

Firstly, the cells to be depleted (CD45RO<sup>+</sup>) are labelled with the specific magnetic micro-beads labelled with anti-CD45RO antibody. Thereafter, the cell suspension is allowed to pass through a MACS LD column, placed within the magnetic field of a MACS separator. The magnetically labelled cells (CD45RO<sup>+</sup> cells) are retained within the column, and the unlabelled cells (CD45RO<sup>-</sup> cells) pass through the column and collected in a tube placed underneath it. This process is known as negative selection.

### **2.12.2 Magnetic labelling and depletion**

Approximately, 50x10<sup>6</sup> cells/ml adenotonsillar MNC were resuspended in 400µl (80µl/10<sup>7</sup>cells) of cold MACS depletion buffer (0.5% BSA in PBS); and 100µl (20µl/10<sup>7</sup>cells) of CD45RO magnetic beads (Miltenyi Biotech) was added into them. After incubation for 15 min at 4°C, cells were washed with 10ml of MACS depletion buffer (400xg for 10 min). The cell pellet was resuspended in 250µl (50µl/10<sup>7</sup>cells)

of MACS depletion buffer; and magnetic separation was carried out in a class-II biosafety cabinet. The MACS LD column (Miltenyi Biotech) was placed in the magnetic field of MACS Separator and rinsed with 3 ml of MACS depletion buffer. Then the cell suspension was added to the column and allowed to pass through. The naive ( $CD45RO^+$  depleted) T cells obtained by this process were collected in a new tube (purity >98%) then resuspended into RPMI medium to a concentration of  $4 \times 10^6$  cells/ml. The depletion of  $CD45RO^+$  cells in adenotonsillar MNC removed the majority of  $T_{FH}$  cells.

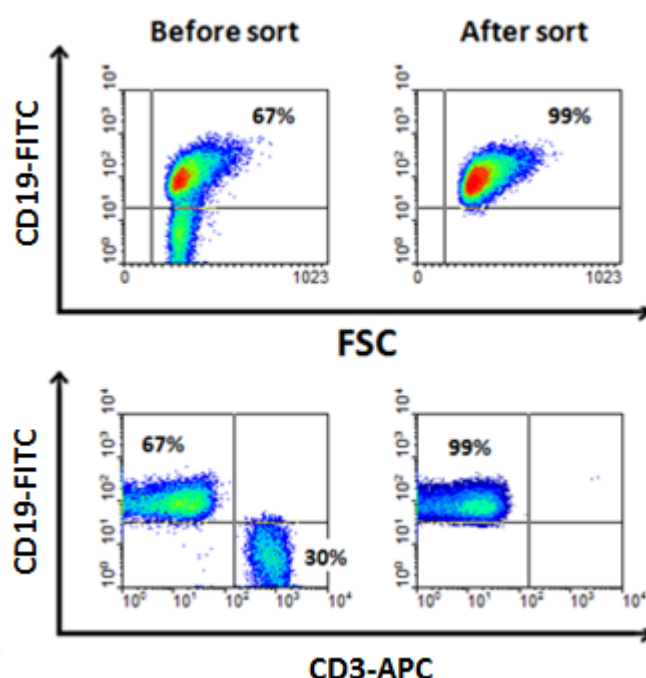
## **2.13 Cell sorting for co-culture experiments**

For co-culture experiments, cells were isolated by magnetic cell sorting using EasySep™ magnetic technology (STEMCELL Technologies Inc.). Adenotonsillar B cells were purified by negative selection using EasySep™ human B cell enrichment kit (purity 99%). Adenotonsillar  $CD4^+$  T cells were also purified by negative selection using EasySep™ human  $CD4^+$  T cell enrichment kit (purity >98%) followed by the isolation of  $CD4^+ CXCR5^{hi}$  cells by positive selection using biotin anti-human CXCR5 antibody (BD bioscience) and EasySep™ biotin selection kit (STEMCELL Technologies Inc.) (purity >90%). Adenotonsillar Plasmacytoid DC (pDC) were also negatively selected using EasySep™ human plasmacytoid DC enrichment kit (purity 96%) as described below.

### **2.13.1 Isolation of B cells from adenotonsillar MNC**

Using sterile 5ml round bottom tubes (BD bioscience),  $50 \times 10^6$  cells/ml of freshly isolated adenotonsillar MNC were resuspended in 1ml of EasySep™ buffer (STEMCELL Technologies Inc.). Then, 50 $\mu$ l/ml of EasySep™ human B cell

enrichment cocktail were added to the cells. After incubating for 10 min in room temperature, 75µl/ml of EasySep™ D magnetic particles were added to the mixture and incubated for 5 min at room temperature. To ensure that the magnetic particles were in a uniform suspension with no visible aggregates they were vortexed for 30 seconds. After that, 1ml of EasySep™ buffer was added to the tube and gently mixed by pipetting up and down 2-3 times. The tube was then placed into the EasySep™ magnet (STEMCELL Technologies Inc.) and incubated for 5 min in room temperature. Finally, the EasySep™ magnet with the tube inserted in it was picked up carefully and in one continuous motion inverted to pour off the desired fraction of B cells into a new 5ml round bottom tube (BD bioscience). The isolated B cells (99% purity) in the new tube are now ready to be used for the co-culture experiments and the magnetically labelled unwanted cells remained bounded inside the original tube, held by the magnetic field from the EasySep™ magnet. (Figure 2.2)

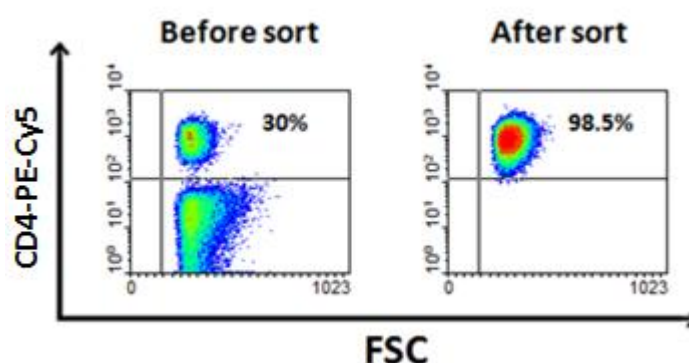


**Figure 2.2. Flow cytometric purity analysis of isolated B cells**

B cells were purified from freshly isolated adenotonsillar MNC using the EasySep™ magnetic cell sorting system resulting in 99% purity.

### 2.13.2 Isolation of CD4<sup>+</sup> T cells from adenotonsillar MNC

Using sterile 5ml round bottom tubes, 50x10<sup>6</sup> cells/ml of freshly isolated adenotonsillar MNC were resuspended in 1ml of EasySep™ buffer. Then, 50µl/ml of EasySep™ human CD4<sup>+</sup> T cell enrichment cocktail were added to the cells. After incubating for 10 min in room temperature, 100µl/ml of EasySep™ D magnetic particles were added to the mixture and incubated for 5 min at room temperature. To ensure that the magnetic particles were in a uniform suspension with no visible aggregates they were vortexed for 30 seconds. After that, 1ml of EasySep™ buffer was added to the tube and gently mixed by pipetting up and down 2-3 times. The tube was then placed into the EasySep™ magnet and incubated for 5 min in room temperature. Finally, the EasySep™ magnet with the tube inserted in it was picked up carefully and in one continuous motion inverted to pour off the desired fraction of CD4<sup>+</sup> T cells into a new 5ml round bottom tube. The isolated CD4<sup>+</sup> T cells (98.5% purity) in the new tube are now ready for the CXCR5 isolation and the magnetically labelled unwanted cells remained bounded inside the original tube, held by the magnetic field from the EasySep™ magnet. (Figure 2.3)

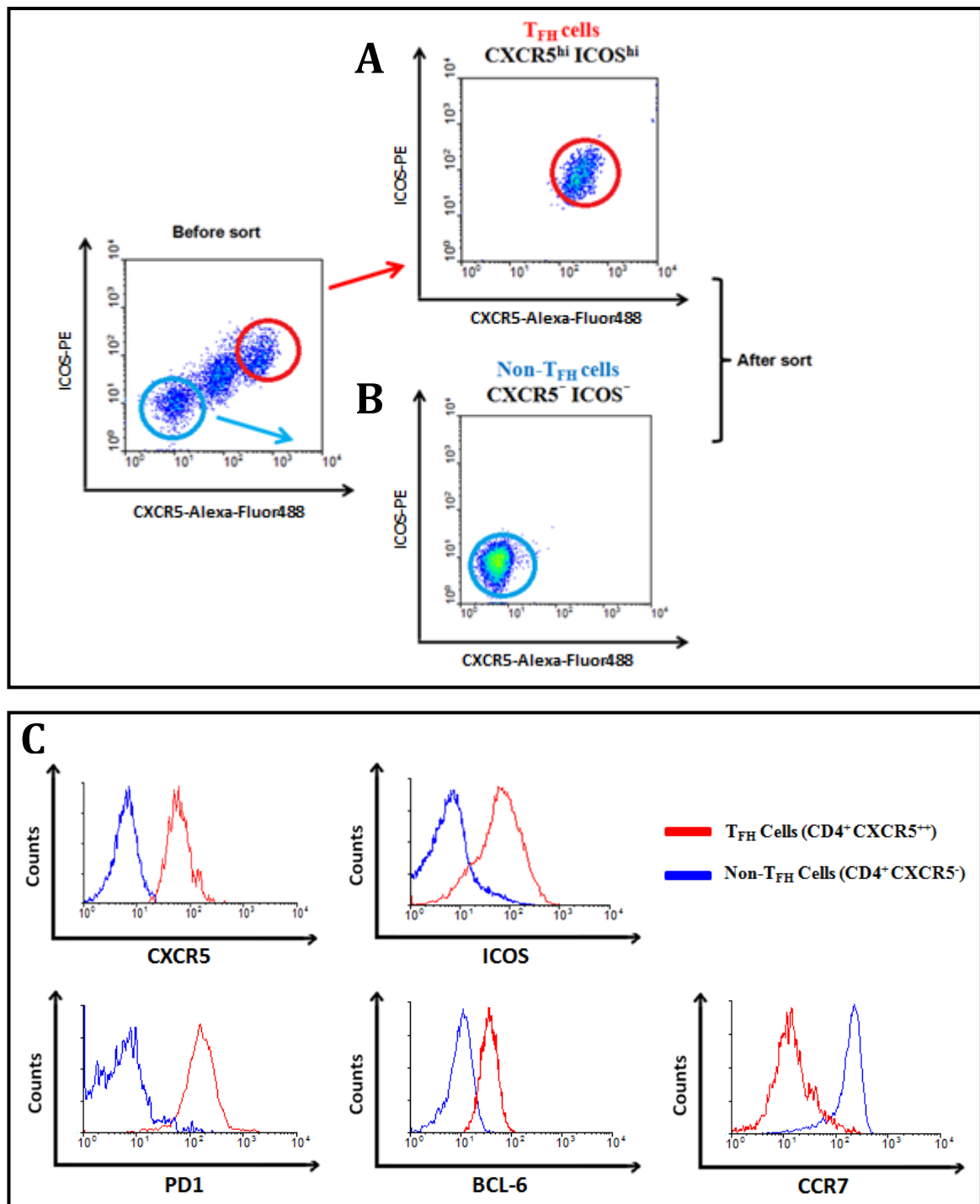


**Figure 2.3. Flow cytometric purity analysis of isolated adenotonsillar CD4<sup>+</sup> T cells**

CD4<sup>+</sup> T cells were purified from freshly isolated adenotonsillar MNC using the EasySep™ magnetic cell sorting system resulting in 98.5% purity.

### 2.13.3 Isolation of T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>hi</sup>) from adenotonsillar MNC

Using sterile 5ml round bottom tubes (BD bioscience), 50x10<sup>6</sup> cells/ml of purified CD4<sup>+</sup> T cells were resuspended in 400μl of EasySep™ buffer. For T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>hi</sup>) purification, CD4<sup>+</sup> T cells were first labelled with biotin anti-human CXCR5 antibody (10μl/10<sup>6</sup>) by incubation at room temperature for 30 min. The cells were washed with 2ml EasySep™ buffer and pelleted by 500xg for 5 min. Cell pellet was then resuspended in 1ml EasySep™ buffer and 50μl/ml of EasySep™ biotin selection cocktail were added to the cells. After incubating for 15 min in room temperature, 50μl/ml of EasySep™ magnetic nanoparticles were added to the mixture and incubated for 10 min at room temperature. After that, 2ml of EasySep™ buffer was added to the tube and gently mixed by pipetting up and down 2-3 times. The tube was then placed into the EasySep™ magnet and incubated for 5 min in room temperature. Then, the EasySep™ magnet with the tube inserted in it was picked up carefully and in one continuous motion inverted to pour off the negative unwanted cells. The isolated CD4<sup>+</sup> CXCR5<sup>hi</sup> T cells (T<sub>FH</sub> cells) are the magnetically labelled cells and remained bound inside the original tube, held by the magnetic field from the EasySep™ magnet. To increase the purity of T<sub>FH</sub> cells, the final step was repeated three more times by adding 2 ml EasySep™ buffer to the tube and placing them in the EasySep™ magnet for 5 min and discarding the unwanted fraction. For non-T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>-</sup> T cells) purification, the same procedure was used, however, higher concentration of biotin anti-human CXCR5 antibody (20μl/10<sup>6</sup>), biotin selection cocktail (100μl/ml) and magnetic nanoparticles (100μl/ml) were used to remove all CXCR5<sup>+</sup> cells (CXCR5<sup>int.</sup> and CXCR5<sup>hi</sup>). Finally, the negatively selected cells (CD4<sup>+</sup> CXCR5<sup>-</sup> T cells) were collected into a new 5ml round bottom tube. (Figure 2.4)



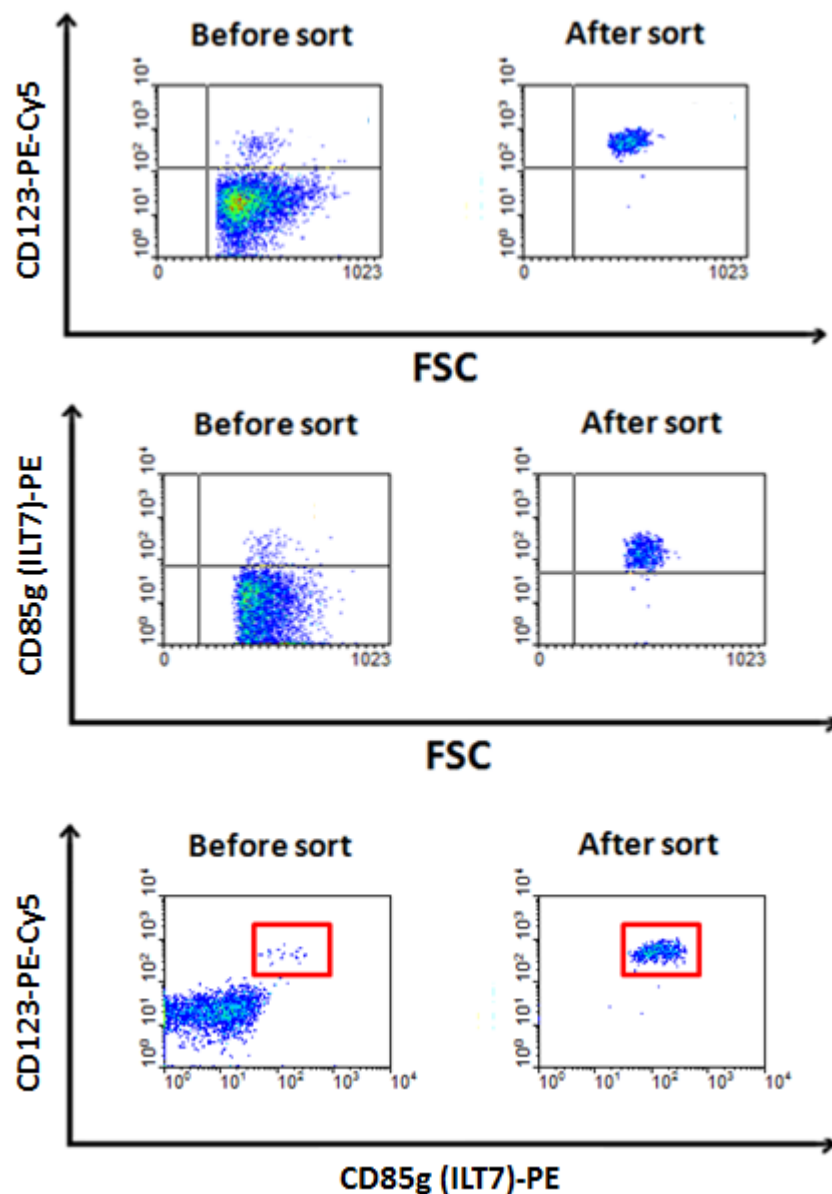
**Figure 2.4. Flow cytometric purity analysis of isolated T<sub>FH</sub> cells and non-T<sub>FH</sub> cells**

T<sub>FH</sub> cells (CD4<sup>+</sup>CXCR5<sup>hi</sup> T cells) and non-T<sub>FH</sub> cells (CD4<sup>+</sup>CXCR5<sup>-</sup> T cells) were purified from freshly isolated CD4<sup>+</sup> T cell population using the EasySep™ magnetic cell sorting system. CXCR5<sup>hi</sup> T<sub>FH</sub> cells (**A**) were positively selected using optimised amount of magnetic beads (>90% purity). CXCR5<sup>-</sup> Non-T<sub>FH</sub> cells (**B**) were negatively selected using optimised quantity of magnetic beads (98.5% purity). (**C**) Representative phenotypic histogram analysis of purified T<sub>FH</sub> cells (red line) and non-T<sub>FH</sub> cells (blue line), showing the difference in expression of T<sub>FH</sub> cells associated markers: CXCR5, ICOS, PD-1, BCL-6 and CCR7.

#### **2.13.4 Isolation of Plasmacytoid DC cells (pDC) from adenotonsillar MNC**

Using sterile 5ml round bottom tubes (BD bioscience),  $50 \times 10^6$  cells/ml of freshly isolated adenotonsillar MNC were resuspended in 1ml of EasySep™ buffer (STEMCELL Technologies Inc.). Then, 50µl/ml of EasySep™ human Plasmacytoid DC enrichment cocktail (STEMCELL Technologies Inc.) were added to the cells and incubated for 30 min in room temperature. Next, 200µl/ml of EasySep™ D magnetic particles (STEMCELL Technologies Inc.) were added to the mixture and incubated for 10 min at room temperature. To ensure that the magnetic particles were in a uniform suspension with no visible aggregates they were vortexed for 30 seconds. After that, 1ml of EasySep™ buffer was added to the tube and gently mixed by pipetting up and down 2-3 times. The tube was then placed into the EasySep™ magnet (STEMCELL Technologies Inc.) and incubated for 5 min in room temperature. Then, the EasySep™ magnet with the tube inserted in it was picked up carefully and in one continuous motion inverted to pour off the desired fraction of pDC into a new 5ml tube (BD bioscience).

To increase pDC purity, the new 5ml tube containing the negatively selected pDC was placed in the EasySep™ magnet once more after adding 2ml of EasySep™ buffer then incubated for 5 min. Finally, the purified pDC was poured from the tube by picking up the EasySep™ magnet and inverting it in a new 5ml tube (BD bioscience). The isolated pDCs in the new tube are now ready for use and the magnetically labelled unwanted cells remained bounded inside the original tube, held by the magnetic field from the EasySep™ magnet. (Figure 2.5)



**Figure 2.5. Flow cytometric purity analysis of isolated adenotonsillar pDC**

Purity of pDC preparations as determined by flow cytometry. Representative density plot for freshly isolated adenotonsillar MNC before and after sorting purified pDC. pDC were purified from freshly isolated adenotonsillar MNC using the EasySep™ magnetic cell sorting system resulting in 96% purity. CD123 and CD85g (ILT7) were used to identify pDC.

#### **2.14 B cell help by T<sub>FH</sub> cells**

The ability of adenotonsillar T<sub>FH</sub> cells to help B cell antibody production was studied using an autologous cell culture system as follows. Adenotonsillar MNC were isolated from adenotonsillar tissues. All three cell types B cells, T<sub>FH</sub> cells and non-T<sub>FH</sub> cells were purified from adenotonsillar MNC of the same donor as described earlier. In co-culture experiments, B cells were cultured with an equal number of either T<sub>FH</sub> cells or non-T<sub>FH</sub> cells at a ratio of 1:1 using a cell concentration of  $5 \times 10^5$  cells/ml. B:T co-culture cells (250  $\mu$ l/well) were cultured in 96-well round bottom plate (Corning Inc., USA) in the presence or absence of different stimulants. These cells were stimulated with (0.5 $\mu$ g/ml) sH1N1 antigen in combination with (0.05 $\mu$ g/ml) CpG-DNA or stimulated with (0.5 $\mu$ l/ml) of LAIV. An unstimulated medium control was included in each experiment. To prevent fungal contamination, all cultures and medium used throughout was treated with 2.5 $\mu$ l/ml amphotericin B (Sigma). Cells were then cultured at 37°C, 5% CO<sub>2</sub>, for 1 day for cytokine measurement and 10 days for antibody production. After incubation, cell culture supernatants were collected from each well and stored at -80°C until further analysis by ELISA.

#### **2.15 Assessment of effect of pDC**

B cells, T<sub>FH</sub> cells and pDC were purified from adenotonsillar MNC of the same donor as described earlier. In co-culture experiments, B cells were cultured with an equal number of T<sub>FH</sub> cells at a ratio of 1:1 using a cell concentration of  $5 \times 10^5$  cells/ml with or without purified pDC. Purified pDC were then added to this mixture using a concentration of  $5 \times 10^4$  cells/ml. B cells + T<sub>FH</sub> cells + pDC co-culture cells (250  $\mu$ l/well) were cultured in 96-well round bottom plate (Corning Inc., USA) in

medium alone or in the presence or absence of different stimulants. These cells were stimulated with (0.5µg/ml) sH1N1 antigen in combination with (0.05µg/ml) CpG-DNA (InvivoGen, USA). An unstimulated medium control was included in each experiment. To prevent fungal contamination, all cultures and medium used throughout was treated with 2.5µl/ml amphotericin B (Sigma). Cells were then cultured at 37°C, 5% CO<sub>2</sub>, for 10 days for antibody production. After incubation, cell culture supernatants were collected from each well and stored at -80°C until further analysis by ELISA.

## **2.16 Health and safety**

All practical work was undertaken in Liverpool University laboratories. All chemicals and reagents used were COSHH assessed. Reagents were prepared, stored and disposed correctly according to the departmental policy and appropriate protective clothing was worn.

## **2.17 Statistical analysis**

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Firstly, data were analysed using the normality test to assess if distribution of data points was normal. Differences between two groups (*i.e.*, children and adults) were analysed by two-tailed un-paired Student's t test (parametric). Differences between stimulated and unstimulated samples in the same group of subjects were analysed by paired t test (parametric). Correlation between two factors was analysed by Pearson's (parametric) correlation test. Throughout the thesis, \* indicates a P-value between 0.01-0.05, \*\* indicates a P-value between 0.001-0.01 and \*\*\* indicates a P-value <0.001.

# **Chapter 3**

## **Characterisation of T<sub>FH</sub> cells in human NALT and PBMC**

### 3.1 Introduction

T<sub>FH</sub> cells in humans have recently been identified as a new T helper subset. Within secondary lymphoid organs, T<sub>FH</sub> cells are specialized to regulate the development of effector and memory B cells and long-lived plasma cells. These cells were first described by several research groups as a unique CD4 T cell phenotype expressing high levels of CXCR5 in tonsils (Breitfeld et al. 2000; Kim et al. 2001). Loss of chemokine receptor CCR7 and high expression of CXCR5 play a major role to drive T<sub>FH</sub> cells migration into B cell follicles in secondary lymphoid tissue, in a CXCL13-dependent manner (Choi et al. 2011; Fazilleau et al. 2009).

It is known that T cell help to B cells is an essential process for antibody-mediated adaptive immunity. The first interaction between T<sub>FH</sub> cells and B cells happens at the T-B cell border leading to B cell differentiation and GC formation within the follicle (Choi et al. 2011). In secondary lymphoid tissue, high expression of the costimulatory molecules including: CD40L, OX40 and ICOS, as well as the inhibitory molecule PD-1 are major players in modifying antigen specific contact of T<sub>FH</sub> cells to B cells expressing peptide MHC-II, CD40, OX40L, ICOSL and PDL-1 (Fazilleau et al. 2009; Kassiotis & O'Garra 2009).

T<sub>FH</sub> cells have been established as a distinct T helper subset based on a set of cell surface markers and cytokine profile. In addition to CD4 and CXCR5, T<sub>FH</sub> cells express high levels of ICOS, PD-1, BCL-6, IL-21R, OX40, CD40L and GL7 (Kerfoot et al. 2011; King 2009; Laurent, Fazilleau & Brousset 2010). Other markers have also been reported in humans including CD25, CD69, CD95 and CD57 (Laurent, Fazilleau & Brousset 2010).

A number of cytokines are produced by T<sub>FH</sub> cells that have major roles in B cell function and antibody production, such as IL-4, IL-10 and IL-21 (King 2009). Most recently, BCL-6 has been identified as the master regulatory transcription factor which controls T<sub>FH</sub> cell differentiation. The up-regulation of BCL-6 in T<sub>FH</sub> cell leads to the down-regulation and blocking of T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17 cell proliferation by suppressing their transcription factors (Figure 1.3) (Laurent, Fazilleau & Brousset 2010; Yu et al. 2009b). Over-expression of BCL-6 in activated T helper cells has been reported to stimulate IL-6 receptor and IL-21 receptor expression and they are both necessary for T<sub>FH</sub> cell differentiation (Laurent, Fazilleau & Brousset 2010).

Recent studies in humans have also shown a circulating CD4 T cell population characterized by high CXCR5 expression in peripheral blood (Chevalier et al. 2011; Ma & Deenick 2014; Morita et al. 2011; Simpson et al. 2010). These circulating cells, termed “peripheral T<sub>FH</sub>” (pT<sub>FH</sub>) share similar functional characteristics to T<sub>FH</sub> cells found in human secondary lymphoid tissues by expressing higher levels of PD-1 and IL-21 compared to CD4<sup>+</sup> CXCR5<sup>-</sup> T cells (Ma & Deenick 2014). However, a main difference between T<sub>FH</sub> cells found in secondary lymphoid organs and pT<sub>FH</sub> cells is the lack of expression of the transcription factor BCL-6 and much lower expression of ICOS by pT<sub>FH</sub> cells (Chevalier et al. 2011; Morita et al. 2011; Simpson et al. 2010).

A study by Morita et al. has shown the ability of these CXCR5<sup>+</sup> pT<sub>FH</sub> cells in providing better help to B cells to differentiate and proliferate compared to their CD4<sup>+</sup> CXCR5<sup>-</sup> counterparts. It also showed that CD4<sup>+</sup> CXCR5<sup>-</sup> cells express higher Blimp-1 mRNA than their CD4<sup>+</sup> CXCR5<sup>+</sup> cell (pT<sub>FH</sub>) counterparts (Morita et al. 2011). These pT<sub>FH</sub> cells have been suggested to represent the memory counterparts

of T<sub>FH</sub> cells outside the lymphoid organs, although it remains unknown whether there is a direct relationship between pT<sub>FH</sub> cells in peripheral blood and T<sub>FH</sub> cells in GC (Morita et al. 2011; Simpson et al. 2010; Vinuesa & Cook 2011).

### **3.2 Aims of Study**

The aims of this study were to:

- Characterize T<sub>FH</sub> cells found in NALT and PBMC.
- Analyse the frequencies of T<sub>FH</sub> cells in NALT and PBMC in children and adults.
- Analyse the correlation between frequencies of GC B cells and T<sub>FH</sub> cells in NALT.

### **3.3 Experimental Design**

To identify T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>high</sup> ICOS<sup>high</sup>), freshly isolated MNC were stained using a combination of CD4, CXCR5 and ICOS antibodies then analyzed by flow cytometry. For further characterization, adenotonsillar MNC were stained intracellularly for the transcription factor BCL-6 and cytokines IL-4, IL-10 and IL-21. The frequencies of T<sub>FH</sub> cells in NALT and PBMC in children and adults was compared. The relationship between frequencies of T<sub>FH</sub> cells and age was analysed. The correlation between numbers of T<sub>FH</sub> cells and GC B cells in NALT was also analysed.

#### **3.3.1 Patients and Samples**

Surgically removed adenotonsillar tissues were obtained from both children and adults (1.5–36 years). Peripheral blood samples were also obtained on the day of operation. Patients who had any known immunodeficiency or were previously vaccinated against influenza were excluded from the study. The study was approved by the local ethics committee (Liverpool Paediatric Research Ethics Committee) and written informed consent was obtained from each patient or their legal guardians as appropriate.

#### **3.3.2 Mononuclear Cell Separation**

Adenotonsillar MNC and PBMC were isolated from adenotonsillar tissues and peripheral blood respectively. These mononuclear cells were isolated using Ficoll density centrifugation following methods described previously in the materials and methods chapter 2. Briefly, adenotonsillar tissues samples were minced by a scalpel to release cells into the medium. Then the cell suspension was filtered through a 70µm cell strainer (BD biosciences, USA). The adenotonsillar cells and peripheral

blood were carefully layered onto 15 ml Ficoll-Paque™ PREMIUM (GE Healthcare Life Sciences, UK) and spun at 400g for 30 min at room temperature. The cloudy layer of mononuclear cells was carefully harvested. MNC were washed twice with sterile PBS solution (Sigma, UK), then the pellet was re-suspended in 5 ml RPMI-1640 culture medium with HEPES (Sigma-Aldrich, UK) supplemented with 10% heat inactivated FBS (Sigma), 1% L-glutamine (Sigma), streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma-Aldrich). After counting, each cell suspension was adjusted to contain  $4 \times 10^6$  cells/ml concentrations.

### **3.3.3 Flow Cytometric Analysis of Cells**

#### **3.3.3.1 Cell Staining for Phenotyping for FACS Analysis**

Anti-human antibodies used for flow cytometric analysis are described in (**Table 2.1**). For T<sub>FH</sub> cell identification, adenotonsillar MNC and PBMC were stained with anti-human-CD3, -CD4, -CXCR5 and -ICOS antibodies. In some samples, PD-1 and CCR7 were also stained to confirm the T<sub>FH</sub> cell phenotype. For GC B cells identification, adenotonsillar MNC were stained with anti-human -CD19, -CD38 and -IgD antibodies. All cell staining was performed in Falcon 5ml round bottom tubes (BD bioscience) as described earlier. Immediately after staining, cells were acquired/analysed using a BD FACS Calibur (BD bioscience) or fixed and permeabilized if needed for intracellular staining.

#### **3.3.3.2 Intracellular Staining for BCL-6 and Cytokines**

Adenotonsillar MNC or PBMC ( $4 \times 10^6$ /ml) were incubated at 37°C in 5% CO<sub>2</sub>. After overnight incubation, 10µg/ml brefeldin A (Sigma) was added to each sample and further incubated for 4 hours at 37°C in 5% CO<sub>2</sub>. Cells were then harvested and washed twice with FACS staining buffer (500xg for 5 min). Firstly, cells were

stained for surface staining markers such as CD4, CXCR5 and ICOS (for T<sub>FH</sub> cells identification) in a same procedure described earlier. Next, the pellets were resuspended and fixed with 100µl intracellular fixation buffer (eBioscience) for 20 min then washed twice with 2 ml 1x permeabilization buffer (500xg for 5 min). Then the proper amount of intracellular fluorochrome-labeled antibody (see Table 2.1) was added to each sample; and incubated for 30 min. After that, cells were washed twice with 1x permeabilization buffer (500xg for 5 min), the stained cells were resuspended in 400µl of FACS staining buffer; and immediately acquired/analysed using the BD FACS Calibur (BD bioscience) and analysed with WinMDI 2.9 software.

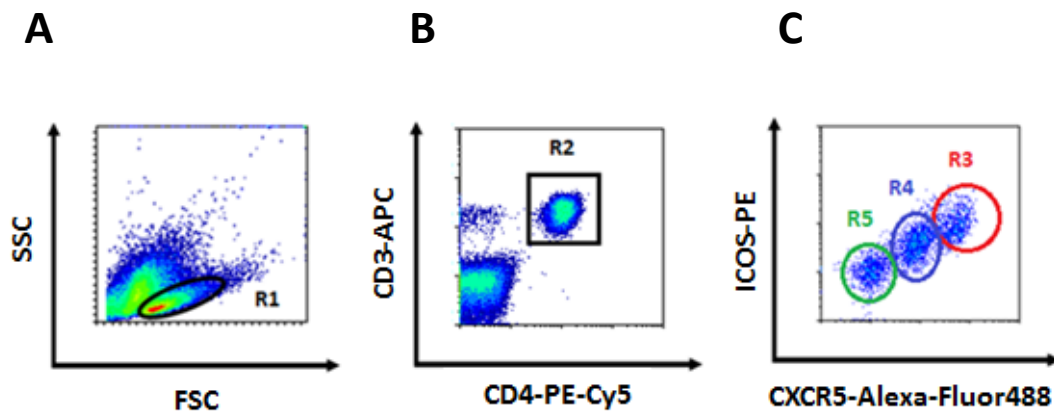
### **3.3.4 Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Differences between two groups (*i.e.*, children and adults) were analysed by two-tailed un-paired Student's t test (parametric). Differences between samples in the same group of subjects were analysed by paired t test (parametric). Correlation between two factors was analysed by Pearson's (parametric) correlation test. Throughout the thesis, \* indicates a P-value between 0.01-0.05, \*\* indicates a P-value between 0.001-0.01 and \*\*\* indicates a P-value <0.001.

### 3.4 Results

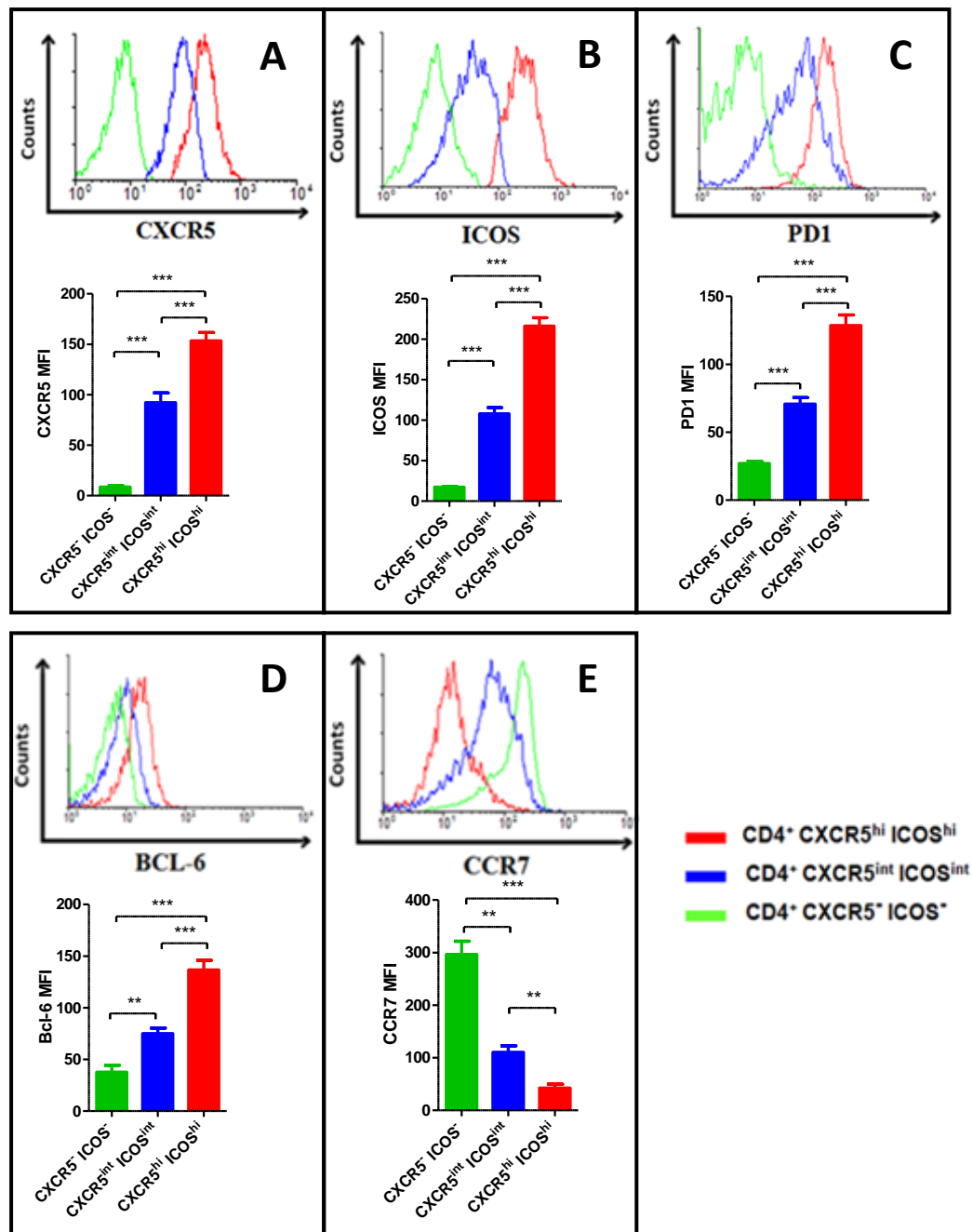
#### 3.4.1 Identification of T<sub>FH</sub> Cells in Adenotonsillar MNC

In order to identify T<sub>FH</sub> cells in adenotonsillar MNC, a combination of fluorescence-labelled anti-human CD4, CXCR5 and ICOS antibodies were used to stain the cells before flowcytometry. After gating on CD3<sup>+</sup> CD4<sup>+</sup> T cells, three populations were observed: CD4<sup>+</sup> CXCR5<sup>high</sup> ICOS<sup>high</sup> (T<sub>FH</sub> cells) (Red) CD4<sup>+</sup> CXCR5<sup>int</sup> ICOS<sup>int</sup> (Blue) and CD4<sup>+</sup> CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> (Green) populations (Figure 3.1). In addition to CXCR5 (Figure 3.2.A), T<sub>FH</sub> cells are characterized by expressing high levels of ICOS (Figure 3.2.B), PD-1 (Figure 3.2.C) and BCL-6 (Figure 3.2.D). In contrast, as seen in figure 3.2.E, T<sub>FH</sub> cells express very low levels of the chemokine receptor CCR7 compared to CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> cells that express high levels of CCR7.



**Figure 3.1. Representative sample analysis showing gating strategy for identification of T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>hi</sup> ICOS<sup>hi</sup>) in adenotonsillar MNC**

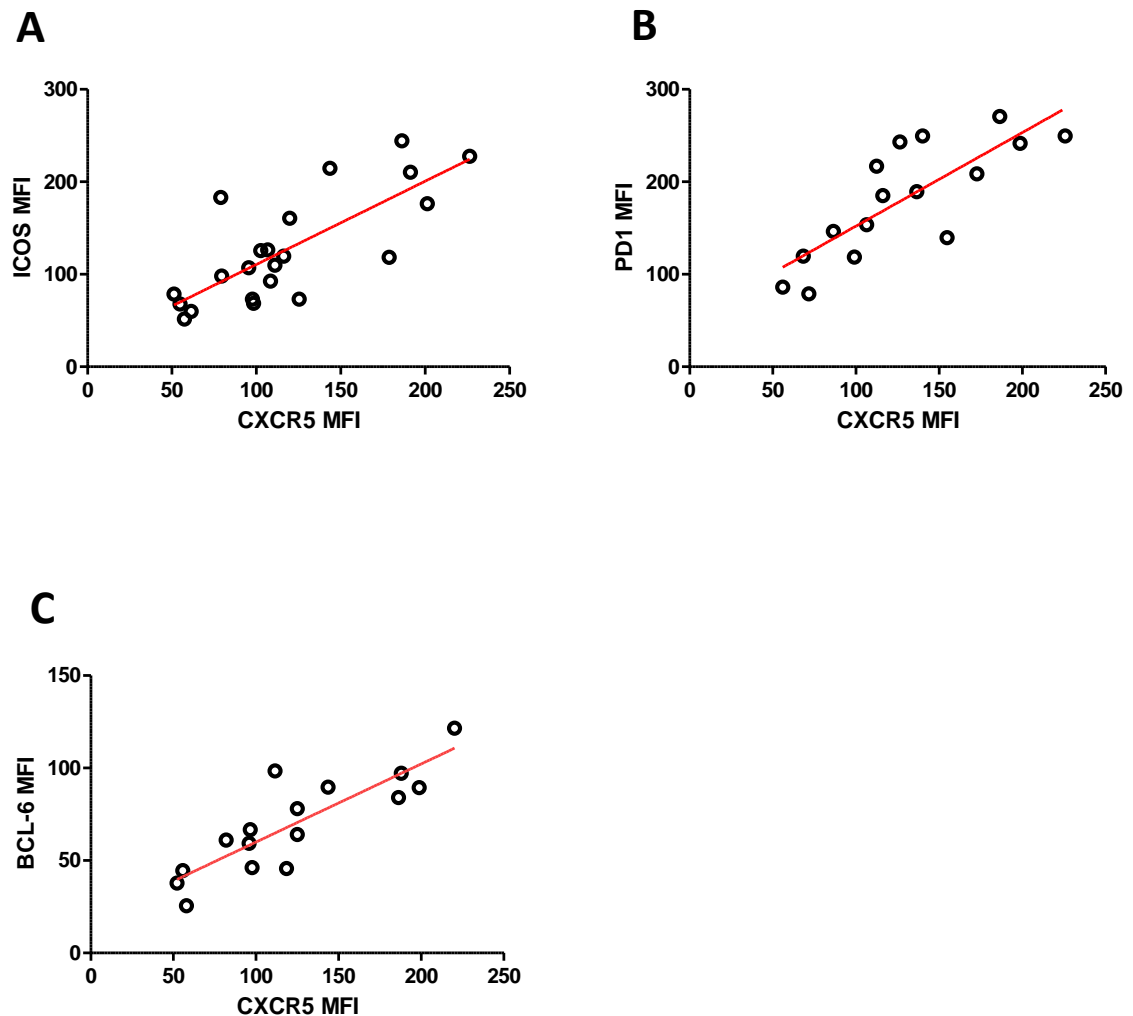
Adenotonsillar MNC were stained by anti-CD3, -CD4, -CXCR5 and -ICOS antibodies followed by flowcytometry. Lymphocytes were defined by typical FSC and SSC (R1) (A). CD4<sup>+</sup> T cells were identified by positive staining of CD3<sup>+</sup> and CD4<sup>+</sup> cells (R2) (B). T<sub>FH</sub> cells were identified by gating on CD3<sup>+</sup> CD4<sup>+</sup> T cells (R2), and positive staining of CXCR5<sup>high</sup> ICOS<sup>high</sup> (T<sub>FH</sub> cells) (R3) CXCR5<sup>int</sup> ICOS<sup>int</sup> (R4) and CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> (R5) populations (C).



**Figure 3.2. T<sub>FH</sub> cells express high amounts of CXCR5, ICOS, PD-1 and BCL-6 in adenotonsillar tissues**

A representative FACS histogram of CXCR5 (A), ICOS (B), PD-1(C), BCL-6 (D) and CCR7 (E) expression in tonsillar CD4<sup>+</sup> T cells. Human tonsil cells were gated on CD4<sup>+</sup> T cells and subsequently divided into CXCR5<sup>high</sup> ICOS<sup>high</sup> (T<sub>FH</sub> cells) (red line) CXCR5<sup>int</sup> ICOS<sup>int</sup> (blue line) and CXCR5<sup>low</sup> ICOS<sup>low</sup> (green line). The bar graph represent MFIs of CXCR5, ICOS, PD-1, BCL-6 and CCR7 expression in three CD4<sup>+</sup> T cell populations analysed by flowcytometry. Data shown as mean  $\pm$  SEM from 12 individual donors. *p*-value derived from Student's t test. \*\*\**P* < 0.001 and \*\**P* < 0.01.

The correlation of CXCR5 MFI with ICOS, PD-1 and BCL-6 MFI from CD4<sup>+</sup> T cells was also analysed. As shown in figure 3.3, CXCR5 was correlated with ICOS (A) ( $r = 0.76$ ,  $P < 0.001$ ,  $n = 20$ ), PD-1 (B) ( $r = 0.8$ ,  $P < 0.001$ ,  $n = 16$ ), and BCL-6 (C) ( $r = 0.85$ ,  $P < 0.001$ ,  $n = 15$ ).

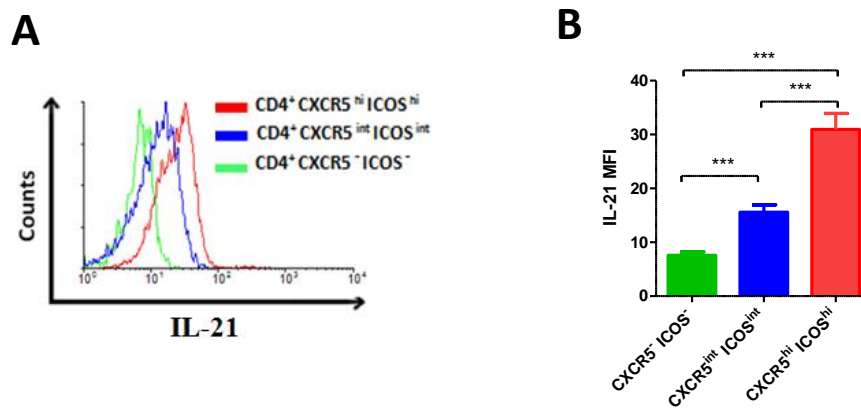


**Figure 3.3. Correlation between levels of expression (MFI) of CXCR5 and ICOS, PD-1 and BCL-6 in CD4<sup>+</sup> T cells**

Correlations between MFIs of CXCR5 and ICOS (A,  $r = 0.76$ ,  $P < 0.001$ ,  $n = 20$ ), MFIs of CXCR5 and PD-1 (B,  $r = 0.8$ ,  $P < 0.001$ ,  $n = 16$ ), and MFIs of CXCR5 and BCL-6 (C,  $r = 0.85$ ,  $P < 0.001$ ,  $n = 15$ ) from CD4<sup>+</sup> T cells in adenotonsillar tissue.

### 3.4.2 Expression of IL-21 by T<sub>FH</sub> Cells

Intracellular cytokine staining for IL-21 was performed to determine the difference in IL-21 expression in three CD4<sup>+</sup> T cell subsets: CXCR5<sup>high</sup> ICOS<sup>high</sup> (Red), CXCR5<sup>int.</sup> ICOS<sup>int.</sup> (Blue) and CXCR5<sup>-/low</sup> ICOS<sup>-/low</sup> (Green). As shown in figure 3.4 T<sub>FH</sub> cells expressed the highest level of IL-21 (mean MFI  $\pm$  SEM, 30.9  $\pm$  2.9) compared to (mean MFI  $\pm$  SEM, 15.6  $\pm$  1.3) produced by CXCR5<sup>int.</sup> ICOS<sup>int.</sup> cells and (mean MFI  $\pm$  SEM, 7.5  $\pm$  0.6) produced by CXCR5<sup>-/low</sup> ICOS<sup>-/low</sup> cells.

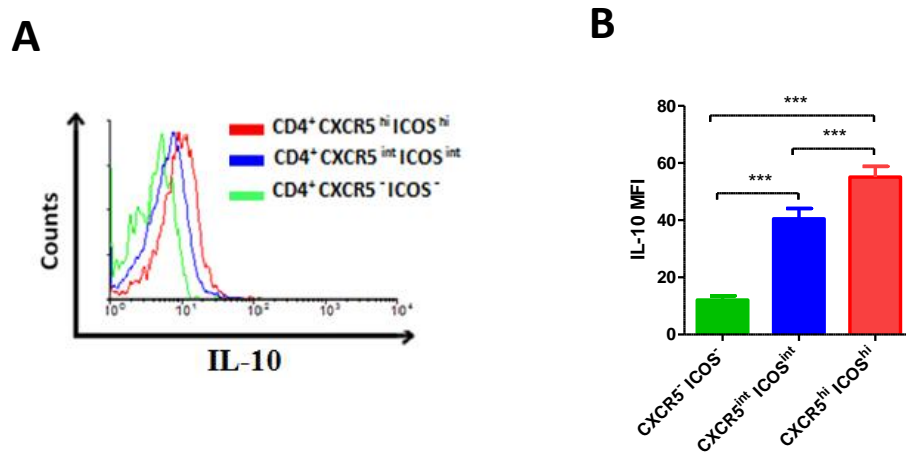


**Figure 3.4. Expression of IL-21 in adenotonsillar T<sub>FH</sub> cells**

Adenotonsillar MNC were gated on CD4<sup>+</sup> T cells and subsequently divided into CXCR5<sup>high</sup> ICOS<sup>high</sup> (T<sub>FH</sub> cells) (Red line) CXCR5<sup>int</sup> ICOS<sup>int</sup> (Blue line) and CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> cells (Green line). (A) A representative FACS histogram showing IL-21 expression in tonsillar CD4<sup>+</sup> T cells. (B) MFI of IL-21 expression in three different CD4<sup>+</sup> T cell subsets analysed by flowcytometry. Data shown as mean MFI  $\pm$  SEM from 15 individual donors. *p*-value derived from Paired two-tailed *t* test. \*\*\**P* < 0.001.

### 3.4.3 Expression of IL-10 by T<sub>FH</sub> Cells

Intracellular cytokine staining for IL-10 was performed to determine the differences in IL-10 expression in three CD4<sup>+</sup> T cell subsets: CXCR5<sup>high</sup> ICOS<sup>high</sup> (Red), CXCR5<sup>int.</sup> ICOS<sup>int.</sup> (Blue) and CXCR5<sup>-/low</sup> ICOS<sup>-/low</sup> (Green). As shown in figure 3.5, T<sub>FH</sub> cells express the highest level of IL-10 (mean MFI  $\pm$  SEM, 55.1  $\pm$  3.7) compared to (mean MFI  $\pm$  SEM, 40.5  $\pm$  3.6) produced by CXCR5<sup>int.</sup> ICOS<sup>int.</sup> cells and (mean MFI  $\pm$  SEM, 12.1  $\pm$  1.4) produced by CXCR5<sup>-/low</sup> ICOS<sup>-/low</sup> cells.

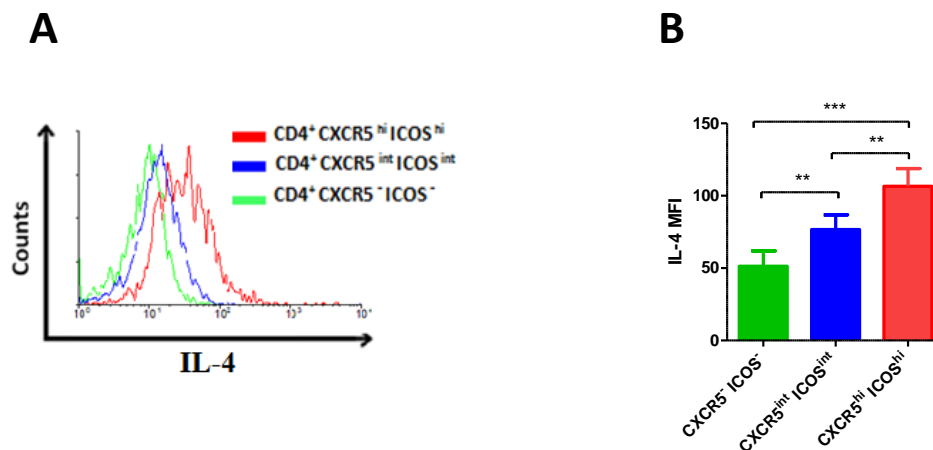


**Figure 3.5. Expression of IL-10 in adenotonsillar T<sub>FH</sub> cells**

Adenotonsillar MNC were gated on CD4<sup>+</sup> T cells and subsequently divided into CXCR5<sup>high</sup> ICOS<sup>high</sup> (T<sub>FH</sub> cells) (Red line) CXCR5<sup>int</sup> ICOS<sup>int</sup> (Blue line) and CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> (Green line). (A) A representative FACS histogram of IL-10 expression in tonsillar CD4<sup>+</sup> T cells. (B) MFI of IL-10 expression in three different CD4<sup>+</sup> T cells analysed by flowcytometry. Data shown as mean  $\pm$  SEM from 10 individual donors. *p*-value derived from Paired two-tailed *t* test. \*\*\**P* < 0.001.

### 3.4.4 Expression of IL-4 by T<sub>FH</sub> Cells

Intracellular cytokine staining for IL-4 was done to determine the differences in IL-4 expression in three CD4<sup>+</sup> T cells: CXCR5<sup>high</sup> ICOS<sup>high</sup> (Red), CXCR5<sup>int.</sup> ICOS<sup>int.</sup> (Blue) and CXCR5<sup>low</sup> ICOS<sup>low</sup> (Green). As shown in figure 3.6, T<sub>FH</sub> cells produces the highest amount of IL-4 (mean MFI  $\pm$  SEM, 106.6  $\pm$  12.2) compared to (mean MFI  $\pm$  SEM, 76.6  $\pm$  10.2) produced by CXCR5<sup>int.</sup> ICOS<sup>int.</sup> cells and (mean MFI  $\pm$  SEM, 51.3  $\pm$  10.6) produced by CXCR5<sup>low</sup> ICOS<sup>low</sup> cells.

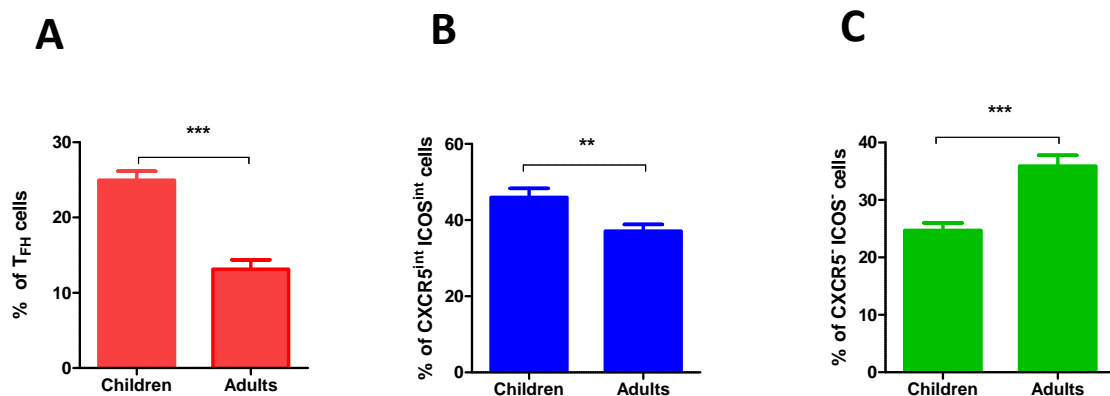


**Figure 3.6. Expression of IL-4 in adenotonsillar T<sub>FH</sub> cells**

Adenotonsillar MNC were gated on CD4<sup>+</sup> T cells and subsequently divided into CXCR5<sup>high</sup> ICOS<sup>high</sup> (T<sub>FH</sub> cells) (Red line) CXCR5<sup>int</sup> ICOS<sup>int</sup> (Blue line) and CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> (Green line). (A) A representative FACS histogram of IL-4 expression in tonsillar CD4<sup>+</sup> T cells. (B) MFI of IL-4 expression in three different CD4<sup>+</sup> T cells analysed by flowcytometry. Data shown as mean  $\pm$  SEM from 10 individual donors. *p*-value derived from Paired two-tailed *t* test. \*\*\**P* < 0.001 and \*\**P* < 0.01.

### 3.4.5 Percentage of T<sub>FH</sub> Cells in Adenotonsillar Tissues in Children and Adults

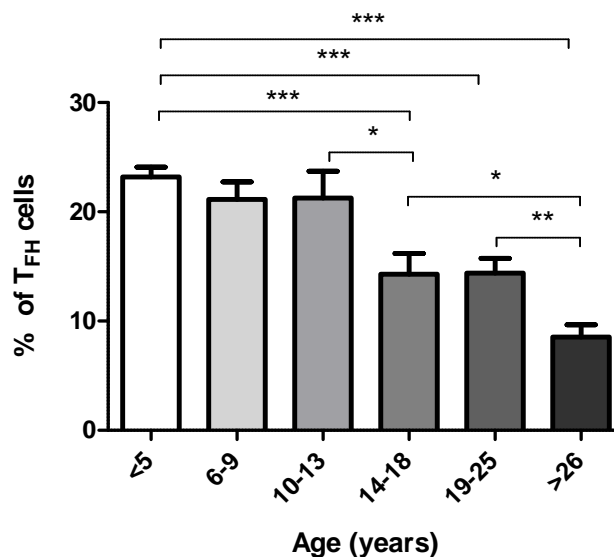
To investigate if there is any association between T<sub>FH</sub> cells and age, freshly isolated adenotonsillar MNC from 82 children and 40 adults were stained for T<sub>FH</sub> cell markers and assessed by flow-cytometry. As shown in figure 3.7.A, higher percentage of T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells) was shown in children (mean  $\pm$  SEM, 24.9%  $\pm$  1.1) compared to that in adults (mean  $\pm$  SEM, 13.4%  $\pm$  1.2). Number of CXCR5<sup>int</sup> ICOS<sup>int</sup> cells was also shown higher in children (mean  $\pm$  SEM, 45.2%  $\pm$  2.3) compared to that in adults (mean  $\pm$  SEM, 39.6%  $\pm$  1.7) (Figure 3.7.B). However, number of CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> cells was lower in children than in adults (mean  $\pm$  SEM, 24.7%  $\pm$  1.2 versus 35.9%  $\pm$  1.8,  $p < 0.0001$ ) (Figure 3.7.C).



**Figure 3.7. Children have higher percentage of T<sub>FH</sub> cells in adenotonsillar tissues than in adults**

Comparisons of percentage of T<sub>FH</sub> cells (represents % of T<sub>FH</sub> cells within CD4<sup>+</sup> T cells) between children and adults (A), percentage of CD4<sup>+</sup> CXCR5<sup>int</sup> ICOS<sup>int</sup> cells between children and adults (B) and percentage of CD4<sup>+</sup> CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> cells between children and adults (C). Student *t*-test, \*\*\* $P < 0.001$  and \*\* $P < 0.01$ .

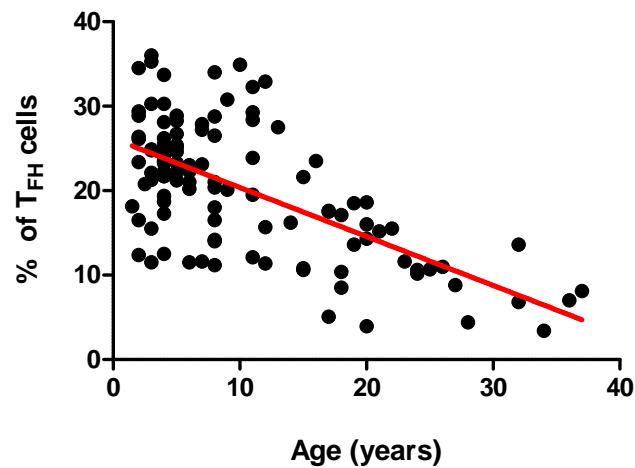
Further analysis showed that there was an age-associated decrease in the percentage of T<sub>FH</sub> cells in adenotonsillar tissue (Figure 3.8). During childhood up to 13 years old, the percentage of T<sub>FH</sub> cells appeared to maintain at a similar level with a mean of around 24%. However, there appeared to be a decrease in percentage in age groups >13 years. A significant decrease in T<sub>FH</sub> cells percentage was seen in the 14-18 years age group compared to 10-13 years age group ( $p < 0.05$ , 23% versus 14%). A further reduction in T<sub>FH</sub> cell percentage was seen in >25 years age group with around only 8% of T<sub>FH</sub> cells.



**Figure 3.8. Percentage of T<sub>FH</sub> cells at different age groups in adenotonsillar tissue**

Percentage of T<sub>FH</sub> cells at different age groups are shown. A total of 120 samples are analysed including: <5 years (n=46), 6-9 years (n=22), 10-13 years (n=16), 14-18 years (n=12), 19-25 years (n=14) and >25 years group (n=10). The results are expressed as the mean percentage of T<sub>FH</sub> cells  $\pm$  SEM. Statistical differences (\*\*\* $P < 0.001$ , \*\* $P < 0.01$ , and \* $P < 0.05$ ) between different groups were calculated by Student *t*-test.

The age-related decrease of T<sub>FH</sub> cells is also presented in figure 3.9. As shown in the figure, in general, there was an inverse correlation between percentage of T<sub>FH</sub> cells in adenotonsillar tissue and age (n=120,  $r = -0.62$ ,  $P < 0.0001$ ).

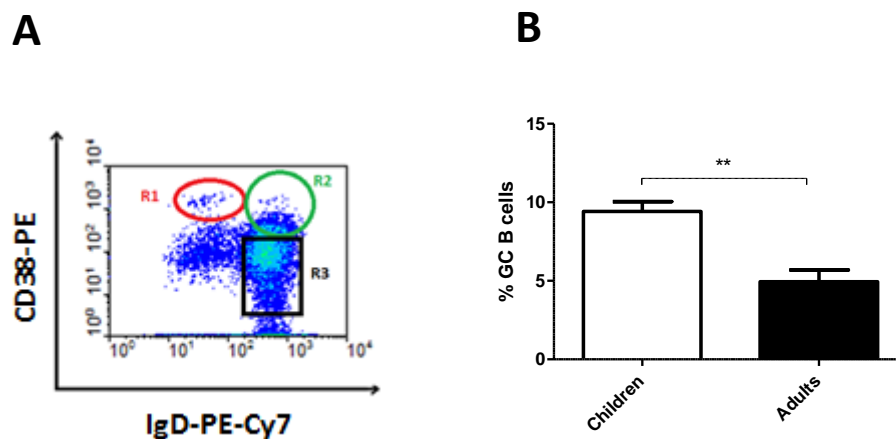


**Figure 3.9. Relationship between percentage of T<sub>FH</sub> cells in tonsillar tissue and age**

A regression linear plot showing the correlation between percentage of T<sub>FH</sub> cells in adenotonsillar tissue and age. Dots (•) represents the percentage of T<sub>FH</sub> cells in CD4<sup>+</sup> T cell population in 120 subjects. ( $r = -0.62$ ,  $P < 0.0001$ ).

### 3.4.6 Number of GC B Cells in Children and Adults

To determine whether there is any relationship between  $T_{FH}$  and GC B cell subsets in adenotonsillar MNC, GC B cell subset was also analysed with a combination of CD19, CD38 and IgD fluorescence-labelled anti-human antibodies. After gating on  $CD19^+$  B cells, the following B cell subsets were shown: GC B cells ( $CD19^+ CD38^{++} IgD^-$ ) R1 (red), pre-GC B cells ( $CD19^+ CD38^{++} IgD^+$ ) R2 (green) and naïve B cells ( $CD19^+ CD38^- IgD^+$ ) R3 (Figure 3.10.A). As can be seen from figure 3.10.B, a higher number of GC B cells was found in children (mean  $\pm$  SEM,  $9.2\% \pm 0.6$ ) than in adults (mean  $\pm$  SEM,  $4.9\% \pm 0.7$ ) ( $p < 0.01$ ).

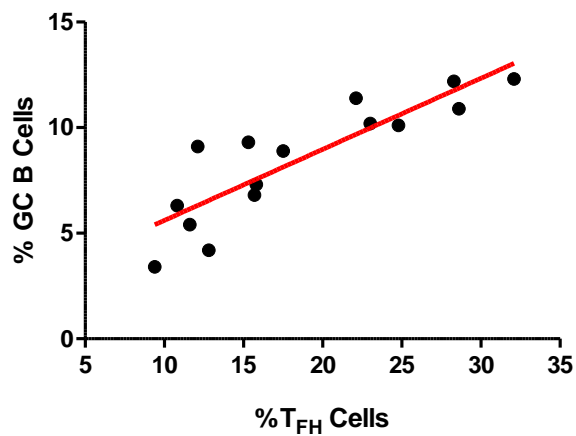


**Figure 3.10. Number of GC B Cells in Children and Adults**

Flowcytometry analysis of B cell subsets in adenotonsillar tissues. (A) Representative FACS dotplot showing B cell subsets including GC B cells ( $CD19^+ CD38^{++} IgD^-$ ) in tonsillar MNC. (B) Comparison of numbers of GC B cells in tonsillar MNC between children ( $n=10$ ) and adults ( $n=7$ ), Student *t*-test,  $**P < 0.01$ .

### 3.4.7 Correlation between percentage of T<sub>FH</sub> Cells and GC B Cells

To investigate if there is any association between numbers of GC B cells and T<sub>FH</sub> cells, freshly isolated adenotonsillar MNC from 15 tonsillar tissue samples were stained for both T<sub>FH</sub> cells and GC B cells and assessed by flow cytometry. As shown in figure 3.11, there was a good correlation between numbers of GC B cells and T<sub>FH</sub> cells ( $r = 0.86$ ,  $P < 0.001$ ).

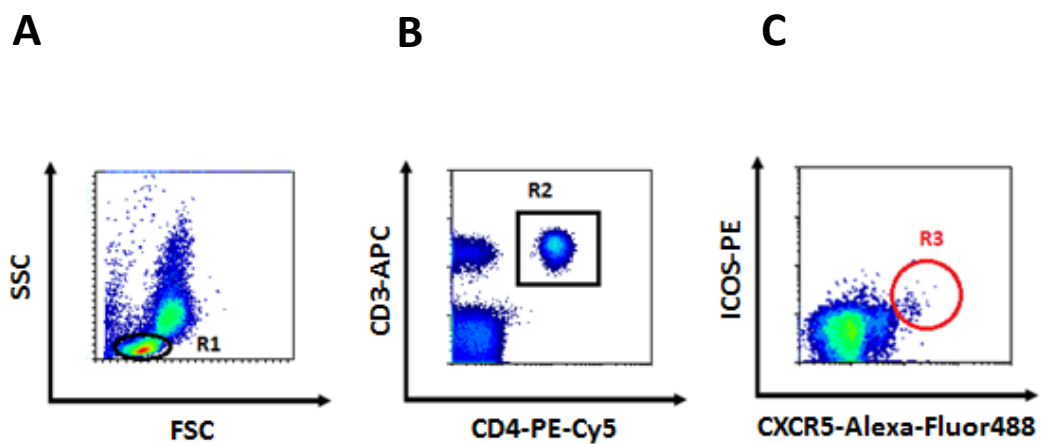


**Figure 3.11. Correlation between numbers of GC B cells and T<sub>FH</sub> cells in adenotonsillar MNC**

A regression linear plot showing the correlation between percentages of GC B cells and T<sub>FH</sub> cells in adenotonsillar MNC ( $r = 0.86$ ,  $P < 0.001$ ,  $n=15$ ).

### 3.4.8 Identification of Peripheral T<sub>FH</sub> Cells (pT<sub>FH</sub>) in PBMC

In order to identify peripheral T<sub>FH</sub> cells (pT<sub>FH</sub>) in PBMC, a combination of CD4, CXCR5 and ICOS fluorescence-labelled anti-human antibodies were used. After gating on CD3<sup>+</sup> CD4<sup>+</sup> T cells, three populations could be seen: CD4<sup>+</sup> CXCR5<sup>high</sup> ICOS<sup>+</sup> (pT<sub>FH</sub> cells) (Red) CD4<sup>+</sup> CXCR5<sup>int</sup> ICOS<sup>-</sup> and CD4<sup>+</sup> CXCR5<sup>-</sup> ICOS<sup>-</sup> populations (Figure 3.12).

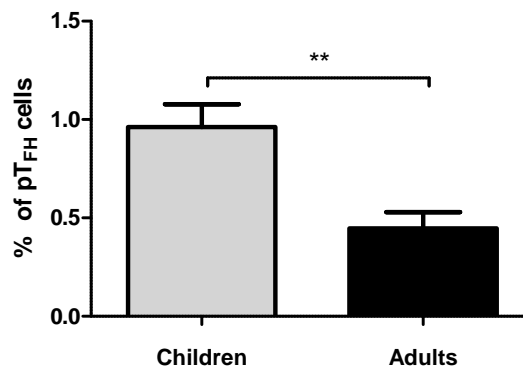


**Figure 3.12.** A representative sample analysis showing gating strategy for identification of pT<sub>FH</sub> cells in PBMC

PBMC were stained with fluorescence-labelled anti-CD3, -CD4, -CXCR5 and -ICOS antibodies followed by flowcytometry. Lymphocytes were gated by typical FSC and SSC (R1) (A). CD4<sup>+</sup> T cells were identified by positive staining of CD3<sup>+</sup> and CD4<sup>+</sup> cells (R2) (B). (C) pT<sub>FH</sub> cells were identified by gating on CD4<sup>+</sup> T cells, showing CXCR5<sup>hi</sup> ICOS<sup>+</sup> (pT<sub>FH</sub> cells) (R3).

### 3.4.9 Percentage of pT<sub>FH</sub> Cells in Children and Adults

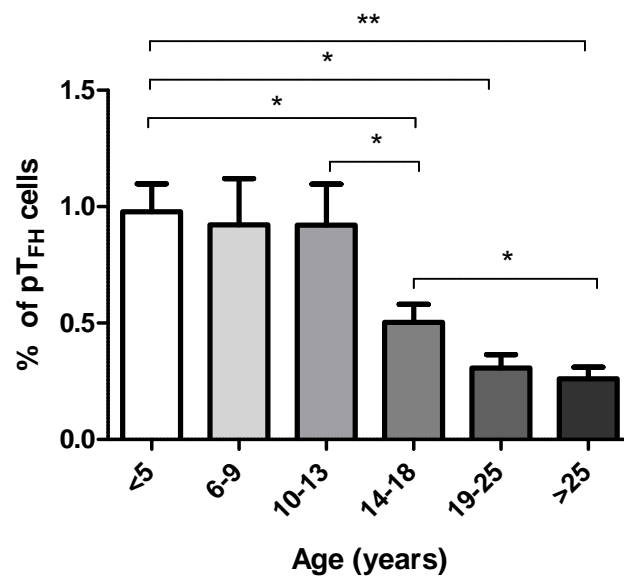
To investigate if there is any association between pT<sub>FH</sub> cells and age, freshly isolated PBMC from 17 children and 14 adults were stained for pT<sub>FH</sub> cell identification and assessed by flow-cytometry. Children were shown to have a higher percentage of pT<sub>FH</sub> cells with a mean  $\pm$  SEM of (0.96%  $\pm$  0.11) compared to adults with a mean  $\pm$  SEM of (0.44%  $\pm$  0.08) pT<sub>FH</sub> cells (Figure 3.13).



**Figure 3.13. Children have higher percentage of pT<sub>FH</sub> cells in PBMC than in adults**

Freshly human PBMC were stained with anti-CD4, -CD3, -CXCR5 and -ICOS antibodies. Data shows that children had higher number of pT<sub>FH</sub> cells than in adults pT<sub>FH</sub> cells. Mean percentages  $\pm$  SEM were shown, n=14 for adults and n=17 for children respectively. (Student *t*-test, \*\*P < 0.01).

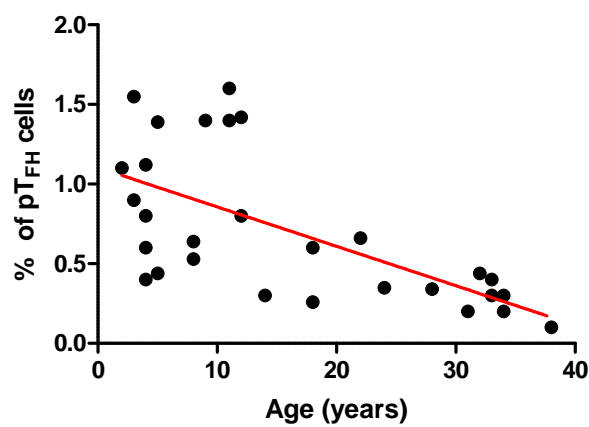
Further analysis showed that there was an age-associated decrease in the percentage of pT<sub>FH</sub> cells in PBMC (Figure 3.14). A decrease in pT<sub>FH</sub> cells percentage was seen in the 14-18 years age group compared to children less than 13 years ( $p < 0.05$ ). A further reduction in pT<sub>FH</sub> cells percentage was seen in >25 years age group with less than 0.3% of pT<sub>FH</sub> cells in human PBMC.



**Figure 3.14. Percentage of pT<sub>FH</sub> cells at different age groups in PBMC**

Percentage of pT<sub>FH</sub> cells at different age groups are shown. A total of 31 samples ( $n=31$ ) are analysed including: <5 years ( $n=9$ ), 6-9 years ( $n=5$ ), 10-13 years ( $n=6$ ), 14-18 years ( $n=3$ ), 19-25 years ( $n=3$ ) and >25 years group ( $n=5$ ). The results are expressed as the mean percentage of pT<sub>FH</sub> cells  $\pm$  SEM. Statistical differences ( $***P < 0.001$ ,  $**P < 0.01$ , and  $*P < 0.05$ ) between different groups were analysed using student *t*-test.

The association between percentage of pT<sub>FH</sub> cells and age is also presented in figure 3.15. As can be seen, overall, there was a negative correlation between percentage of pT<sub>FH</sub> cell and age in human PBMC ( $n=31$ ,  $r = -0.60$ ,  $P < 0.0001$ ).



**Figure 3.15. Relationship between percentage of pT<sub>FH</sub> cells in PBMC and age**

A regression linear plot showing the correlation between percentages of pT<sub>FH</sub> cells and age in PBMC. ( $r = -0.60$ ,  $P < 0.0001$ ,  $n=31$ ).

### 3.5 Discussion

Secondary lymphoid organs including tonsillar tissues have been reported to be highly enriched with T<sub>FH</sub> cells (Rasheed et al. 2006; Yu & Vinuesa 2010a). In this study, we have observed a prominent percentage of T<sub>FH</sub> cells in adenotonsillar samples from both children and adults, using a combination of surface markers of CD4, CXCR5 and ICOS. With these markers, three populations were identified in CD4<sup>+</sup> T lymphocytes: CD4<sup>+</sup> CXCR5<sup>high</sup> ICOS<sup>high</sup> (T<sub>FH</sub> cells), CD4<sup>+</sup> CXCR5<sup>int</sup> ICOS<sup>int</sup> and CD4<sup>+</sup> CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> populations. The frequencies of T<sub>FH</sub> as expressed as proportions in CD4<sup>+</sup> T cells range from 12% to 36% (in children) and from 5% to 20% (in adults). These results are generally consistent with previous reports (Bentebibel et al. 2011; Rasheed et al. 2006; Yu & Vinuesa 2010a).

In addition to the use of CXCR5 and ICOS markers, additional markers for T<sub>FH</sub> cells were also used to confirm the identification of T<sub>FH</sub> in this study. These additional markers included PD-1, BCL6, CCR7 were used, and we confirmed that the CD4<sup>+</sup> CXCR5<sup>high</sup> ICOS<sup>high</sup> (T<sub>FH</sub>) was also expressing PD-1<sup>hi</sup>, BCL-6<sup>hi</sup> and CCR7<sup>low</sup>. These are in agreement with previous reports by other groups (Ma et al. 2009).

Among these T<sub>FH</sub> markers, CXCR5 was the first and most widely used cell surface marker to identify T<sub>FH</sub> cells (Breitfeld, Ohl et al. 2000). CXCR5 specifically allows migration of T<sub>FH</sub> cells towards the follicular chemokine ligand CXCL13, a chemokine responsible for cell entry into follicle (Laurent, Fazilleau & Brousset 2010). Together, the CXCR5-CXCL13 interaction and down-regulation of CCR7 expression are responsible for T<sub>FH</sub> cells follicular homing. This leads to regulation of antigen-specific B-cell responses using co-stimulatory molecules including ICOS, CD28 and OX40 (Gómez-Martín et al. 2010; Laurent, Fazilleau & Brousset 2010).

ICOS is generally used in combination with CXCR5 to identify T<sub>FH</sub>. ICOS is co-stimulatory molecules that regulate T cell activation and function expressed primarily on activated human T cells including T<sub>FH</sub> cells. ICOS regulates T<sub>FH</sub> control of B cell function by inducing cytokine secretion (especially IL-4, IL-10 and IL-21) and up-regulating T-B interaction molecules (Hutloff et al. 1999; Simpson, Quezada & Allison 2010). Both murine and human studies have shown the importance of ICOS in the development and function of T<sub>FH</sub> cells. It has recently been reported that ICOS or ICOS-L deficiency is correlated with fewer T<sub>FH</sub> cells and a decrease in GC formation suggesting a major role of ICOS in T<sub>FH</sub> cell differentiation (Akiba et al. 2005; Bauquet et al. 2009; Laurent, Fazilleau & Brousset 2010).

PD-1 was also shown to be highly expressed on the T<sub>FH</sub> cells identified in adenotonsillar cells in this study. This is consistent with previous reports (Xu et al. 2014). PD-1 is an important molecule for T cell tolerance (Haynes et al. 2007; Iwai et al. 2002). PD-1 provide an inhibitory signal to GC T<sub>FH</sub> cells to prevent excess proliferation of CD4 T cells in GCs (Crotty 2011). PD-1 is suggested to contribute to GC B cell survival and the formation and affinity of long-lived plasma cells through interaction with its ligands PD-L1 and PD-L2 which are highly expressed on GC B cells (Good-Jacobson et al. 2010). Haynes and colleagues have shown that within CXCR5<sup>high</sup> CCR7<sup>low</sup> (T<sub>FH</sub> cells) the PD-1 expression levels and the abundance of IL-4 were the highest (Haynes et al. 2007).

In our study, T<sub>FH</sub> cells was shown to express very low levels of CCR7 compared to CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> cells that express high levels of CCR7. Down-regulation of CCR7 expression is crucial for T<sub>FH</sub> cells follicular homing (Gómez-Martín et al. 2010; Laurent, Fazilleau & Brousset 2010). By down-regulation of CCR7, activated

T<sub>H</sub> cells become less reactive to both CCL19 and CCL21, which are main T-zone chemokines, leading T<sub>FH</sub> cells to move inside GC (Haynes 2008).

The T<sub>FH</sub> cells identified in this study expressed high level of BCL-6 detected by flowcytometry. This is consistent with previous studies (Kroenke et al. 2012; Ma et al. 2009; Nurieva et al. 2009). BCL-6 has been considered the master regulatory transcription factor that controls T<sub>FH</sub> cell differentiation (Crotty, Johnston & Schoenberger 2010; Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009b). Over-expression of BCL-6 in activated T helper cells has been reported to stimulate IL-6 receptor and IL-21 receptor expression and they are both necessary for T<sub>FH</sub> cells differentiation (Laurent, Fazilleau & Brousset 2010). Recent studies have shown that T<sub>FH</sub> differentiation does not occur in vivo in the absence of BCL-6 (*Bcl6*<sup>-/-</sup>). However, the differentiation of other CD4 T cell subsets is relatively unaffected by the lack of BCL-6 (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009b).

Several cytokines are produced by T<sub>FH</sub> cells that have major roles in B cell function and antibody production, and these include IL-4, IL-10 and IL-21 (Kim et al. 2001; King 2009). Here we showed that T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>hi</sup> ICOS<sup>hi</sup>) in human NALT expressed highest levels of IL-4, IL-10 and IL-21 as compared to CXCR5<sup>Int</sup> ICOS<sup>Int</sup> and CXCR5<sup>low/-</sup> ICOS<sup>low/-</sup> cell populations. These findings are consistent with the previous report that CD4<sup>+</sup> CXCR5<sup>hi</sup> cells express higher amounts of IL-4, IL-10 and IL-21 compared to CD4<sup>+</sup> CXCR5<sup>low</sup> T cells (Ma et al. 2009). It was previously reported that with the acquisition of CXCR5, the production of IL-4 and IL-21 increases (Ma et al. 2009). Kim et al. reported in human tonsillar tissues that T<sub>FH</sub> cells were more efficient in production of IL-4 and IL-10 as evidenced by intracellular staining and ELISA compared to non-T<sub>FH</sub> cells (Kim et al. 2001).

Using an IL-21 reporter mouse, Luthje and co-workers reported that the expression of IL-10 mRNA was restricted to IL-21<sup>+</sup> T<sub>FH</sub> cells (Luthje et al. 2012). Chevalier et al. reported that CXCR5<sup>+</sup> T cells expressed significantly higher levels of IL-10 as compared with CXCR5<sup>-</sup> T cells in human blood. (Chevalier et al. 2011). A study by Yusuf et al demonstrated that IL-4 production was observed by T<sub>FH</sub> cells but not non-T<sub>FH</sub> cells. The majority of the IL-4 was produced by the highest CXCR5 expressing cells and no IL-4 was made by non-T<sub>FH</sub> CD4 T cells (Yusuf et al. 2010). Our study also shows the importance of IL-21 in T<sub>FH</sub> cell-mediated B cell antibody production in adenotonsillar cells. Blocking the IL-21R by a recombinant neutralizing antibody significantly reduced the numbers of T<sub>FH</sub> cell and that was correlated with the reduction of specific antibody production by adenotonsillar B cells. Further details are described in detail separately in result chapters 4 and 5.

Research from human subjects and animal models suggest that there may be differences in innate and adaptive immunity in different age populations. However, it is unknown whether there is an age-associated change in T<sub>FH</sub> cell repertoire. In this study, we have found that there was an age-associated difference in T<sub>FH</sub> cell percentage in human NALT. A significant difference in the percentage of T<sub>FH</sub> cells between children and adults was seen, i.e. the mean T<sub>FH</sub> percentage was higher in children than in adults. Furthermore, in adults, there appeared to be a significant decrease in T<sub>FH</sub> percentage with age. However, when we further analysed the percentage of T<sub>FH</sub> cells in different age groups in children, no significant difference in the percentage was seen from age 1.5 to 13 years. The finding that the percentage of T<sub>FH</sub> cells in adenotonsillar tissues stayed at a relatively high level from the age 1.5 to 13 in children that were higher than in adults suggests that T<sub>FH</sub> cells were primed fairly early and rapidly in young childhood. It is possible that immediately after

birth, the intensive exposure to foreign agents eg. microbes during infancy rapidly primed T<sub>FH</sub> cells during which GC in secondary lymphoid tissues form intensively.

Studies in animal models suggest neonates are deficient in T<sub>FH</sub> priming. In a mouse study following immunisation, numbers of T<sub>FH</sub> cells (CXCR5<sup>high</sup> PD-1<sup>high</sup> CD4<sup>+</sup> cells) in draining LN in neonates (1-week-old) were significantly lower than in adult mice (6-8 weeks old) (Mastelic et al. 2012). Another study showed that the numbers of T<sub>FH</sub> cell in neonate mice were considerably lower than that in adults before immunization (baseline); and also neonates had significantly lower antibody production, affinity maturation, and GC reactions compared with adults following immunization (Debock et al. 2013).

Although the results from mouse studies suggest neonates are deficient in T<sub>FH</sub> priming, repeated and/or frequent exposure of adenotonsillar tissue to various antigens during infancy in children may enable NALT to rapidly develop T<sub>FH</sub> numbers at later infancy. It is known that young infants' response to immunisation is generally low and needs booster immunisations. There are evidences to support that at the age of 12 months, children respond to immunisations more efficiently than younger infants (Demirjian & Levy 2009; Wood & Siegrist 2011). It is difficult for this study to determine the time during infancy that effective priming of T<sub>FH</sub> occurs, as no children <1 year were able to be recruited into this study. There may be differences between human and mouse studies in that children are exposed to natural infections intensively during infancy whereas mice do not.

It is now known that T<sub>FH</sub> works closely together with GC B cells for optimal B cell response. Therefore, we expected there maybe a correlation between the numbers of GC B cell and T<sub>FH</sub> cell. Indeed, we show that there was a good correlation between the two. This finding is corroborated by several previous reports in mice and

humans. Baumjohann and colleagues using a mouse model showed that the proportions of both T<sub>FH</sub> cell (defined as CXCR5<sup>+</sup> PD-1<sup>+</sup> or CXCR5<sup>+</sup> ICOS<sup>+</sup>) and GC B cell were positively correlated with increasing doses of antigens (Baumjohann et al. 2013). A more recent study using human tonsils and LN reported that numbers of GC B cell (CD19<sup>+</sup> CD38<sup>+</sup> IgD<sup>-</sup>) were correlated with numbers of T<sub>FH</sub> cell (Renand et al. 2013).

T<sub>FH</sub> and GC B cell interactions are considered to be crucial in GC B cell response. In the GC, T<sub>FH</sub> cells interact with GC B cells via a range of molecular pairings including ICOS and ICOS ligand, PD-1 and PD-1 ligand, and IL-21 and its receptor (IL-21R). These interactions leads to the secretion of IL-4, IL-10 and IL-21 by T<sub>FH</sub> which are received by the GC B cells for optimal GC function in the form of high affinity memory B cells and long-lived plasma cells (Nutt & Tarlinton 2011).

Recent studies suggest there may be T<sub>FH</sub> equivalent in the circulation i.e. peripheral blood (pT<sub>FH</sub>) that express CXCR5 and ICOS markers and have similar B cell-help functions (Bentebibel et al. 2013; Morita et al. 2011; Pallikkuth et al. 2012; Simpson et al. 2010; Vinuesa & Cook 2011). Using these markers, we demonstrated the presence of such pT<sub>FH</sub> cells by flowcytometry. Similar to percentages of T<sub>FH</sub> in NALT, there appeared to be an age-associated difference in the percentage of pT<sub>FH</sub> cells. A lower percentage of pT<sub>FH</sub> cells were observed in 14-18 years group compared to children less than 13 years. There was a further reduction in pT<sub>FH</sub> cell percentage in older individuals. The similar correlation of these T<sub>FH</sub> percentages with age may suggest that these pT<sub>FH</sub> cells in the circulation are related to T<sub>FH</sub> cells found in secondary lymphoid tissues. It is plausible that these circulating pT<sub>FH</sub> cells may be originated from the same cellular precursors with the typical T<sub>FH</sub> in secondary lymphoid tissues.

A number of reports offer support to the hypothesis that these peripheral blood CD4<sup>+</sup> CXCR5<sup>+</sup> T cells are related to T<sub>FH</sub> cells found in NALT. These circulating CXCR5<sup>+</sup> CD4 T cells are absent from the peripheral blood of human ICOS-deficient patients which coincide with a disturbed GC formation in secondary lymphoid tissue (Bossaller et al. 2006). Recently, Morita et al. report that pT<sub>FH</sub> cells (CXCR5<sup>+</sup> CD4<sup>+</sup> T cells) from human peripheral blood provide better help to B cells than CXCR5<sup>-</sup> CD4 T cells. Circulating CXCR5<sup>+</sup> T cells are shown to be more effective in providing help to naïve B cells to differentiate toward plasmablasts at least in the presence of the superantigen SEB (Morita et al. 2011). Similar to tonsillar T<sub>FH</sub> cells, a recent study reported that blood CXCR5<sup>+</sup> CD4 T cells secreted IL-21 upon contact with naïve B cells, whereas CXCR5<sup>-</sup> CD4 T cells barely secreted any IL-21 (Morita et al. 2011). B cells were able to produce IgG, IgM and IgA only when co-cultured with CXCR5<sup>+</sup> cells in an IL-21- and ICOS-dependent manner. In contrast, CXCR5<sup>-</sup> CD4 T cells were unable to induce any switched immunoglobulin and only small amounts of IgM were produced (Morita et al. 2011).

In summary, we have shown the presence of a prominent number of T<sub>FH</sub> cells in NALT of children which were higher than adults. The fact that young children at 1.5-2 years already appeared to have developed a high number of T<sub>FH</sub> suggests that T<sub>FH</sub> priming by antigens during infancy (<1 year) is fairly efficient. T<sub>FH</sub> in adenotonsillar MNC expressed high levels of IL-4, IL-10 and IL-21 which are important for B cell antibody production. We also shown a correlation between the numbers of GC B cell and T<sub>FH</sub> cell in human NALT. Since T<sub>FH</sub> provides crucial help for B cell immune response, novel vaccine adjuvants to boost T<sub>FH</sub> number or function may be an attractive vaccination strategy to enhance vaccine efficacy in young children.

# **Chapter 4**

## **CpG-DNA promotes T<sub>FH</sub> cells in human NALT and PBMC**

## 4.1 Introduction

It is known that T cell help to B cells is an essential process for T cell-dependent antibody response. In the last decade,  $T_{FH}$  cells have been identified as a new T helper subset. Within secondary lymphoid organs, including tonsils,  $T_{FH}$  cells are considered to be specialized to regulate the development of effector and memory B cells and long-lived plasma cells. The interaction between  $T_{FH}$  and B cells leads to B cell differentiation and GC formation (Choi et al. 2011).

For better future vaccine design, there has been an increasing interest in understanding the mechanisms that controls the differentiation and maturation of  $T_{FH}$  cells. It has been reported that some vaccine adjuvants were able to promote antigen-specific  $T_{FH}$  cells. These antigen-specific  $T_{FH}$  cells were shown to help B cells to produce higher affinity antibodies (Fazilleau et al. 2009). Other studies using nanoparticle vaccines to malaria have shown enhanced humoral responses with robust GC formation in parallel with an expansion of antigen-specific  $T_{FH}$  cells (Moon et al. 2012).

In recent years, a number of novel adjuvants and vaccine delivery systems aimed to enhance vaccine immunogenicity have been under investigation (Spensieri et al. 2013). CpG-DNA is a TLR-9 ligand and has been suggested as a candidate vaccine adjuvant capable of enhancing antibody responses when combined to antigens (Klinman 2006; McCluskie & Davis 1999; Weeratna et al. 2000). However, it remains unclear whether CpG-DNA could act on  $T_{FH}$  through which to mediate B cell function.

CpG-DNA are short single-stranded synthetic oligonucleotides containing unmethylated Cytosine-Guanine (CpG, where the p indicates the phosphodiester bond) dinucleotides and also known as CpG motifs. CpG-DNA is recognized by

TLR-9 and activation of TLR-9 leads to strong immunostimulatory effects. Three types of stimulatory CpG-DNA have been suggested based on their immunostimulatory activities named as CpG-DNA type A, B and C (Klinman 2006; McCluskie & Davis 1999). In this study type B CpG-DNA was chosen to be examined as a candidate adjuvant. CpG-DNA class B was first identified in 1995 by Krieg and colleges (Krieg et al. 1995). Members of this class of CpG-DNA have been shown to induce B cell proliferation, differentiation and immunoglobulin and cytokine secretion. They induce pDC expression of costimulatory molecules, including CD86, CD54, MHC II and CD40 (Hartmann & Krieg 2000; Krieg & Yi 2000; Krieg et al. 1995).

DC are considered to be the most effective APCs and provide an important link between the innate and adaptive immunity (Stockwin et al. 2000). DCs are usually found where pathogens are most likely to first be encountered. Upon antigen encounter, DCs migrates to the draining LN via lymphatic vessels where they interact with T cells. The common nominator of several agents with adjuvant activity, including, CpG-DNA, is that they stimulate immune responses by inducing the maturation of DCs (Ramírez-Pineda et al. 2004). Recently, it has been reported that antigen presentation by DC is necessary to initiate T<sub>FH</sub> cell development (Choi et al. 2011; Goenka et al. 2011). pDC represents a small subset of DC and have been previously described in the T-cell area of human mucosa, especially in tonsils (Polak et al. 2008; Rescigno 2013). In humans, pDC uniquely express both TLR-7 and TLR-9 which are not found on other DC types (Fuchsberger, Hochrein & O'Keeffe 2005; Hornung et al. 2002).

Influenza virus is an important cause of respiratory tract infection and responsible for 3–5 million clinical infections and 250,000–500,000 fatal cases annually worldwide

(Stöhr 2002). Influenza virus infection induces host immune responses that help to reduce virus replication and prevent further virus spread. Protection against influenza virus infection has been shown to be mediated largely by anti-HA neutralizing antibodies, and their induction at high and sustained titres is essential for successful vaccination. The surface HA glycoprotein of influenza virus is a major target for antiviral activity as immune response to HA offer neutralizing antibodies following vaccination or natural infection (Wilks et al. 2012).

CpG-DNA is known to have a strong stimulatory effect on B cells. This TLR-9 ligand was also reported to enhance T cell-mediated help through DC maturation and differentiation (Krieg 2002). Accordingly, for working with T<sub>FH</sub> cells and B cells, CpG-DNA class B was chosen to be examined as a candidate adjuvant.

Giving that we showed there is a significant number of T<sub>FH</sub> in human NALT and it seems to be primed early in childhood, in this study, we investigated whether a candidate adjuvant CpG-DNA has any effect on T<sub>FH</sub> in NALT and subsequently on B cell antibody production.

## **4.2 Aims of Study**

More specifically, this study will investigate:

- Whether CpG-DNA promotes T<sub>FH</sub> cell frequencies in human NALT and PBMC.
- Whether CpG-DNA enhances B cell immunity to Influenza virus through T<sub>FH</sub> cells in human NALT.
- The role of IL-21 in T<sub>FH</sub> cell-mediated B cell antibody production.
- The role of pDC in the T<sub>FH</sub> cell mediated B cell antibody production.

### **4.3 Experimental Design**

Adenotonsillar MNC were stimulated with CpG-DNA, an influenza virus antigen (a partially purified seasonal H1N1 virus (A/H1N1Brisbane/2007)), or in combination of the two. The effects of these stimulations on T<sub>FH</sub> cells number were analysed by flowcytometry. HA-specific B cell responses were measured using ELISpot assay and cell culture supernatants were analysed for antibody production by ELISA. Additionally, the role of IL-21 in T<sub>FH</sub> cell-mediated B cell antibody production in adenotonsillar MNC was analysed by blocking IL-21 receptor. The role of pDC in the T<sub>FH</sub> cell mediated B cell antibody production was also analysed.

#### **4.3.1 Patients and Samples**

Surgically removed adenotonsillar tissues were obtained from both children and adults (1.5-36 years). Peripheral blood samples were also obtained. Patients who were previously vaccinated against influenza or who had any known immunodeficiency were excluded from the study.

#### **4.3.2 Mononuclear Cell Separation**

Mononuclear cells were isolated using Ficoll density centrifugation following methods described previously in the materials and methods chapter 2. Briefly, adenotonsillar tissues were minced by a scalpel to release cells into the medium. Then the cell suspension was filtered through a 70µm cell strainer. The adenotonsillar cells and peripheral blood were carefully layered onto 15 ml Ficoll-Paque™ PREMIUM and spun at 400g for 30 min at room temperature. The cloudy layer of mononuclear cells was carefully harvested. MNC were washed with sterile PBS solution, then the pellet was re-suspended in 5 ml RPMI-1640 culture medium with HEPES (Sigma-Aldrich, UK) supplemented with 10% heat inactivated FBS

(Sigma), 1% L-glutamine (Sigma), streptomycin (50µg/ml) and penicillin (50U/ml) (Sigma-Aldrich). After counting, each cell suspension was adjusted to contain  $4 \times 10^6$  cells/ml concentrations.

### **4.3.3 Influenza Virus Antigens and CpG-DNA**

#### **4.3.3.1 sH1N1 Virus Antigen**

The seasonal H1N1 influenza virus (sH1N1) antigen used in this study for cell stimulation experiments was derived from A/Brisbane/59/2007 strains. This Influenza antigen was  $\beta$ -propiolactone inactivated, partially purified A/Brisbane/59/2007 virus antigens from National Institute for Biological Standards and Control (NIBSC, UK).

#### **4.3.3.2 Recombinant HA**

The recombinant HA was derived from a seasonal H1N1 influenza virus (A/Brisbane/59/2007) (BEI Resources, ATCC, USA). This recombinant HA was used as the coating antigen in ELISA and ELISpot assays.

#### **4.3.3.3 CpG-DNA**

In this study, a type B CpG-DNA (CpG 2006, InvivoGen, USA) was chosen to be examined as a candidate adjuvant. CpG-DNA class B was first identified in 1995 by Krieg and colleges (Krieg et al. 1995).

### **4.3.4 Stimulation by Influenza Virus Antigen and CpG-DNA for Antibody and IL-21 Production**

The cell suspension was adjusted to contain  $4 \times 10^6$  cells/ml concentrations in RPMI-1640 medium. 500 µl/well of cells were cultured in 48-well flat bottom culture plates then stimulated with (1µg/ml) sH1N1 antigen alone or in combination with (0.1µg/ml) CpG-DNA, or CpG-DNA alone. Cells then were cultured for 24 hours for cytokine production and 14 days for antibody production at 37°C, 5% CO<sub>2</sub>. After

that, cell culture supernatants were collected from each well and stored at -80°C until further analysis by ELISA.

#### **4.3.5 Measurement of HA Specific IgG/IgM/IgA ELISA Assay**

ELISA assay in this study was designed and developed to detect influenza HA-specific IgG, IgM and IgA antibodies found in cell culture supernatants using a procedure as previously described (Mahallawi et al. 2013). In brief, ELISA plates were coated with recombinant HA purified protein then incubated overnight at 4°C. The following day, plates were washed then blocked for 1 hour at room temperature. Samples were then added and incubated for 2 hours at room temperature. Plates were washed and followed by the addition of alkaline phosphatase-conjugated mouse anti-human IgG or IgM or IgA. After 2 hours, plates were washed and PNPP substrate buffer were added then incubated at room temperature in dark. Finally, the OD at 405nm was measured using a microtiter plate reader and data were analysed using DeltaSoft software.

#### **4.3.6 Measurement of B Cell Antibody Response by ELISpot Assay**

ELISpot assay was used to measure the frequencies of antibody-producing B cells specific to HA of sH1N1 (A/Brisbane/59/2007) using a protocol previously described. Briefly, ELISpot plates were coated overnight at 4°C with recombinant HA of sH1N1. Plates were washed and blocked by incubation with RPMI containing 10% FBS at room temperature for 1 hour. Stimulated MNC were then added to the plates and incubated overnight at 37°C. Plates were washed and incubated with goat anti-human IgG biotin for 30 minutes at room temperature. After washing, Horseradish peroxidase Avidin D conjugate was added and incubated. Coloured spots were developed with the addition of substrate and counted using an automated ELISpot reader.

#### **4.3.7 CD45RO<sup>+</sup> Cell Depletion From Adenotonsillar MNC**

To evaluate the CpG-DNA stimulation to induce T<sub>FH</sub> cells from naïve T (CD45RO<sup>-</sup>) cells, effector and memory (CD45RO<sup>+</sup>) cells were depleted from the adenotonsillar MNC. The cells to be depleted (CD45RO<sup>+</sup>) were labelled with the specific magnetic micro-beads binding with the target surface molecules (CD45RO). The cell suspension is then allowed to pass through a MACS LD column, placed within the magnetic field of a MACS separator. The magnetically labelled cells (CD45RO<sup>+</sup> cells) are retained within the column, and the unlabelled cells (CD45RO<sup>-</sup> MNC) pass through the column and collected in a tube placed underneath it. The depletion of CD45RO<sup>+</sup> cells in adenotonsillar MNC removed the majority of T<sub>FH</sub> cells.

#### **4.3.8 Flow Cytometric Analysis of Cells**

##### **4.3.8.1 Cell Stimulation for Flowcytometry Analysis**

The cell suspension was adjusted to contain 4x10<sup>6</sup> cells/ml concentrations in RPMI-1640. 500 µl/well of cells were cultured in 48-well flat bottom culture plates and stimulated with (1µg/ml) sH1N1 antigen alone or in combination with (0.1µg/ml) CpG-DNA and CpG-DNA alone. For T<sub>FH</sub> cells analysis, PBMC and unfractionated adenotonsillar MNC were cultured for 3 days and CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC were cultured for 7 days at 37°C, 5% CO<sub>2</sub>. After that, cells were harvested for FACS staining.

##### **4.3.8.2 Cell Staining for Phenotyping for FACS Analysis**

For T<sub>FH</sub> cells identification, adenotonsillar MNC and PBMC were stained with anti-human-CD3, -CD4, -CXCR5 and -ICOS antibodies. All cell staining was performed as described earlier. Immediately after staining, cells were acquired/analysed using a BD FACS Calibur or fixed and permeabilized if needed for intracellular staining.

#### **4.3.8.3 Intracellular Staining for BCL-6, IL-21 and TLR-9**

For BCL-6 analysis, stimulated cells were incubated for 3 days. For IL-21 analysis, stimulated cells were incubated overnight, then 10µg/ml brefeldin A was added to each sample and further incubated for 4 hours at 37°C in 5% CO<sub>2</sub>. For TLR-9 analysis, freshly isolated cells were used for staining. After that, cells were harvested and washed twice with FACS staining buffer followed by surface staining with fluorochrome-labelled anti-human antibodies depending on the phenotype (Table 2.1) in the same procedure described earlier. Next, cells were fixed with intracellular fixation buffer. After incubation for 20 min, cells were washed twice with permeabilization buffer. Then the proper amount of either BCL-6, IL-21 or TLR-9 anti-human antibodies was added and incubated for 30 min. Cells were then washed with permeabilization buffer then resuspended in FACS staining buffer; and immediately acquired/analysed by flow cytometry.

#### **4.3.9 T cell Proliferation Assay**

T cell proliferation was analysed by CFSE staining followed by flow cytometry. Briefly, adenotonsillar MNC were labelled with CFSE (at 37°C, for 8 min) and the reaction was quenched with ice-cold media. After washing, cells were resuspended in RPMI 1640 media and stimulated with (1µg/ml) sH1N1 antigen alone or in combination with (0.1µg/ml) CpG-DNA and CpG-DNA alone, and incubated (at 37°C, in 5% CO<sub>2</sub>) for 5 days. Then cells were harvested, washed and resuspended in 0.02% BSA in PBS and anti-CD4-PE-Cy7, -CXCR5-PE and -ICOS-APC were added to stain CD4<sup>+</sup> CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells (T<sub>FH</sub> cells). T<sub>FH</sub> cell proliferation index was examined by gating from T<sub>FH</sub> cells (CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells) then analysed by flow cytometry using BD FACS Calibur (BD bioscience).

#### **4.3.10 Purification of T<sub>FH</sub> cells, B cells and pDC for Co-culture Experiments**

For co-culture experiments, cells were isolated by magnetic cell sorting using EasySep™ magnetic technology (STEMCELL Technologies Inc.) as described in details in material and method chapter 2. Briefly, adenotonsillar B cells were negatively selected using EasySep™ human B cell enrichment kit (purity >99%). Adenotonsillar CD4<sup>+</sup> T cells were also negatively selected using EasySep™ human CD4<sup>+</sup> T cell enrichment kit (purity >98%). Followed by the isolation of CD4<sup>+</sup> CXCR5<sup>hi</sup> (T<sub>FH</sub> cells) by positive selection using biotin anti-human CXCR5 antibody and EasySep™ biotin selection kit (purity >90%). Non-T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>-</sup>) was negatively selected from CD4<sup>+</sup> T cells gaining a purity of >99%. Adenotonsillar pDC were also negatively selected using EasySep™ human plasmacytoid DC enrichment kit (purity 96%).

#### **4.3.11 B Cell Help by T<sub>FH</sub> Cells**

The ability of adenotonsillar T<sub>FH</sub> cells to induce antibody production by adenotonsillar B cells was studied using an autologous system as follows. In co-culture experiments, B cells were cultured with an equal number of either T<sub>FH</sub> cells or non-T<sub>FH</sub> cells at a ratio of 1:1 using a cell concentration of 5x10<sup>5</sup> cells/ml. 250 µl/well of B:T co-culture cells were cultured in 96-well round bottom plate in the presence or absence of a combination of CpG-DNA and influenza virus antigen. Cells were then cultured at 37°C, 5% CO<sub>2</sub>, for 10 days for antibody production.

#### **4.3.12 Assessment of Effect of pDC**

In co-culture experiments, B cells were cultured with an equal number of T<sub>FH</sub> cells at a ratio of 1:1 using a cell concentration of 5x10<sup>5</sup> cells/ml with or without pDC. Purified pDC were then added to this mixture using a concentration of 5x10<sup>4</sup> cells/ml. 250 µl/well of B cells + T<sub>FH</sub> cells + pDC co-culture cells were cultured in

96-well round bottom plate in medium alone or in the presence or absence of a combination of CpG-DNA and influenza virus antigen. Cells were then cultured at 37°C, 5% CO<sub>2</sub> for 10 days for antibody production.

#### **4.3.13 Anti-IL-21R Blocking Experiments**

Adenotonsillar MNC suspension was adjusted to contain  $4 \times 10^5$  cells/ml concentrations in RPMI-1640 medium. Cells were cultured in 96-well round bottom plate in the presence or absence of different stimulants with or without the recombinant human anti-IL-21R Fc blocking antibody ( $\alpha$ -IL-21R). Firstly,  $\alpha$ -IL-21R was added to the designated wells and incubated for 1 hour at 37°C in a 5% CO<sub>2</sub> incubator. These cells were then stimulated with (0.5µg/ml) sH1N1 antigen in combination with (0.05µg/ml) CpG-DNA. Cells were then cultured at 37°C, 5% CO<sub>2</sub> for 3 days for T<sub>FH</sub> cell analysis by FACS and 10 days for antibody production. After 3 days, cells were harvested and stained for surface staining markers such as CD4, CXCR5 and ICOS in a same procedure described earlier. Furthermore, after 10 days incubation, cell culture supernatants were collected from each well and stored at -80°C until further analysis by ELISA to measure the specific HA of H1N1 IgM and IgA.

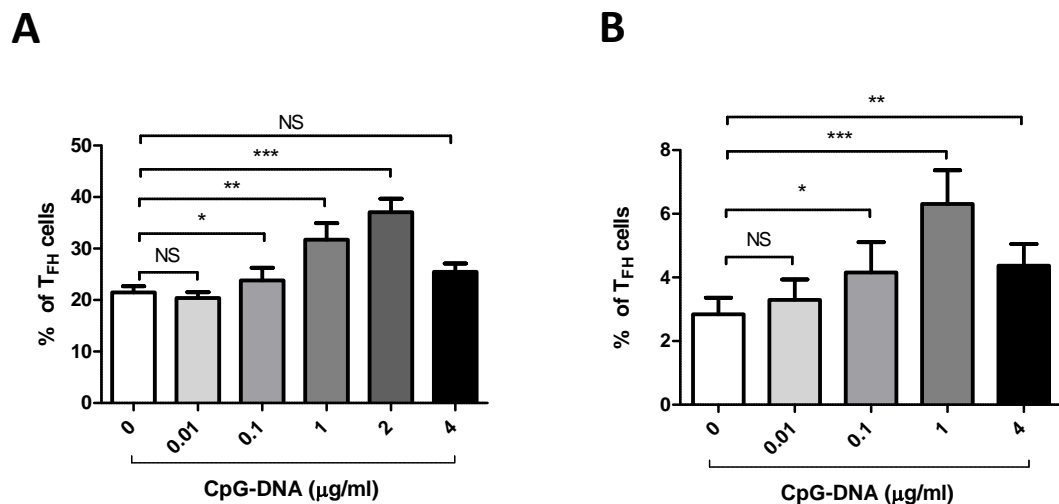
#### **4.3.14 Statistical Analysis**

Statistical analysis was carried out using GraphPad Prism 5 software. Differences between stimulated and unstimulated samples in the same group of subjects were analysed by Student's paired t test (parametric). Correlation between two factors was analysed by Pearson's (parametric) correlation test. Throughout the thesis, \* indicates a P-value between 0.01-0.05, \*\* indicates a P-value between 0.001-0.01 and \*\*\* indicates a P-value <0.001.

## 4.4 Results

### 4.4.1 CpG-DNA Promotes T<sub>FH</sub> Cells in Adenotonsillar Cells

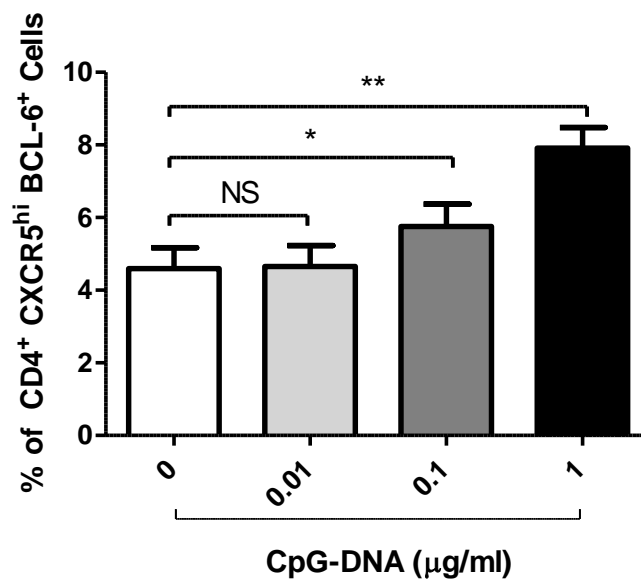
To determine whether CpG-DNA has any effect on T<sub>FH</sub> cells, both unfractionated adenotonsillar MNC (A) and CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC (B) were stimulated with different concentrations of CpG-DNA (0, 0.01, 0.1, 1, 2 and 4 µg/ml), followed by staining CD4/CXCR5/ICOS and flowcytometric analysis for T<sub>FH</sub> percentage. As shown in figure 4.1.A and B, CpG-DNA induced a significant increase in percentage of T<sub>FH</sub> cells in a dose dependent manner for both unfractionated MNC and CD45RO<sup>+</sup> cell depleted MNC.



**Figure 4.1. CpG-DNA promotes T<sub>FH</sub> cells in adenotonsillar cells**

Effect of CpG-DNA on percentage of T<sub>FH</sub> cells in unfractionated adenotonsillar MNC (A) and CD45RO<sup>+</sup> cell depleted MNC (B) following stimulation for 3 and 7 days respectively with different concentrations of CpG-DNA. The values represent the mean  $\pm$  SEM from 10 individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\*\**P* < 0.001, \*\**P* < 0.01, and \**P* < 0.05, NS: not significant).

Further analysis using  $CD4^{+} CXCR5^{hi} BCL-6^{+}$  as a  $T_{FH}$  cell marker following stimulation of adenotonsillar MNC for 72 hours with different concentrations of CpG-DNA (0, 0.01, 0.1 and 1  $\mu\text{g/ml}$ ) showed similar results. As shown in figure 4.2, CpG-DNA induced a significant increase in percentage of  $CD4^{+} CXCR5^{hi} BCL-6^{+}$  cells in a dose dependent manner.

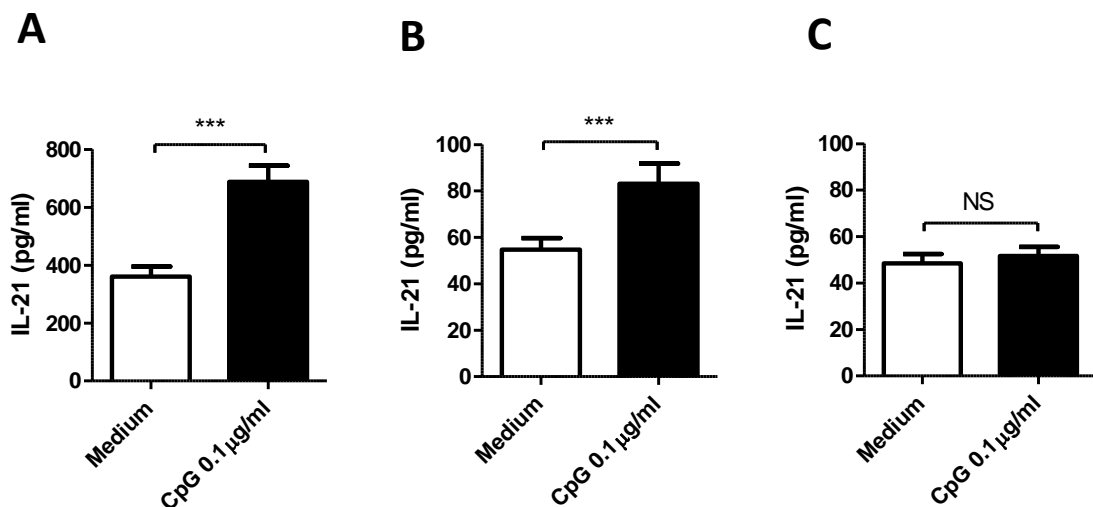


**Figure 4.2. CpG-DNA promotes  $CD4^{+} CXCR5^{hi} BCL-6^{+}$  cells in adenotonsillar cells**

Effect of CpG-DNA on percentage of  $CD4^{+} CXCR5^{hi} BCL6^{+}$  cells in MNC stimulated for 3 days with different concentrations of CpG-DNA. The values represent the mean  $\pm$  SEM from 7 individual tonsils (*p-value* derived from Paired two-tailed *t* test, \*\* $P < 0.01$ , and \* $P < 0.05$ , NS: not significant).

#### 4.4.2 Effect of CpG-DNA on IL-21 Production in Relation to T<sub>FH</sub> cells in NALT

As T<sub>FH</sub> was shown to correlate with a high level of IL-21 expression (results chapter 3), the effect of CpG-DNA on IL-21 production in cell culture supernatants were analysed following stimulation of unfractionated adenotonsillar MNC for 24 hours with CpG-DNA. As shown in figure 4.3.A, CpG-DNA induced a significant increase in IL-21 concentration. To ascertain the effect of CpG-DNA on T<sub>FH</sub> production of IL-21 purified CXCR5<sup>hi</sup> CD4<sup>+</sup> T cells (T<sub>FH</sub>) were co-cultured with purified B cells in the presence of CpG-DNA for 24 hours. IL-21 concentration was shown to increase significantly following CpG-DNA stimulation in the presence of T<sub>FH</sub> cells (Figure 4.3.B) but not in the presence of CXCR5<sup>-</sup> CD4<sup>+</sup> T cells (non-T<sub>FH</sub>) (Figure 4.3.C).



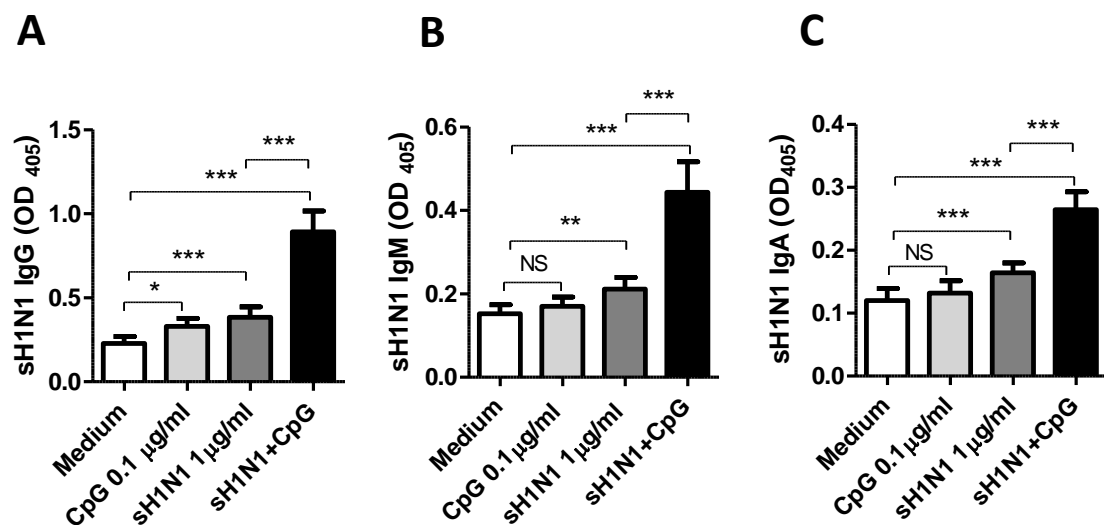
**Figure 4.3. IL-21 concentration increases in the presence of CXCR5<sup>hi</sup>, but not of CXCR5<sup>-</sup> CD4<sup>+</sup> T cells following stimulation with CpG-DNA**

Unfractionated adenotonsillar MNC were stimulated with CpG-DNA (A) and purified CXCR5<sup>+</sup> CD4<sup>+</sup> T cells (T<sub>FH</sub>) (B) or CXCR5<sup>-</sup> CD4<sup>+</sup> T cells (non-T<sub>FH</sub>) (C) were co-cultured with B cells in the presence of CpG-DNA. After 24 hours, cell culture supernatants were collected and analysed for IL-21 using ELISA. The value represents the mean  $\pm$  SEM from 22 (Unfractionated adenotonsillar MNC) and 17 (Purified cells) individual tonsils (*p-value* derived from Paired two-tailed *t* test, \*\*\**P* < 0.001, NS: not significant).

#### 4.4.3 Effect of CpG-DNA as an Adjuvant on B cell Antibody Production in

##### Relation to T<sub>FH</sub> Cells

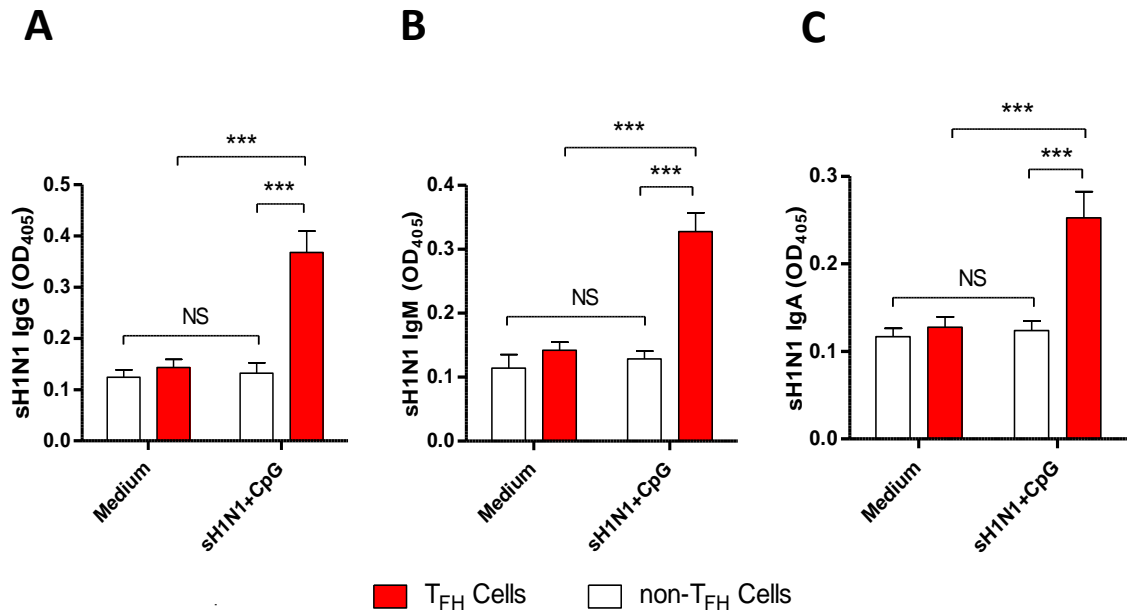
To investigate the adjuvanting effect of CpG-DNA on B cell antibody production in association with T<sub>FH</sub> cells, influenza HA-specific antibody response was studied following influenza virus antigen stimulation. Adenotonsillar MNC were stimulated with sH1N1 virus antigen with or without CpG-DNA for 14 days. Cell culture supernatants were collected and analysed for anti-sH1N1 HA IgG, IgM and IgA antibody production using ELISA. As shown in figure 4.4, the addition of CpG-DNA significantly enhanced the production of HA-specific IgG (Figure 4.4.A), IgM (Figure 4.4.B) and IgA (Figure 4.4.C) antibodies compared to sH1N1 virus antigen alone.



**Figure 4.4. Anti-sH1N1 HA-specific antibody production in adenotonsillar MNC following CpG-DNA and influenza antigen stimulations**

Adenotonsillar MNC were stimulated with sH1N1 virus antigen with or without CpG-DNA. After 14 days, cell culture supernatants were collected and analysed for anti-sH1N1 HA IgG (A), IgM (B) and IgA (C) antibody production using ELISA. Results are expressed as mean antibody titre (OD)  $\pm$  SEM from 34 individual tonsils. The OD was chosen to be used because no proper standard for IgM and IgA was found for anti-sH1N1 HA (*p*-value derived from Paired two-tailed *t* test, \*\*\**P* < 0.001 and \*\**P* < 0.01, NS: not significant).

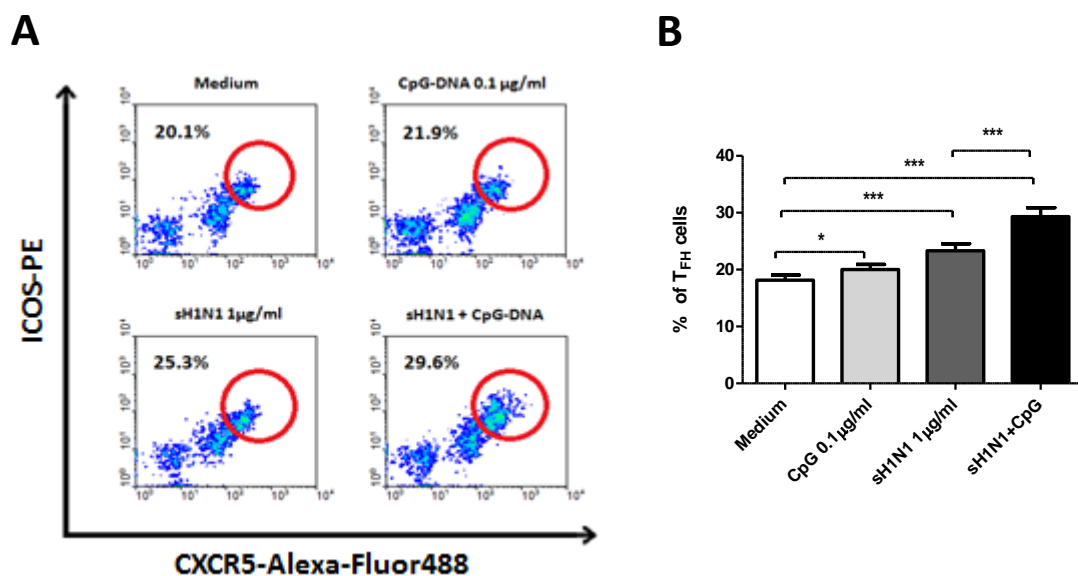
To ascertain whether T<sub>FH</sub> are required for the antibody induction induced by sH1N1 virus antigen and CpG-DNA, T<sub>FH</sub> (CD4<sup>+</sup> CXCR5<sup>hi</sup>) cells were purified and co-cultured with purified B cells in the presence of CpG-DNA and sH1N1 antigen. As shown in figure 4.5, the co-cultured cells stimulated by CpG-DNA and sH1N1 antigen induced significant antibody production only in the presence of CD4<sup>+</sup> CXCR5<sup>hi</sup> (T<sub>FH</sub>) cells, but not in the presence of CD4<sup>+</sup> CXCR5<sup>-</sup> (non-T<sub>FH</sub>) cells; i.e. T<sub>FH</sub> but not non-T<sub>FH</sub> cells were able to help B cells for the production of HA-specific IgG (Figure 4.5.A), IgM (Figure 4.5.B) and IgA (Figure 4.5.C) antibodies.



**Figure 4.5. HA-specific sH1N1 IgG, IgM and IgA titre are produced from B cells co-cultured with purified T<sub>FH</sub> cells but not with non-T<sub>FH</sub> cells**

Purified B cells were co-cultured with either purified T<sub>FH</sub> cells or non-T<sub>FH</sub> cells (at a ratio of 1:1). These cells were stimulated with sH1N1 virus antigen with or without CpG-DNA and CpG-DNA alone. After 10 days, cell culture supernatants were collected and analysed for anti-sH1N1 HA IgG (A), IgM (B) and IgA (C) antibody production using ELISA. The value represents the mean  $\pm$  SEM from 22 individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\*\**P* < 0.001, and NS: not significant).

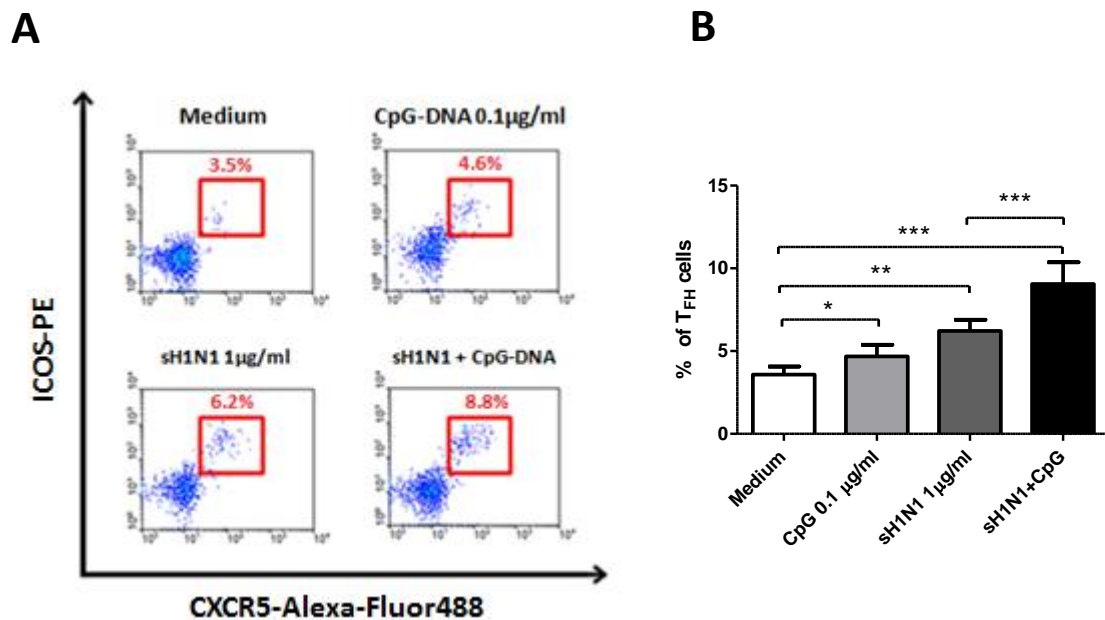
To find out whether the antibody production by adenotonsillar MNC following stimulation by CpG-DNA and sH1N1 virus antigen is correlated with changes in  $T_{FH}$ , percentage of  $T_{FH}$  were analysed. As shown in figure 4.6.A CpG-DNA alone, at a concentration of 0.1  $\mu\text{g/ml}$ , induced a small increase in percentage of  $T_{FH}$  cells. The sH1N1 virus antigen was also shown to increase the percentage of  $T_{FH}$  cells compared to unstimulated medium control (25.3% versus 20.1%). The addition of CpG-DNA to sH1N1 virus antigen significantly enhanced the percentage of  $T_{FH}$  cells elicited by sH1N1 antigen (29.6%).



**Figure 4.6. Effect of CpG-DNA on  $T_{FH}$  percentage in adenotonsillar MNC in the presence of an influenza virus antigen**

(A) A representative density plot of flowcytometric analysis of MNC adenotonsillar stimulated for 72 hours with a sH1N1 antigen with or without CpG-DNA. The value shown is the percentage of  $T_{FH}$  cells in  $CD4^+$  T cells. (B) Mean percentage of  $T_{FH}$  cells in adenotonsillar MNC stimulated with sH1N1 virus antigen with or without CpG-DNA. The values represent the mean  $\pm$  SEM from 37 individual tonsils ( $p$ -value derived from Paired two-tailed  $t$  test, \*\*\* $P < 0.001$  and \* $P < 0.05$ ).

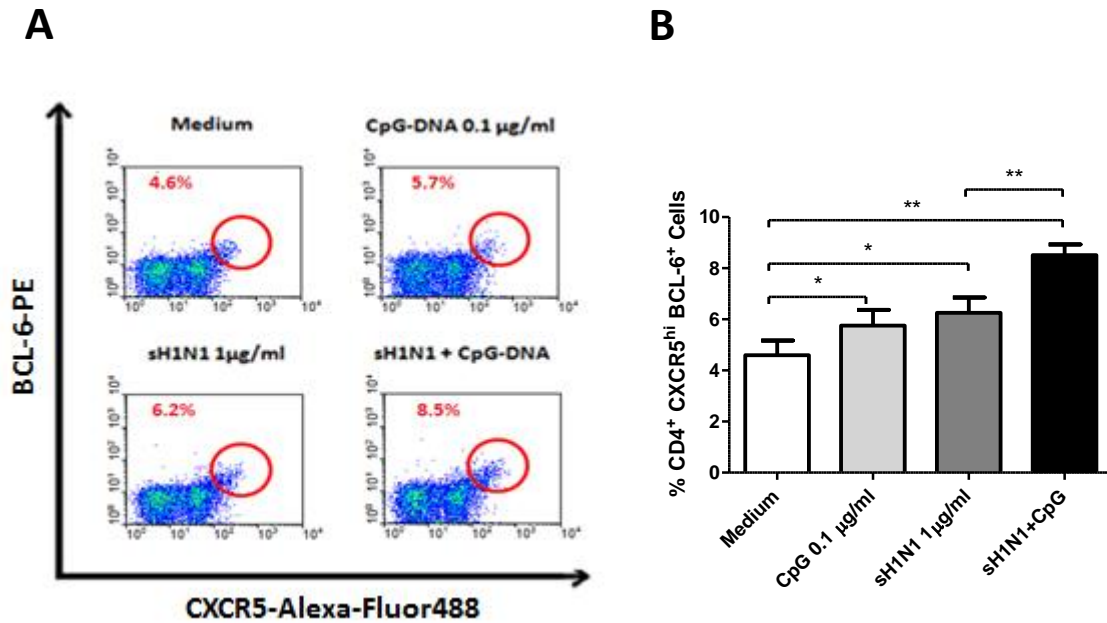
To investigate the induction of T<sub>FH</sub> cells from naïve CD4<sup>+</sup> T cells, adenotonsillar MNC samples were analysed after magnetic depletion of effector and memory (CD45RO<sup>+</sup>) T cell population. Further analysis using CD45RO<sup>+</sup> cell depleted adenotonsillar MNC showed similar results following stimulation with sH1N1 antigen with or without CpG-DNA. As shown in figure 4.7.A, CpG-DNA together with the sH1N1 virus antigen induced a marked increase in the percentage of T<sub>FH</sub> (mean, 8.8%), higher than CpG-DNA or sH1N1 antigen stimulation alone (means 4.6% and 6.2% respectively).



**Figure 4.7. Effect of CpG-DNA on T<sub>FH</sub> percentage in CD45RO<sup>+</sup> cell depleted MNC in the presence of an influenza virus antigen**

(A) A representative density plot showing effect of CpG-DNA and sH1N1 antigen on T<sub>FH</sub> percentage in CD45RO<sup>+</sup> cell depleted MNC following stimulation for 7 days. The value shown is the percentage of T<sub>FH</sub> cells in CD45RO<sup>+</sup> cell depleted MNC. (B) Mean percentage of T<sub>FH</sub> cells from CD45RO<sup>+</sup> cell depleted MNC stimulated with sH1N1 virus antigen and CpG-DNA. The values represents the mean ± SEM from 16 individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\*\**P* < 0.001, \*\**P* < 0.01, and \**P* < 0.05).

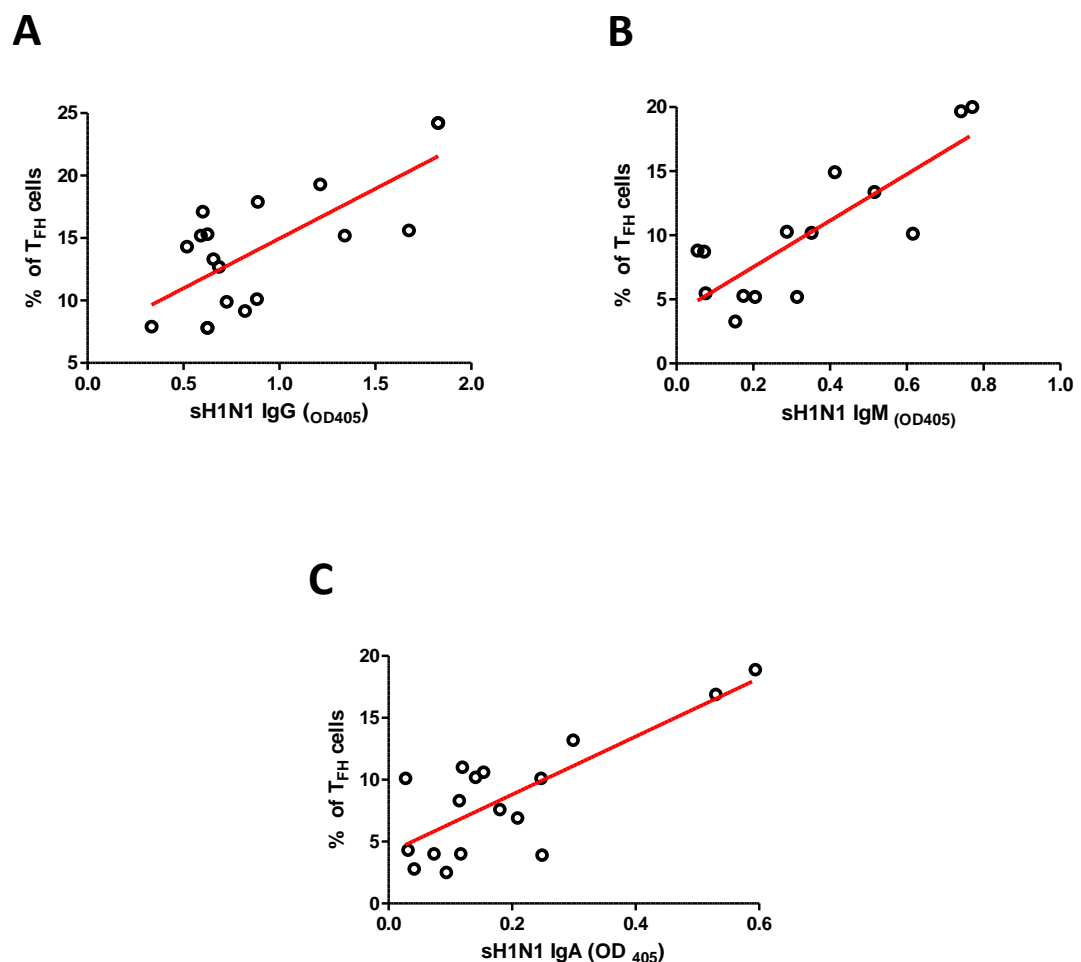
When  $CD4^+ CXCR5^{hi} BCL-6^+$  was used as a marker for  $T_{FH}$  cells, the effect of CpG-DNA and influenza antigen on  $T_{FH}$  cells was similar to above (Figure 4.8).



**Figure 4.8. CpG-DNA promotes  $CD4^+ CXCR5^{hi} BCL-6^+$  cells in adenotonsillar cells**

(A) A representative density plot flowcytometry analysis of MNC stimulated for 3 days with sH1N1 virus antigen with or without CpG-DNA and CpG-DNA alone. The value shown is the percentage of  $CD4^+ CXCR5^{hi} BCL6^+$  cells. (B) Summary percentage of  $CD4^+ CXCR5^{hi} BCL6^+$  cells from MNC stimulated with sH1N1 virus antigen with or without CpG-DNA and CpG-DNA alone. The values represent the mean  $\pm$  SEM from seven individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\*\**P* < 0.001, \*\**P* < 0.01, and \**P* < 0.05).

To determine whether there is a correlation between the enhanced antibody production induced by CpG-DNA and  $T_{FH}$ , percentage of  $T_{FH}$  cells were analysed in association with HA-specific sH1N1 IgG, IgM and IgA production. As shown in figure 4.9, the percentage of  $T_{FH}$  cells were correlated well with HA-specific sH1N1 IgG (Figure 4.9.A) ( $r = 0.72$ ,  $P < 0.001$ ), IgM (Figure 4.9.B) ( $r = 0.82$ ,  $P < 0.001$ ) and IgA levels (Figure 4.9.C) ( $r = 0.78$ ,  $P < 0.001$ ).

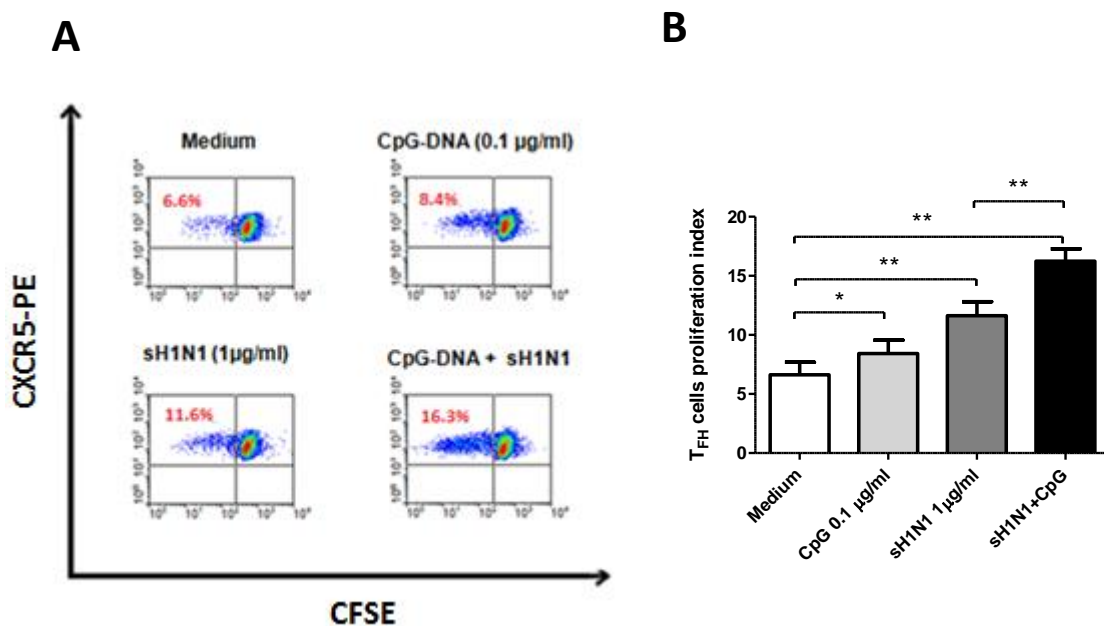


**Figure 4.9. Correlation between HA-specific sH1N1 antibody levels and percentage of  $T_{FH}$  cell in adenotonsillar MNC**

A regression linear plot showing the correlation between percentage of  $T_{FH}$  cell to the HA-specific sH1N1 IgG (**A**) ( $r = 0.72$ ,  $P < 0.001$ ,  $n=17$ ), HA-specific sH1N1 IgM (**B**) ( $r = 0.82$ ,  $P < 0.001$ ,  $n=17$ ) and HA-specific sH1N1 IgA levels (**C**) ( $r = 0.78$ ,  $P < 0.001$ ,  $n=17$ ).

#### 4.4.4 Effect of CpG-DNA and Influenza Antigen on T<sub>FH</sub> Cell Proliferation in Adenotonsillar MNC

To study whether T<sub>FH</sub> proliferate upon stimulation by CpG-DNA and influenza antigen, adenotonsillar MNC were stained with CFSE followed by stimulation with CpG-DNA and sH1N1 virus antigen for 5 days before flowcytometry. As shown in figure 4.10.A, CpG-DNA together with sH1N1 antigen induced marked T<sub>FH</sub> cell proliferation, with a mean T<sub>FH</sub> cell proliferation index of 16.3%, significantly higher than CpG-DNA alone and sH1N1 virus antigen alone (means 8.4% and 11.6% respectively).



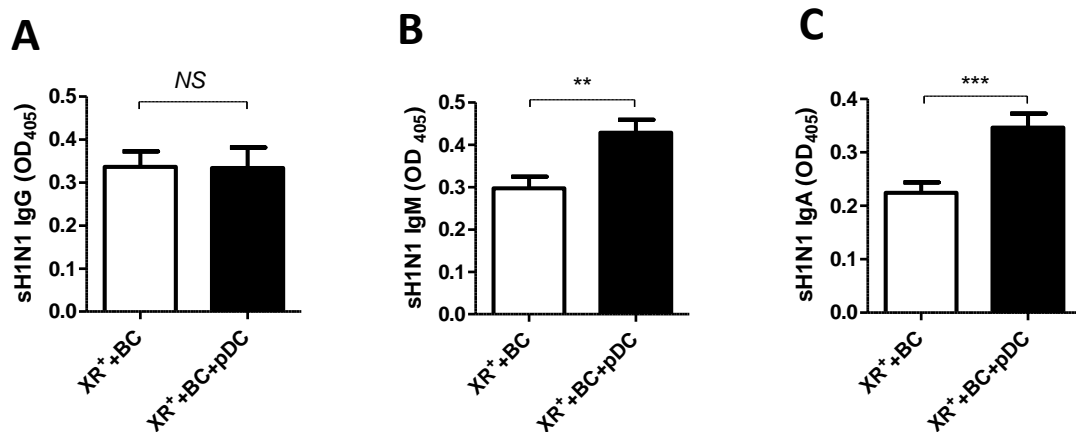
**Figure 4.10. Analysis of T<sub>FH</sub> cell proliferation in the presence of an influenza virus antigen and CpG-DNA**

(A) A representative CFSE staining analysis of MNC stimulated for 5 days with sH1N1 virus antigen with or without CpG-DNA, the value shown is the percentage of T<sub>FH</sub> cells proliferation in CD4<sup>+</sup> T cells. (B) Summary percentage of T<sub>FH</sub> cell proliferation from MNC stimulated with sH1N1 virus antigen with or without CpG-DNA. The values represent the mean  $\pm$  SEM of T<sub>FH</sub> cell proliferation index gated from CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells from 10 individual tonsils (*p-value* derived from Paired two-tailed *t* test, \*\*\*P < 0.001, \*\*P < 0.01, and \*P < 0.05).

#### 4.4.5 Effect of Plasmacytoid DC on T<sub>FH</sub>-mediated Help for B cell Antibody

##### Production

Having shown that T<sub>FH</sub> cells contribute to the B cell antibody production, efforts were made to identify the mechanisms for this action. As T<sub>FH</sub> did not show significant expression of TLR-9 (shown later in figure. 4.14), whereas pDC is known to express high levels of TLR-9, and also a significant number of pDC exists in tonsillar tissue, possible effect of pDC was studied. Purified B cells were co-cultured with purified T<sub>FH</sub> cells with or without purified pDC cells. These cells were stimulated with sH1N1 virus antigen combined with CpG-DNA. As shown in figure 4.11, the presence of pDC in the co-culture significantly enhances anti-sH1N1 HA-specific IgM (Figure 4.11.B) and IgA antibody production (Figure 4.11.C), although no difference was shown for IgG antibody (Figure 4.11.A).

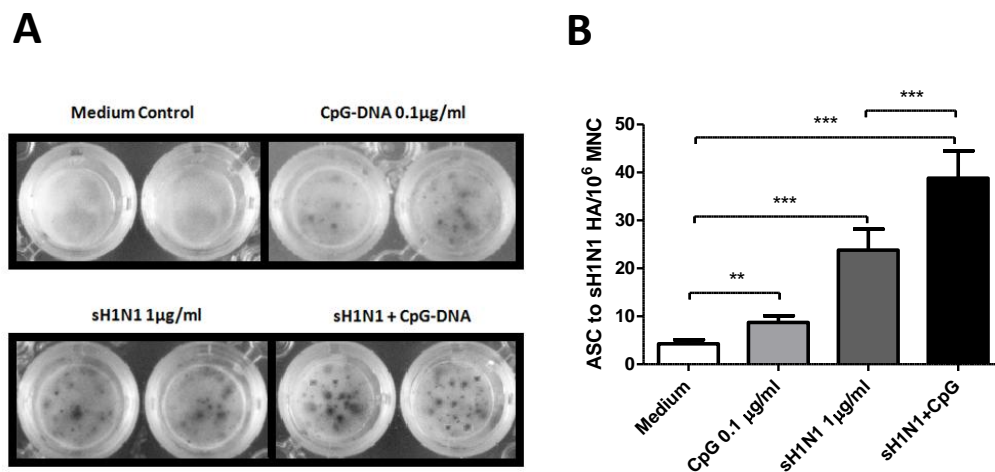


**Figure 4.11. pDC enhances the antibody production when added to purified T<sub>FH</sub> cells co-cultured with purified B cells**

Purified B cells were co-cultured with purified T<sub>FH</sub> cells (at a ratio of 1:1) with or without purified pDC cells. These cells were stimulated with sH1N1 virus antigen combined with CpG-DNA. After 10 days, cell culture supernatants were collected and analysed for anti-sH1N1 HA IgG (A), IgM (B) and IgA (C) antibody production using ELISA. The value represents the mean  $\pm$  SEM from 10 individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\**P* < 0.01, and \**P* < 0.05, NS: not significant). XR<sup>+</sup>: CXCR5<sup>hi</sup> cells, BC: B cells, pDC: plasmacytoid DC.

#### 4.4.6 Detection of Antibody Secreting Cells (ASC) Following Stimulation by CpG-DNA and Influenza Virus Antigen in Adenotonsillar cells

To confirm antibody production measured by ELISA, ELISpot assay was performed to enumerate the numbers of anti-sH1N1 HA B cell responses following stimulation by sH1N1 virus antigen with or without CpG-DNA. As shown in figure 4.12.A that CpG-DNA alone induced a small increase in numbers of HA-specific sH1N1 IgG ASC compared to unstimulated medium control (mean  $\pm$  SEM,  $7.8 \pm 1.1$  and  $3.2 \pm 0.35$  respectively). sH1N1 virus antigen was also shown to induce numbers of HA-specific sH1N1 IgG ASC (mean  $\pm$  SEM,  $23.8 \pm 3.7$ ). The addition of CpG-DNA to sH1N1 virus antigen significantly enhanced the numbers of HA-specific sH1N1 IgG ASC (mean  $\pm$  SEM,  $38.8 \pm 4.3$ ) in human adenotonsillar tissues (Figure 4.12.A and B).



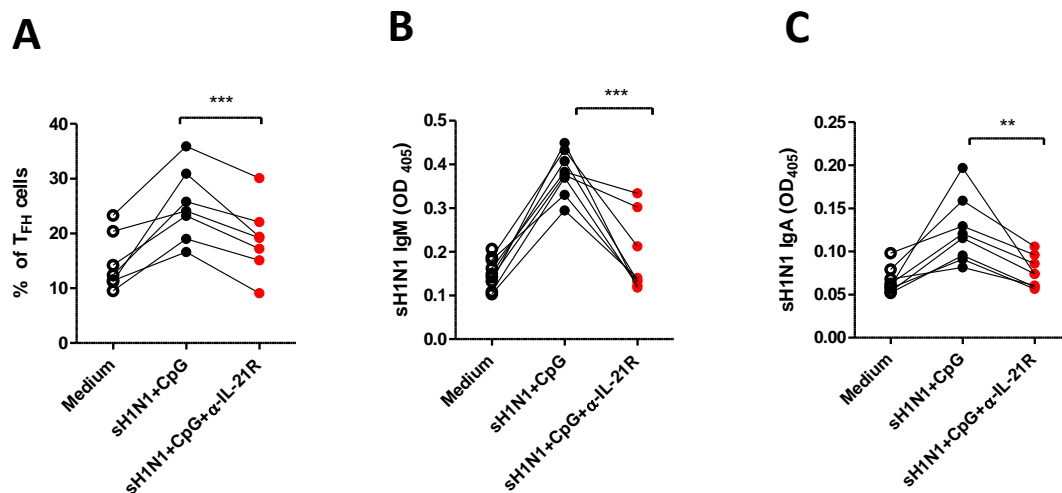
**Figure 4.12. ELISpot assay to enumerate antibody secreting B cell response showing number of HA-specific ASC**

Adenotonsillar MNC were stimulated with sH1N1 virus antigen with or without CpG-DNA and CpG-DNA alone. **(A)** Representative sample showing the number of HA-specific ASC. **(B)** Summary of the number of HA-specific ASC from MNC stimulated with sH1N1 virus antigen with or without CpG-DNA and CpG-DNA alone. The value represents the mean  $\pm$  SEM from 10 individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\*\**P* < 0.001, \*\**P* < 0.01, and \**P* < 0.05).

#### 4.4.7 IL-21 is Important in T<sub>FH</sub> cell-mediated B cell Antibody Production in

##### Adenotonsillar cells

In order to study the importance of IL-21 in T<sub>FH</sub> cell-mediated B cell antibody production, anti-IL-21R blocking antibody ( $\alpha$ -IL-21 R) was added to adenotonsillar MNC and incubated for 1 hour at 37 °C, then stimulated with CpG-DNA and sH1N1 antigen for 3 days (for T<sub>FH</sub> analysis) and 10 days (for antibody production). As shown in figure 4.13.A blocking IL-21 receptor reduced the percentage of T<sub>FH</sub> cells compared to un-blocked controls. Further, the antibody level (sH1N1 IgM and sH1N1 IgA) was also significantly reduced when IL-21 receptor was blocked as shown in figures 4.13.B and C respectively.

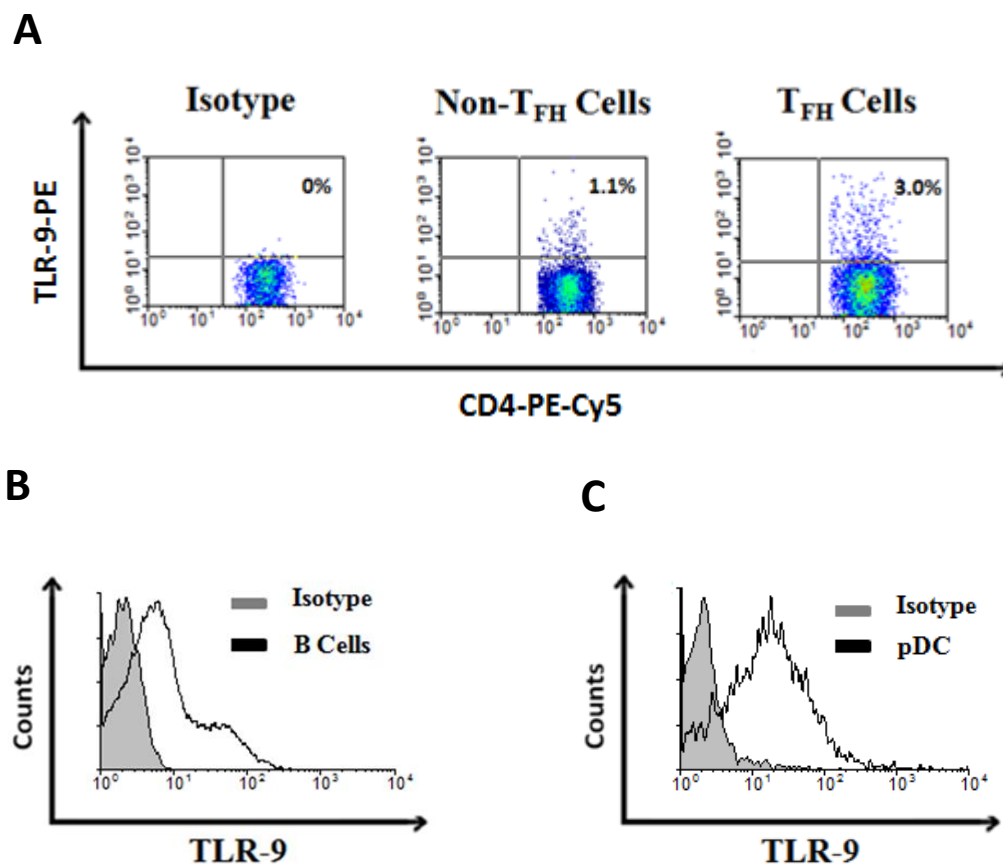


**Figure 4.13. Blocking IL-21R leads to a significant reduction in percentage of T<sub>FH</sub> cells and antibody production in human adenotonsillar tissue**

Anti-IL-21R blocking antibody ( $\alpha$ -IL-21R) was added to adenotonsillar MNC and incubated for 1 hour/37 °C, then stimulated with CpG-DNA and sH1N1 virus antigen. (A) Shows the percentage of T<sub>FH</sub> cells with and without  $\alpha$ -IL-21R. The specific sH1N1 IgM (B) and IgA (C) OD level from adenotonsillar MNC stimulated with sH1N1 virus antigen + CpG-DNA with and without  $\alpha$ -IL-21R after 10 days measured by ELISA. The values represent the mean  $\pm$  SEM from eight individual tonsils (*p*-value derived from Paired two-tailed t test, \*\*\**P* < 0.001 and \*\**P* < 0.01).

#### 4.4.8 TLR-9 Expression on Purified T<sub>FH</sub> cells, non-T<sub>FH</sub> cells, B cells and pDC

Purified T<sub>FH</sub> cells, non-T<sub>FH</sub> cells, B cells and pDC were analysed for the expression of TLR-9 by staining them with intracellular anti-TLR-9-PE. T<sub>FH</sub> cells show a higher number of TLR-9<sup>+</sup> cells compared to non-T<sub>FH</sub> cells (Figure 4.14.A). B cells show a high TLR-9 expression compared to isotype control (Figure 4.14.B). Further, a high TLR-9 MF1 was also seen in purified pDC (Figure 4.14.C).



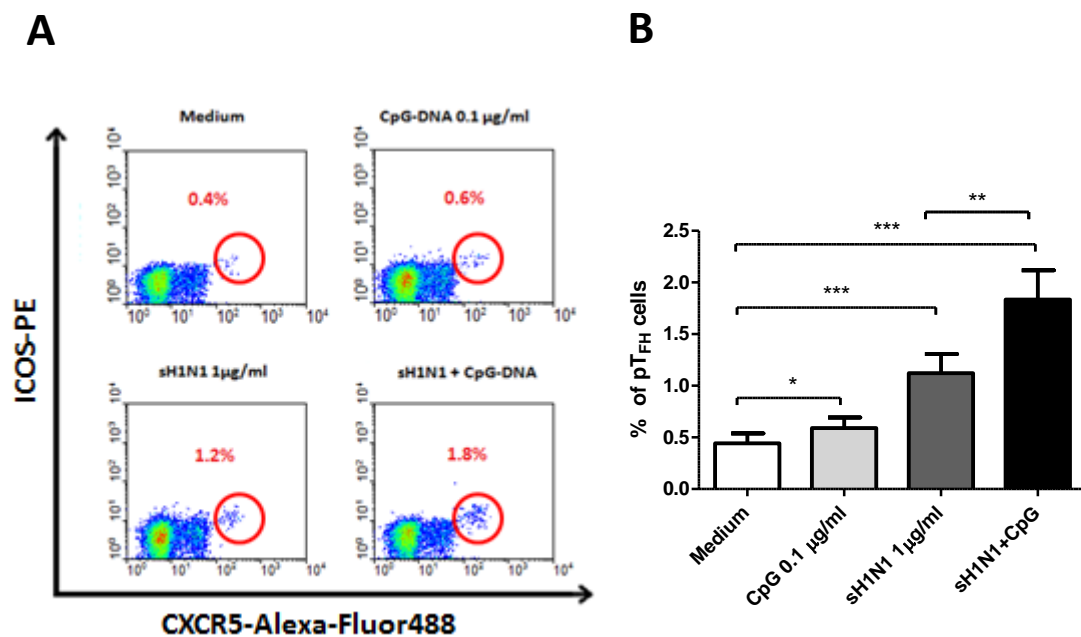
**Figure 4.14. TLR-9 expression in T<sub>FH</sub>, non-T<sub>FH</sub>, B cells and pDC**

(A) Representative density plot analysis of purified T<sub>FH</sub> cells and non-T<sub>FH</sub> cells compared to an isotype control. The values represented in (A) are the mean percentage of CD4<sup>+</sup> TLR-9<sup>+</sup> cells from five individual tonsils.

Representative histogram analysis of purified B cells (black line) (B) and purified pDC (black line) (C) compared to an isotype control (gray shade), showing the difference in TLR-9 expression from five individual tonsils.

#### 4.4.9 Effect of CpG-DNA on pT<sub>FH</sub> cells in PBMC

In order to study the effect of CpG-DNA on pT<sub>FH</sub> cells, freshly isolated PBMC were stimulated for 72 hours with CpG-DNA with or without sH1N1 antigen. As shown in figure 4.15.A, CpG-DNA alone induced a small increase in pT<sub>FH</sub> percentage (0.6%). In combination with sH1N1 antigen, CpG-DNA induced a marked increase in the percentage of pT<sub>FH</sub> (1.8%).

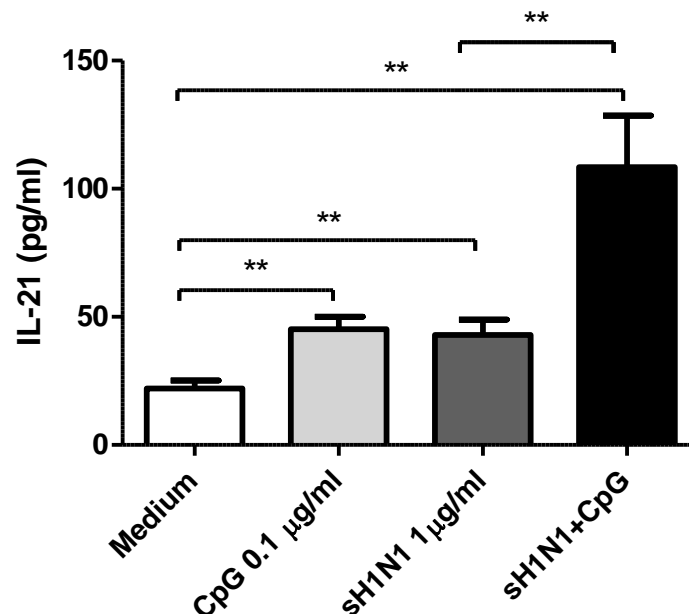


**Figure 4.15. CpG-DNA can promote pT<sub>FH</sub> cells in PBMC**

(A) Representative density plot flowcytometry analysis of PBMC stimulated for 72 hours with sH1N1 virus antigen with or without CpG-DNA and CpG-DNA alone. The value shown is the percentage of pT<sub>FH</sub> cells in CD4<sup>+</sup> T cells. (B) Summary percentage of pT<sub>FH</sub> cells from PBMC stimulated with sH1N1 virus antigen with or without CpG-DNA. The values represent the mean  $\pm$  SEM from 12 individual samples (*p*-value derived from Paired two-tailed *t* test, \*\*\**P* < 0.001, \*\**P* < 0.01, and \**P* < 0.05).

#### 4.4.10 Induction of IL-21 in PBMC with Influenza virus antigen and CpG-DNA

Induction of IL-21 was analysed in PBMC culture supernatants, 24 hours after stimulation with sH1N1 virus antigen with or without CpG-DNA and CpG-DNA alone. Production of IL-21 was shown to be higher with CpG-DNA combined with sH1N1 virus antigen stimulation (mean  $\pm$  SEM,  $108.4 \pm 20.1$ ) than that induced by either CpG-DNA (mean  $\pm$  SEM,  $45.1 \pm 4.9$ ) or sH1N1 virus antigen alone (mean  $\pm$  SEM,  $42.9 \pm 5.9$ ) (Figure 4.16).



**Figure 4.16. IL-21 Levels increases after stimulation with CpG-DNA in PBMC**

PBMC were stimulated with sH1N1 virus antigen with or without CpG-DNA and CpG-DNA alone. After 24 hours, cell culture supernatants were collected and analysed for IL-21 levels using ELISA. CpG-DNA combined with sH1N1 virus antigen induced high levels of IL-21 compared to sH1N1 virus antigen alone. The value represents the mean  $\pm$  SEM from 11 individual samples (*p-value* derived from Paired two-tailed *t* test, \*\**P* < 0.01).

## 4.5 Discussion

It is recognised now that  $T_{FH}$  cells are important in GC B cell differentiation and antibody responses, therefore promoting  $T_{FH}$  frequency and function may be a good vaccination strategy. Recently, it has been reported that certain adjuvants were able to enhance the generation and development of  $T_{FH}$  (Moon et al. 2012; Su et al. 2013). As CpG-DNA has been identified as a vaccine adjuvant capable of enhancing B cell responses when combined to different antigens (Cooper et al. 2004; Halperin et al. 2003; Rynkiewicz et al. 2011; Sagara et al. 2009; Siegrist et al. 2004), we asked whether CpG-DNA has an effect on  $T_{FH}$  and thereby enhance specific antibody production.

In this study, we found that the percentage of tonsillar  $T_{FH}$  cells (Figure 4.1) was increased by CpG-DNA stimulation in a dose dependent manner in both unfractionated and  $CD45RO^{+}$  cell-depleted adenotonsillar MNC. This suggests that CpG-DNA may activate and/or promote the induction of  $T_{FH}$  cells.

We also showed that co-incubation of CpG-DNA with influenza virus antigen induced a much enhanced increase in the percentage of  $T_{FH}$  cells. The addition of CpG-DNA to sH1N1 virus antigen also significantly enhanced the HA-specific IgG, IgM and IgA antibody production compared to the influenza virus antigen alone. This enhancement of  $T_{FH}$  cells percentage strongly correlated with the levels of the influenza HA-specific IgG, IgM and IgA antibodies. To ascertain whether  $T_{FH}$  were required for this antibody induction,  $T_{FH}$  cells and non- $T_{FH}$  cells were purified and co-cultured with purified B cells in the presence of CpG-DNA and sH1N1 antigen. We reported that the co-cultured cells induced significant antibody production only in the presence of  $CD4^{+} CXCR5^{hi}$  ( $T_{FH}$ ) cells, but not in the presence of  $CD4^{+}$

CXCR5<sup>+</sup> (non-T<sub>FH</sub>) cells; i.e. T<sub>FH</sub> but not non-T<sub>FH</sub> cells were able to help B cells for the production of HA-specific antibodies. These results suggest that the enhanced antibody production by CpG-DNA may be mediated, at least in part, by T<sub>FH</sub> cells, and our results are also corroborated by several recent studies.

Using a murine model Mastelic et. al reported that adjuvantation with CpG-DNA increased T<sub>FH</sub> and GC B cell neonatal responses, up to adult levels. These mice were immunized with tetanus toxoid adsorbed to aluminium hydroxide with or without a class B CpG-DNA. The addition of CpG-DNA significantly enhanced the numbers of T<sub>FH</sub> cells especially in neonatal mice (Mastelic et al. 2012). The study also reported that the increase in tetanus toxoid-specific IgG antibody production was associated with an increase in the numbers of neonatal GC B cells (Mastelic et al. 2012). More recently, Chakarov and Fazilleau reported that mice vaccinated with a peptide in incomplete Freund's adjuvant (IFA) combined with CpG-DNA induced a significant increase in antigen-specific T<sub>FH</sub> cells compared to mice vaccinated with IFA only. This CpG-DNA effect on T<sub>FH</sub> cells was dose dependent and was seen only in CpG-DNA type B and not type A or C (Chakarov & Fazilleau 2014). Furthermore, adjuvantation of IFA with CpG-DNA type B increased both the antigen specific GC B cells and plasma cells in correlation with a raise in serum antigen-specific IgG (Chakarov & Fazilleau 2014).

In line with these findings, Rookhuizen and DeFranco reported a robust expansion of total and antigen-specific GC B cells and high titre of antigen specific IgG antibodies in mice vaccinated with an antigen adjuvanted with CpG-DNA (Rookhuizen & DeFranco 2014). CpG-DNA enhanced the total number of T<sub>FH</sub> cells per LN and also

increased the proportion of  $T_{FH}$  in activated  $CD4^{+}$  T cells (Rookhuizen & DeFranco 2014).

Using an ELISpot assay, we also detected significant numbers of IgG ASC upon stimulation by the sH1N1 virus antigen and CpG-DNA. Co-incubation of CpG-DNA with sH1N1 antigen significantly augmented the antigen-specific IgG ASC to influenza HA in adenotonsillar cells compared to the antigen stimulation alone. It is likely that influenza HA- specific memory B cells are abundant in NALT, which respond to the antigen stimulation and CpG-DNA. Our results also demonstrated the predominance of IgG ASC that is concordant with previous studies showing memory B cell responses to protein antigens in human NALT (Boyaka et al. 2000; Nadal et al. 1992).

A significant increase in  $CD4^{+}$   $CXCR5^{hi}$   $BCL-6^{+}$  was seen after CpG-DNA stimulation in a dose dependent manner and this was correlated with the increase in numbers of  $T_{FH}$  cells. BCL-6 is the master regulatory transcription factor that controls  $T_{FH}$  cell differentiation (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009b). In this study BCL-6 expression was assessed by flowcytometry. The BCL-6 expression in  $CD4^{+}$  T cells was shown to be increased consistently by stimulation with CpG-DNA and sH1N1 antigen.

A rise in number of  $T_{FH}$ -IL-21<sup>+</sup> cells after stimulation with CpG-DNA (data not shown), that was correlated with an enhanced IL-21 production in cell culture supernatant. We further showed that mainly  $T_{FH}$  cells but not non- $T_{FH}$  cells produced IL-21 following stimulation with CpG-DNA. It is known that IL-21 plays an important role in  $T_{FH}$  cell differentiation and B cell immunity *in vivo* (Eto et al. 2011).

We showed in this study that tonsillar T<sub>FH</sub> cells were able to proliferate following stimulation with CpG-DNA and sH1N1 virus antigen. This may suggest that there are antigen-specific T<sub>FH</sub> (eg. Influenza antigen-specific T<sub>FH</sub>) which proliferate upon antigenic stimulation. The fact CpG-DNA enhances T<sub>FH</sub> proliferation would indicate that it is possible to use CpG-DNA to modulate T<sub>FH</sub> function to enhance B cell response. Studies by Morita et al. showed the induction of blood CXCR5<sup>+</sup> CD4<sup>+</sup> T cells to proliferate after stimulation with CMV or an inactivated influenza virus (Morita et al. 2011). A study by Rasheed and colleagues reported that a polyclonal stimulation of tonsillar CD4<sup>+</sup> T cells with anti-CD3/CD28 antibodies induced T<sub>FH</sub> cell proliferation (Rasheed et al. 2006). Bendigs et al. showed that the addition of CpG-DNA to anti-CD3-stimulated T cells induced a proliferative responses in the absence of APC and suggested that CpG-DNA could directly co-stimulate T cells (Bendigs et al. 1999). Lacroix-Lamandé *et al.* reported in mouse model, CpG-DNA administered orally induced a significantly increased numbers of CD4<sup>+</sup> T cell observed in the intestinal mucosa and the draining lymph nodes (Lacroix-Lamandé et al. 2009).

TLR-9 expression was examined by intracellular staining followed by flowcytometry, and we found that TLR-9 was detectable only at a low level on T<sub>FH</sub> cells. TLR expression in T cells remains inconclusive, however, several studies have reported the expression of TLR-9 on CD4<sup>+</sup> T cells and the direct co-stimulatory effects of CpG-DNA on primary T cell activation (Kabelitz 2007). A previous study by Bendigs et al. showed that TLR-9 ligand (CpG-DNA) was able to co-stimulate T cell proliferation and cytokine production in the absence of APCs (Bendigs et al. 1999). Another study demonstrated in murine purified CD4<sup>+</sup> T cells that CpG-DNA directly enhances CD4<sup>+</sup> T cell proliferation and IL-2 production. They also reported

that MyD88 expression particularly in CD4<sup>+</sup> T cells was required for the CpG-DNA-mediated antibody response to a T cell dependent antigen in vivo (Gelman et al. 2006). Treatment of highly purified activated mouse CD4<sup>+</sup> T cells with dsRNA synthetic analog poly (I:C) and CpG-DNA upregulated the expression of TLR-3 and TLR-9 respectively, although peptidoglycan and LPS (respective ligands for TLR-2 and TLR-4) showed no effect (Gelman et al. 2004).

As expected, TLR-9 was expressed in tonsillar B cells. The TLR-9 expression by B cells is well documented (Bourke et al. 2003; Jiang et al. 2007). pDC was also found to express TLR-9. In humans, the expression of TLR-9 by pDC is unique in the DC system and other DC types including conventional DC were not found to express TLR-9 (Fuchsberger, Hochrein & O'Keeffe 2005; Hornung et al. 2002).

We have shown that T<sub>FH</sub> express high levels of IL-21 and that were important for B cell antibody production. T<sub>FH</sub> cells also express IL-21 receptor (IL-21R), and this coexpression reflects an autocrine loop (Nurieva et al. 2008; Silver & Hunter 2008). Recently it has been reported that IL-21 acts as growth factor and it is required for the generation and/or maintenance of T<sub>FH</sub> cells (Vogelzang et al. 2008). Our study showed the importance of IL-21 in T<sub>FH</sub> cell-mediated B cell antibody production in adenotonsillar cells. Blocking the IL-21R by a neutralizing antibody significantly reduced the numbers of T<sub>FH</sub> cells. The reduction of T<sub>FH</sub> cells was correlated with a significant reduction of HA-specific IgM and IgA production. Because the Fc portion of IL-21R-Fc protein was from human IgG, the control for these experiments was human IgG. Consequently, specific-HA IgG secretion could not be analyzed in these cultures because of the cross-reactivity of the Fc portion of IL-21R-Fc in the IgG ELISA.

In agreement with these findings, Vogelzang and colleagues reported that IL-21 deficient mice exhibited a marked reduction in GC B cells and GC formation compared with wild type mice 7 days after immunization. Further, major defects in IgG1 production was reported in these IL-21 deficient mice (Vogelzang et al. 2008). Analyses of T<sub>FH</sub> cells 7 days after immunization shown a defect in the generation of T<sub>FH</sub> cells, defined as CD4<sup>+</sup> CXCR5<sup>+</sup> ICOS<sup>+</sup> T cells, in IL-21 deficient mice (Vogelzang et al. 2008). Nurieva et al. reported that the numbers of both T<sub>FH</sub> cells and GC B cells were significantly lower in IL-21 deficient mice immunized with keyhole limpet hemocyanin (KLH) compared with wild type mice (Nurieva et al. 2008).

Using purified T<sub>FH</sub> cells and B cells from human tonsillar tissues, Berglund et al. have reported a major reduction in IgM production when IL-21 was blocked by neutralizing antibody (IL-21R-Fc) compared to unblocked controls (Berglund et al. 2013). Rodríguez-Bayona et al. studied purified tonsillar T<sub>FH</sub> cells and plasma cells co-cultured in the presence or absence of neutralizing antibody (IL-21R-Fc). This study showed that secretion of IgA by plasma cells was significantly reduced when IL-21R was neutralized as a result of blocking T<sub>FH</sub> cell-derived IL-21. Further, blocking IL-10 did not reduce the T<sub>FH</sub> cell effect on plasma cell antibody production, indicating that T<sub>FH</sub> cell-derived IL-21 was the active factor (Rodríguez-Bayona et al. 2012). Therapies aimed at blocking the IL-21/IL-21R signalling, either directly on IL-21/IL-21R or indirectly targeting T<sub>FH</sub> cells to control the differentiation of autoreactive B cells into plasma cells, was suggested as a potential strategy for treatment of autoimmune diseases (Moens & Tangye 2014).

To determine whether pDC cells contribute to the adjuvant effect of CpG-DNA in the T<sub>FH</sub> cell mediated B cell antibody response, purified pDC were added to T<sub>FH</sub> cells and co-cultured with B cells. We found that the addition of pDC to T<sub>FH</sub>-B cell co-culture significantly enhanced the influenza HA-specific antibody response. This suggests that pDC may contribute significantly to CpG-DNA-mediated T<sub>FH</sub> help to B cell antibody response. There has been no data available on any potential effect of pDC on T<sub>FH</sub> in the published literature. However, it has been shown that antigen presentation by myeloid DC is necessary to initiate T<sub>FH</sub> cell development (Choi et al. 2011; Goenka et al. 2011), despite that in most cases, antigen presentation by B cells is responsible for promoting the full differentiation program of T<sub>FH</sub> cells (Ballesteros-Tato & Randall 2014; Johnston et al. 2009; Nurieva et al. 2009). As it is known that pDC express high levels of TLR-9, and we showed there is a prominent number of pDC in adenotonsillar tissue, pDC may play an important part in the T<sub>FH</sub>-mediated B cell help for antibody response in human NALT. Recently, Chakarov and Fazilleau reported a significant increase in T<sub>FH</sub> cell development upon the addition of CpG-DNA to other vaccine adjuvant and that was depended on TLR-9 signalling in CD11c<sup>+</sup> DC. They also demonstrated an important role played by monocyte-derived DCs on the T<sub>FH</sub> cell response after CpG-DNA vaccination (Chakarov & Fazilleau 2014).

CpG-DNA has strong immunostimulatory effects on pDC through TLR-9 recognition and signalling (Krug et al. 2001a; Krug et al. 2001b; Rothenfusser et al. 2002). Moseman et al. reported that CpG-DNA type B promoted pDC to prime allogeneic naïve CD4<sup>+</sup> T cells (Moseman et al. 2004). Recently, Deal et al. reported that rotavirus-specific serum and mucosal antibody responses were enhanced upon the addition of purified pDC (Deal et al. 2013). Another study showed that the

depletion of pDC impaired the specific influenza antibody response (Jego et al. 2003). Knowing that NALT are enriched with pDC (Polak et al. 2008; Rescigno 2013), this important role played by pDC in the T<sub>FH</sub> cell mediated B cell antibody response might be an important strategy in modulating the immune response against respiratory pathogens.

In summary, we show that CpG-DNA enhanced B cell antibody response that was correlated with induction of T<sub>FH</sub> cells in human NALT, suggesting that CpG-DNA may be able to enhance vaccine immunogenicity through modulating T<sub>FH</sub> function. IL-21 is crucial in T<sub>FH</sub> cell-mediated B cell antibody production in adenotonsillar cells. We also show an important role of pDC in enhancing the antibody levels in our tonsillar T<sub>FH</sub> - B cells co-cultures. Enhancing vaccine immunogenicity through modulation of T<sub>FH</sub> function in human NALT using immunological adjuvants such as CpG-DNA may be an effective vaccination strategy against respiratory pathogens.

# **Chapter 5**

## **Effect of $T_{FH}$ on Live Attenuated Influenza Vaccine (LAIV)-induced antibody response in NALT**

## 5.1 Introduction

Influenza virus is highly contagious and it is transmitted through airborne droplets and via the nasal mucosa. The virus infects by binding their surface glycoprotein HA to sialic acid receptors on the host epithelia cell surface (Barbey-Martin et al. 2002). Intranasal vaccination has been proposed for a more effective and biologically relevant way of immunization against influenza. Intranasal vaccination most likely relies on the local mucosal immune tissue for both local and systemic immune responses. Mucosal immunity in the upper respiratory tract is considered the first line of defence against a number of pathogens of both bacterial and viral origin. Human adenoids and tonsils are major components of NALT and known to be main induction sites for both mucosal and systemic immunity against upper respiratory tract pathogens including influenza infection (Kiyono & Fukuyama 2004; Tamura & Kurata 2004; Wu & Russell 1997; Zuercher et al. 2002).

In the 1940s, inactivated influenza vaccines were first presented and they are still considered the main formulation of influenza vaccines. The aim of influenza vaccination is to induce an immunological protection against influenza infection. Protection following natural infection with influenza is primarily mediated by anti HA-specific antibodies in serum and mucosa, and T-cell responses associated with reduced disease severity (Brokstad et al. 2001). Influenza vaccination is the main preventative method against influenza infection and its associated complications (Brokstad et al. 2001; Cox & Subbarao 1999).

Live Attenuated Influenza Vaccine (LAIV) is a live, trivalent intranasal spray vaccine that produces the HA and NA surface antigens from a number of influenza viruses including one influenza A (H1N1) virus, one influenza A virus (H3N2), and

one influenza B virus (WHO). Studies have reported LAIV vaccines delivered intranasally were able to induce strong immune responses including induction of mucosal IgA and IgG, and serum IgG and enhanced local cytokine responses, which may provide better protection than intramuscular injection of inactivated influenza vaccines. LAIV vaccines were also shown to stimulate stronger cellular immune response by inducing influenza specific memory T cells and B cells (De Filette et al. 2006; Vajdy et al. 2007). Intranasal vaccination with LAIV has been used successfully in several countries including USA and Canada (under the commercial name FluMist<sup>®</sup>) with good efficacy (Belshe 2004). Most recently, LAIV Intranasal vaccination has also been licensed in Europe including the UK (under the commercial name Fluenz<sup>™</sup>). Both live attenuated vaccine and inactivated vaccines are currently in use. At present, seasonal influenza vaccination are recommended to be taken every year using TIV for all individuals in particular, children aged 6 months or older, those with a diversity of chronic illnesses and health care workers. In addition, LAIV is also recommended for healthy non-pregnant people aged 2-49 years (Osterholm et al. 2012).

Intramuscular vaccinations with TIV mainly induce serum HAI antibody responses, in older children and adults who have significant immunological memory to influenza virus. In contrast, intranasal administration of LAIV induces both serum and mucosal antibody responses, particularly in young, non-immune children (Belshe 2004; Cox & Subbarao 1999; De Filette et al. 2006). Recent studies reported that LAIV intranasal influenza vaccine consistently showed higher efficacy in young children when compared to TIV delivered intramuscularly (Osterholm et al. 2012).

Although it has been shown to be safe and effective in humans, not much research has been done to evaluate the local mucosal immunity induced by these LAIV intranasal vaccines. As these vaccines contain live attenuated viruses and administered through the nasal mucosa mimicking natural infection, it may induce an immune response resembling natural immunity. NALT components, including adenotonsillar tissues are local mucosal immune organs in the upper respiratory tract; therefore, intranasal vaccines are likely to depend on these immune tissues to induce specific immune responses.

Within secondary lymphoid organs, T<sub>FH</sub> cells have been identified as a new T helper subset specialized to regulate the development of effector and memory B cells and long-lived plasma cells. The interaction between T<sub>FH</sub> cells and B cells leads to B cells differentiation and GC formation within the follicle (Choi et al. 2011). Immunological events that lead to the development of protective immunity after vaccinations remain largely unknown. For better future vaccine design, there has been an increasing interest in understanding the mechanisms that controls the differentiation and maturation of T<sub>FH</sub> cells.

In this study, an *in vitro* cell culture model was used to study the effect of T<sub>FH</sub> mediated B cell immune responses induced by a LAIV intranasal vaccine, which contains live attenuated influenza viruses including A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B influenza strains.

## **5.2 Aims of Study**

To investigate:

- Whether LAIV intranasal vaccine promotes T<sub>FH</sub> cells in human NALT.
- Whether LAIV enhances B cell immunity to Influenza virus through T<sub>FH</sub> cells in human NALT.
- The role of IL-21 in T<sub>FH</sub> cell-mediated B cell antibody production in NALT.

## **5.3 Experimental Design**

Adenotonsillar MNC were stimulated with a LAIV intranasal vaccine. The effect of LAIV stimulation on T<sub>FH</sub> cell numbers were analysed by flow cytometry. HA-specific memory B cell responses were measured using ELISpot and cell culture supernatants were analysed for antibody production by ELISA. Additionally, the role of IL-21 in T<sub>FH</sub> cell-mediated B cell antibody production in NALT was analysed by blocking IL-21 receptor.

### **5.3.1 Patients and Samples**

Surgically removed adenotonsillar tissues were obtained from children and adults (1.5–36 years). Peripheral blood samples were also obtained from each patient on the same day of operation. Patients who were previously vaccinated against influenza or who had any known immunodeficiency were excluded from the study.

### **5.3.2 Mononuclear Cell Separation**

Mononuclear cells were isolated using Ficoll density centrifugation following methods described previously in the materials and methods chapter 2. Briefly, adenotonsillar tissue samples were minced by a scalpel to release cells into the medium. Then the cell suspension was filtered through a 70µm cell strainer. The

adenotonsillar cells and peripheral blood were carefully layered onto 15 ml Ficoll-Paque™ PREMIUM and spun at 400g for 30 min at room temperature. The interface layer of mononuclear cells was carefully harvested. MNC were washed twice with sterile PBS solution, then the pellet was re-suspended in 5 ml RPMI-1640 culture medium with HEPES supplemented 10% heat inactivated FBS, 1% L-glutamine, streptomycin (50µg/ml) and penicillin (50U/ml). After counting, each cell suspension was adjusted to contain  $4 \times 10^6$  cells/ml concentrations.

### **5.3.3 Influenza Virus Antigen and LAIV**

#### **5.3.3.1 Intranasal Live Attenuated Influenza Vaccine (LAIV)**

Intranasal LAIV (FluMist formula 2009-10) is a live, trivalent intranasal spray vaccine that produces the HA and NA surface antigens from a number of influenza viruses including A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B influenza strains. These three viruses are characterized as being temperature sensitive, cold adapted and attenuated viruses (FluMist, BEI resources ATCC).

#### **5.3.3.2 Recombinant HA**

The recombinant HA was derived from a seasonal H1N1 influenza virus (A/Brisbane/59/2007) (BEI Resources, ATCC, USA). This recombinant HA was used as the coating antigen in ELISA and ELISpot assays.

### **5.3.4 Stimulation by LAIV for Antibody and IL-21 Production**

Adenotonsillar MNC were isolated from adenotonsillar tissues as described earlier. The cell suspension was adjusted to contain  $4 \times 10^6$  cells/ml concentrations in RPMI-1640 medium. 500 µl/well of cells were cultured in 48-well flat bottom culture plates then stimulated with 2µl/well LAIV (FluMist, BEI resources, ATCC). An unstimulated medium control was included in each experiment. Cells then were

cultured for 24 hours for cytokine production and 14 days for antibody production at 37°C, 5% CO<sub>2</sub>. Cell culture supernatants were collected from each well and stored at -80°C until further analysis by ELISA.

### **5.3.5 Measurement of HA Specific IgG/IgM/IgA ELISA Assay**

ELISA assay was used to detect influenza HA -specific IgG, IgM and IgA antibodies found in cell culture supernatants using a procedure as previously described (Mahallawi et al. 2013). In brief, ELISA plates were coated with purified recombinant HA and incubated overnight at 4°C. The following day, plates were washed then blocked for 1 hour at room temperature. Samples were then added and incubated for 2 hours at room temperature. Plates were washed and followed by the addition of alkaline phosphatase-conjugated mouse anti-human IgG or IgM or IgA. After 2 hours, plates were washed and PNPP substrate buffer were added then incubated at room temperature and in dark. Finally, the OD at 405nm was measured using a microtiter plate reader and data were analysed using DeltaSoft software.

### **5.3.6 Measurement of B cell Antibody Response by ELISpot Assay**

ELISpot assay was used to measure the frequencies of antibody-producing B cells specific to HA of sH1N1 (A/Brisbane/59/2007) using a protocol previously described. Briefly, ELISpot plates were coated overnight at 4°C with recombinant HA of sH1N1. Plates were washed and blocked by incubation with RPMI containing 10% FBS at room temperature for 1 hour. LAIV stimulated MNC were then added to the plates and incubated overnight at 37°C. Plates were washed and incubated with goat anti-human IgG biotin for 30 min at room temperature. After washing, Horseradish peroxidase Avidin D conjugate was added and incubated. Coloured

spots were developed with the addition of substrate and counted using an automated ELISpot reader.

### **5.3.7 CD45RO<sup>+</sup> Cell Depletion from Adenotonsillar MNC**

To evaluate the LAIV stimulation on CD45RO<sup>-</sup> cells, effector memory (CD45RO<sup>+</sup>) cells were depleted from the adenotonsillar MNC. The cells to be depleted (CD45RO<sup>+</sup>) were labelled with the specific magnetic micro-beads binding with the target surface molecules (CD45RO). The cell suspension is then allowed to pass through a MACS LD column, placed within the magnetic field of a MACS separator. The magnetically labelled cells (CD45RO<sup>+</sup> cells) are retained within the column, and the unlabelled cells (CD45RO<sup>-</sup> MNC) pass through the column and collected in a tube placed underneath it. The depletion of CD45RO<sup>+</sup> cells in adenotonsillar MNC removed the majority of T<sub>FH</sub> cells.

### **5.3.8 Flow Cytometric Analysis**

#### **5.3.8.1 Cell Stimulation for Flowcytometry Analysis**

Adenotonsillar MNC were adjusted to contain  $4 \times 10^6$  cells/ml concentrations in RPMI-1640. 500  $\mu$ l/well of cells were cultured in 48-well flat bottom culture plates and stimulated with 2 $\mu$ l/well LAIV. An unstimulated control was included in each experiment. For T<sub>FH</sub> cells analysis, unfractionated adenotonsillar MNC were cultured for 3 days and CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC were cultured for 7 days at 37°C, 5% CO<sub>2</sub>. After that, cells were harvested for FACS staining.

#### **5.3.8.2 Cell Staining for Phenotyping for FACS Analysis**

For T<sub>FH</sub> cells identification, adenotonsillar MNC and PBMC were stained with anti-human-CD3, -CD4, -CXCR5 and -ICOS antibodies. For GC B cells identification, adenotonsillar MNC were stained with anti-human CD19, -CD38 and -IgD

antibodies. All cell staining was performed as described earlier. Immediately after staining, cells were acquired/analysed using a BD FACS Calibur or fixed and permeabilized if needed for intracellular staining.

#### **5.3.8.3 Intracellular Staining for BCL-6 and IL-21**

For BCL-6 analysis, stimulated CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC were incubated for 7 days. For IL-21 analysis, stimulated cells were incubated overnight, and then 10µg/ml brefeldin A was added to each sample and further incubated for 4 hours at 37°C in 5% CO<sub>2</sub>. After that, cells were harvested and washed twice with FACS staining buffer followed by surface staining with fluorochrome-labelled anti-human antibodies depending on the phenotype (Table 2.1) in a same procedure described earlier. Next, cells were fixed with intracellular fixation buffer. After incubation for 20 min, cells were washed twice with permeabilization buffer. Then the proper amount of BCL-6 or IL-21 anti-human antibodies was added and incubated for 30 min. Cells were then washed with permeabilization buffer then resuspended in FACS staining buffer; and immediately acquired/analysed by flow cytometry.

#### **5.3.9 T cell Proliferation Assay**

T cell proliferative response was analysed by CFSE staining followed by flow cytometry. Briefly, adenotonsillar MNC were labelled with CFSE (at 37°C, for 8 min) and the reaction was quenched with ice-cold media. After washing, cells were resuspended in RPMI 1640 media and stimulated with 2µl/well LAIV, and incubated (at 37°C, in 5% CO<sub>2</sub>) for 5 days. Then cells were harvested, washed and resuspended in 0.02% BSA in PBS and anti-CD4-PE-Cy7, -CXCR5-PE and -ICOS-APC were added to stain CD4<sup>+</sup> CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells (T<sub>FH</sub> cells). T<sub>FH</sub> cell proliferation was

then examined by analysis of CFSE dilution and by gating from T<sub>FH</sub> cells (CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells) followed by flow cytometry using BD FACS Calibur.

#### **5.3.10 Purification of T<sub>FH</sub> cells and B cells for Co-culture Experiments**

For co-culture experiments, cells were isolated by magnetic cell sorting using EasySep™ magnetic technology (STEMCELL Technologies Inc.) as described in details in material and method chapter 2. Briefly, adenotonsillar B cells were negatively selected using EasySep™ human B cell enrichment kit (purity >99%). Adenotonsillar CD4<sup>+</sup> T cells were negatively selected using EasySep™ human CD4<sup>+</sup> T cell enrichment kit (purity >98%). CD4<sup>+</sup> CXCR5<sup>hi</sup> (T<sub>FH</sub> cells) were isolated by positive selection using biotin anti-human CXCR5 antibody and EasySep™ biotin selection kit (purity >90%). Non-T<sub>FH</sub> cells (CD4<sup>+</sup> CXCR5<sup>-</sup>) was negatively selected from CD4<sup>+</sup> T cells gaining a purity of >99%. Details described previously in the materials and methods chapter 2.

#### **5.3.11 B cell Help by T<sub>FH</sub> Cells**

The ability of adenotonsillar T<sub>FH</sub> cells to help B cell antibody production was studied using an autologous system as follows. In co-culture experiments, B cells were cultured with an equal number of either T<sub>FH</sub> cells or non-T<sub>FH</sub> cells at a ratio of 1:1 using a cell concentration of 5x10<sup>5</sup> cells/ml. 250 µl/well of B:T cells were co-cultured in 96-well round bottom plate in the presence or absence of LAIV (0.5µl/ml). Cells were then cultured at 37°C, 5% CO<sub>2</sub>, for 10 days followed by analysis for antibodies by ELISA.

#### **5.3.12 Anti-IL-21R Blocking Experiments**

Adenotonsillar MNC suspension was adjusted to contain 4x10<sup>5</sup> cells/ml concentrations in RPMI-1640 medium. Cells were cultured in 96-well round bottom

plate in the presence or absence of LAIV with or without the recombinant human  $\alpha$ -IL-21R. Firstly, recombinant human  $\alpha$ -IL-21R was added to the designated wells and incubated for 1 hour at 37°C in a 5% CO<sub>2</sub> incubator. These cells were then stimulated with LAIV (0.5 $\mu$ l/ml). Cells were then cultured in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> for 3 days for T<sub>FH</sub> cell analysis and 10 days for antibody production. After 3 days, cells were harvested and stained for surface staining markers such as CD4, CXCR5 and ICOS in a same procedure described earlier. Furthermore, after 10 days incubation, cell culture supernatants were collected from each well and stored at -80°C until further analysis by ELISA to measure the HA-specific H1N1 IgM and IgA antibodies.

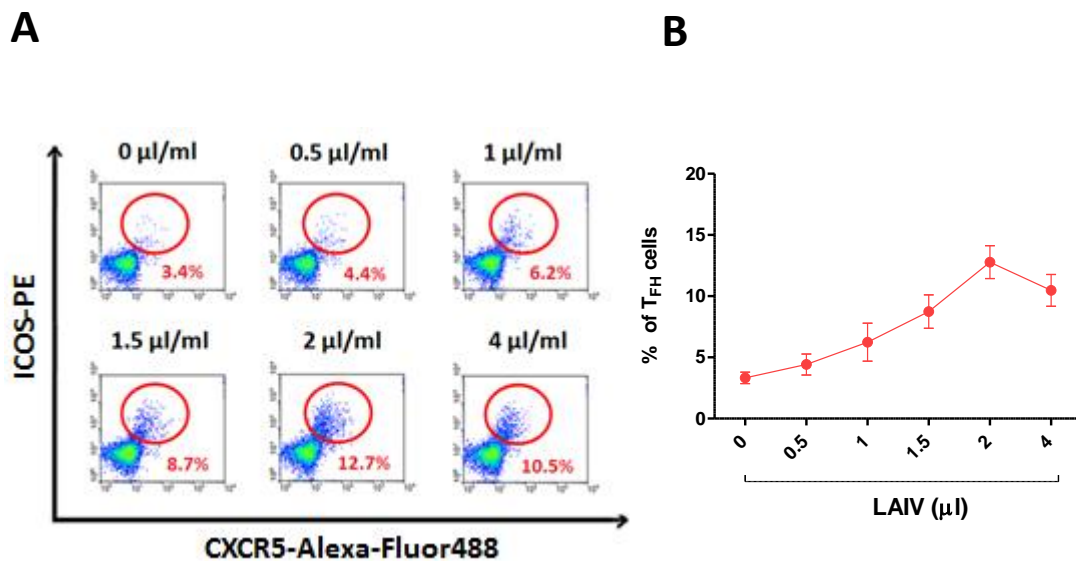
### **5.3.13 Statistical Analysis**

Statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Differences between stimulated and unstimulated samples in the same group of subjects were analysed by Student's paired t test (parametric). Throughout the thesis, \* indicates a P-value between 0.01-0.05, \*\* indicates a P-value between 0.001-0.01 and \*\*\* indicates a P-value <0.001.

## 5.4 Results

### 5.4.1 Optimisation of LAIV concentration for T<sub>FH</sub> cell stimulation in adenotonsillar MNC

The optimal concentration of LAIV required for induction of T<sub>FH</sub> cells in CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC was determined by stimulation of 5 samples with different concentrations (0, 0.5, 1.0, 1.5, 2 and 4  $\mu$ l/ml) of LAIV. As shown in figure 5.1, 2 $\mu$ l/ml of LAIV showed to give the highest stimulatory effect compared to the unstimulated (0  $\mu$ l/ml) controls. This concentration (2  $\mu$ l/ml) was used to stimulate adenotonsillar MNC in subsequent experiments. Similar findings were also seen in unfractionated adenotonsillar MNC (See appendix-I).

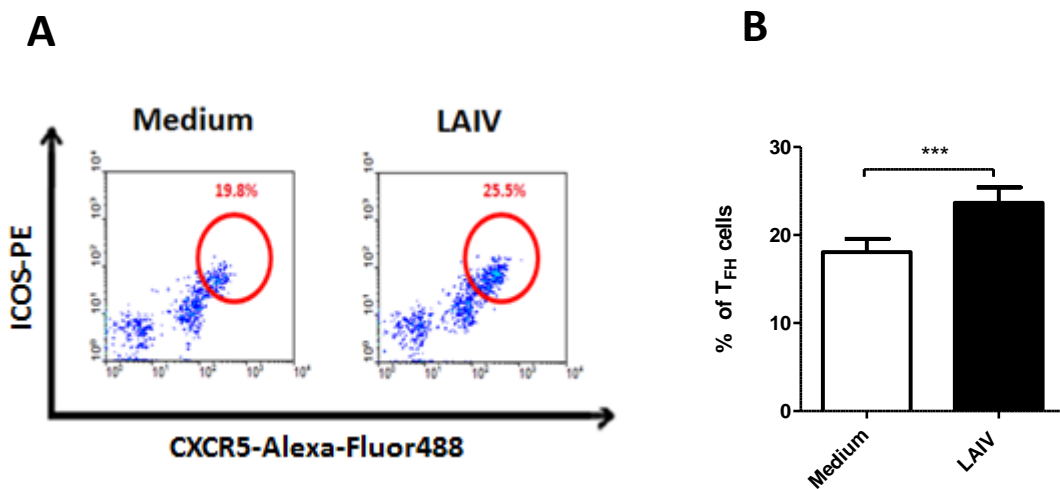


**Figure 5.1. Dose-response curve for T<sub>FH</sub> cell induction by stimulation with LAIV**

(A) A representative density plot flowcytometry analysis of CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC stimulated for 7 days with different concentrations of LAIV. The value shown is the percentage of T<sub>FH</sub> cells in CD4<sup>+</sup> T cells. (B) Summary percentage of T<sub>FH</sub> cells from CD45RO<sup>+</sup> cell-depleted adenotonsillar MNC stimulated with LAIV. The values represent the mean  $\pm$  SEM from five individual tonsils.

#### 5.4.2 LAIV can promote T<sub>FH</sub> cells in adenotonsillar cells

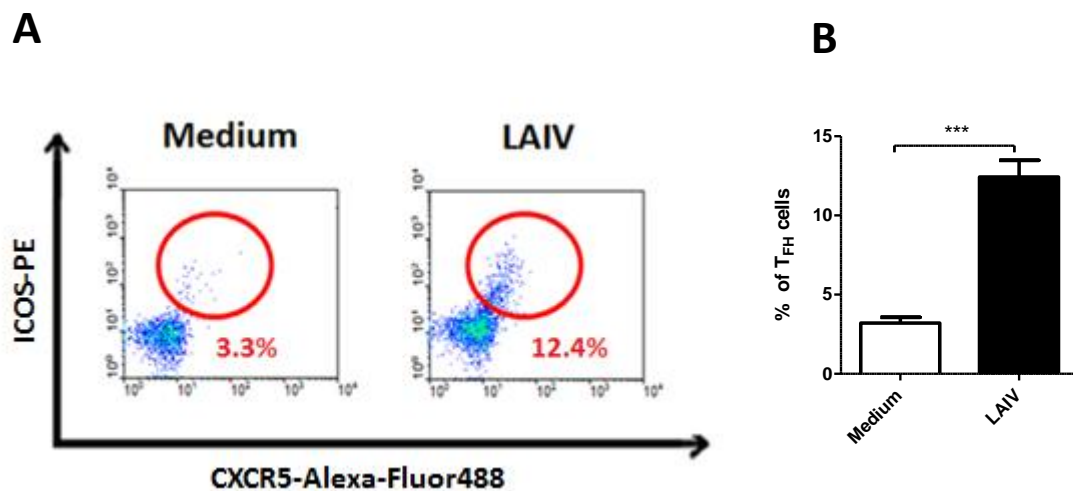
In order to study the effect of LAIV on T<sub>FH</sub> cells, freshly isolated adenotonsillar MNC were stimulated for 72 hours with LAIV (2 $\mu$ l/ml). It is shown in figure 5.2.A that LAIV elicited a significant increase in the percentage of T<sub>FH</sub> cells compared to unstimulated medium control (25.5% versus 19.8%).



**Figure 5.2. LAIV can promote T<sub>FH</sub> cells in adenotonsillar cells**

(A) Representative density plot flowcytometry analysis of MNC stimulated for 72 hours with LAIV. The value shown is the percentage of T<sub>FH</sub> cells in CD4<sup>+</sup> T cells. (B) Summary percentage of T<sub>FH</sub> cells from MNC stimulated with LAIV (2 $\mu$ l/ml). The values represent the mean  $\pm$  SEM from 15 individual tonsils (*p-value* derived from Paired two-tailed *t* test, \*\*\**P* < 0.001).

In order to study the effect of LAIV on T<sub>FH</sub> induction from naive CD4<sup>+</sup> T cells, adenotonsillar MNC depleted of activated and memory (CD45RO<sup>+</sup>) T cells were stimulated for 7 days with LAIV (2μl/ml). As shown in figure 5.3.A, LAIV induced a marked increase in the percentage of T<sub>FH</sub> cells (mean, 12.4%) higher than unstimulated medium control (mean 3.3%).

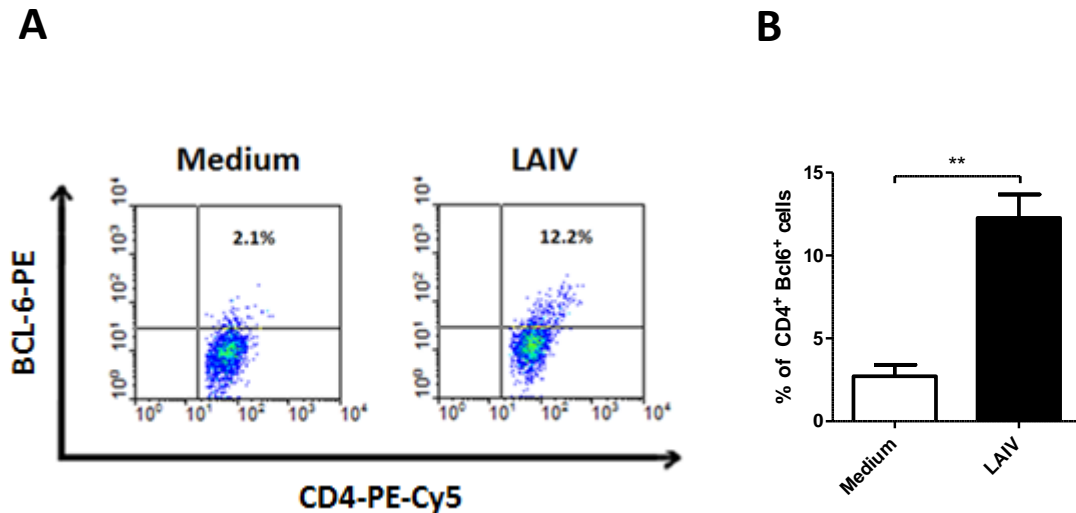


**Figure 5.3. LAIV induced T<sub>FH</sub> cells in CD45RO<sup>+</sup> depleted adenotonsillar MNC**

(A) A representative density plot showing induction T<sub>FH</sub> following cell stimulation for 7 days with LAIV. The value shown is the percentage of T<sub>FH</sub> cells in CD4<sup>+</sup> CD45RO<sup>-</sup> T cells. (B) Summary percentage of T<sub>FH</sub> cells from MNC stimulated with 2μl/ml LAIV. The values represent the mean ± SEM from 12 individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\*\**P* < 0.001).

### 5.4.3 Induction of CD4<sup>+</sup> BCL-6<sup>+</sup> cells in adenotonsillar cells by LAIV

Effect of LAIV on T<sub>FH</sub> induction was also analysed by staining for CD4<sup>+</sup> BCL-6<sup>+</sup> T<sub>FH</sub> cells, adenotonsillar MNC depleted of activated and memory (CD45RO<sup>+</sup>) T cells were stimulated with LAIV (2µl/ml). These cells were then harvested and stained for BCL-6 intracellular staining following a protocol described earlier. It is shown in figure 5.4.A that LAIV induced a significant increase in the number of CD4<sup>+</sup> BCL-6<sup>+</sup> cells (12.2%) compared to unstimulated medium control (2.1%) within T<sub>FH</sub> cells in human adenotonsillar tissues.

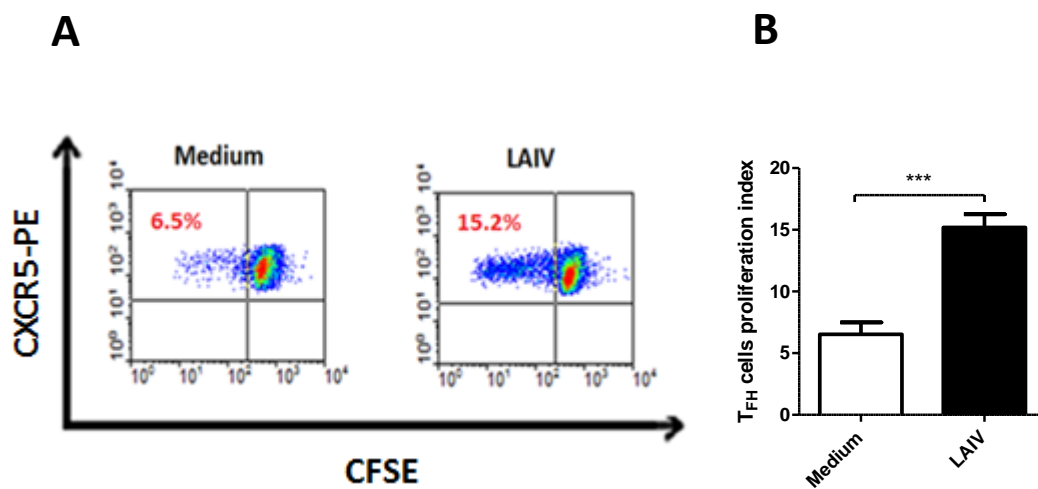


**Figure 5.4. CD4<sup>+</sup> BCL-6<sup>+</sup> cells induction by LAIV**

(A) A representative density plot flowcytometry analysis of MNC (CD45RO<sup>+</sup>) depleted cells stimulated for 7 days with LAIV. The value shown is the percentage of CD4<sup>+</sup> BCL-6<sup>+</sup> gated from T<sub>FH</sub> cells. (B) Summary of CD4<sup>+</sup> BCL-6<sup>+</sup> cells from MNC stimulated with LAIV. The values represent the mean  $\pm$  SEM from eight individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\**P* < 0.01).

#### 5.4.4 Effect of LAIV on T<sub>FH</sub> cell proliferation in adenotonsillar MNC

To determine whether T<sub>FH</sub> cells proliferate upon stimulation by LAIV, adenotonsillar MNC were stained with CFSE followed by stimulation with LAIV for 5 days before flowcytometry. As shown in figure 5.5.A, LAIV induced a marked T<sub>FH</sub> cell proliferation, with a mean T<sub>FH</sub> cell proliferation index of 15.2%, significantly higher than unstimulated medium control (mean 6.5%).

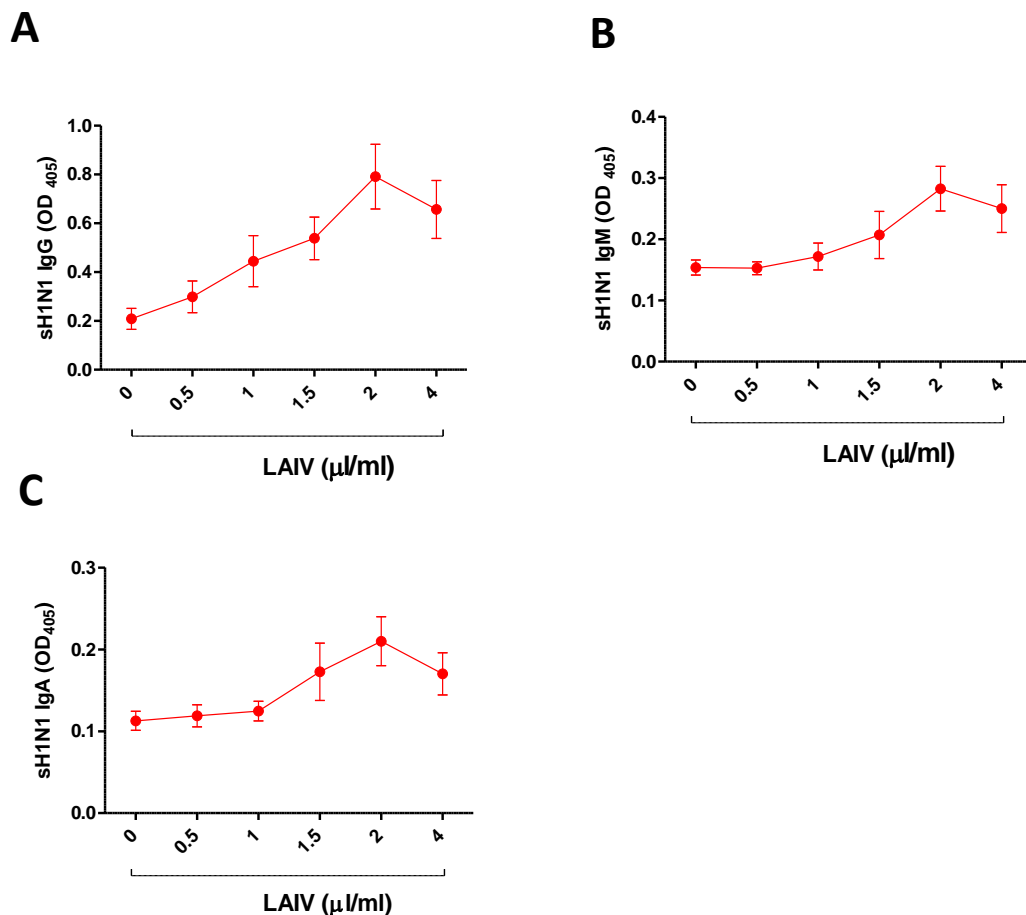


**Figure 5.5. T<sub>FH</sub> cell proliferative response in adenotonsillar MNC after stimulation with LAIV**

(A) Representative CFSE staining analysis of MNC stimulated for 5 days with LAIV, the value shown is the mean T<sub>FH</sub> cell proliferation index in CD4<sup>+</sup> T cells. (B) Summary analysis of T<sub>FH</sub> cell proliferation from MNC stimulated with LAIV. The value represents the mean  $\pm$  SEM of T<sub>FH</sub> cell proliferation index gated from CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells from 10 individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\**P* < 0.01).

#### 5.4.5 Induction of anti-sH1N1 HA antibodies in adenotonsillar cells by LAIV

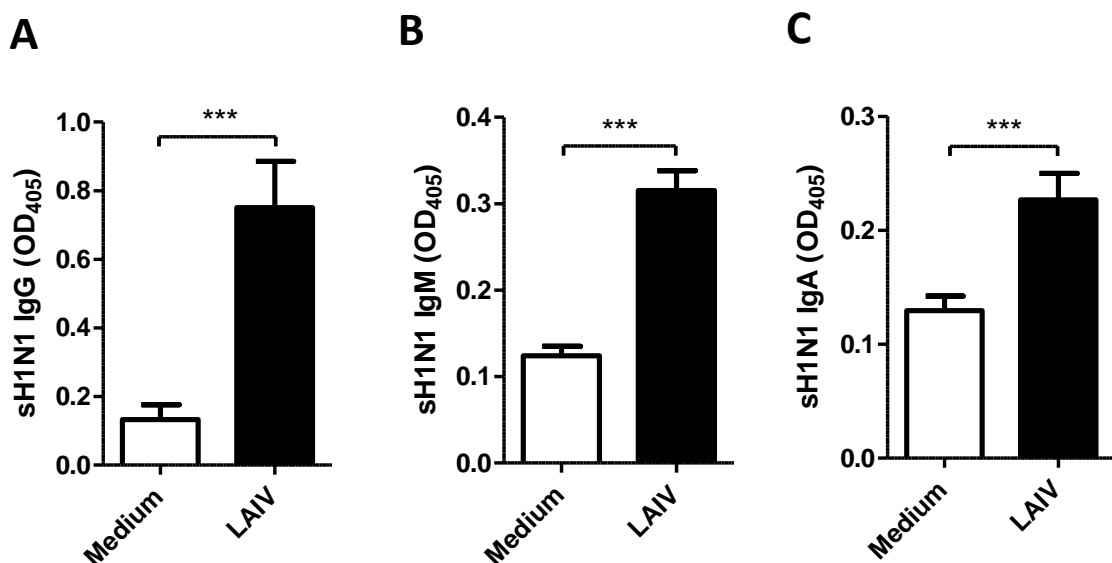
Induction of HA-specific antibody production to sH1N1 in adenotonsillar MNC culture was analysed following LAIV stimulation with different doses (0, 0.5, 1.0, 1.5, 2 and 4  $\mu\text{l/ml}$ ) of LAIV. As seen in figure 5.6, HA-specific sH1N1 antibody production (IgG, IgM and IgA) was highest when stimulated with LAIV at 2  $\mu\text{l/ml}$ , the same as the optimal concentration shown earlier for  $T_{\text{FH}}$  induction. This concentration was therefore used to stimulate adenotonsillar MNC in subsequent experiments.



**Figure 5.6. Optimisation of LAIV concentration for in vitro stimulation of adenotonsillar MNC for specific antibody response**

Dose response of LAIV in the induction of anti-sH1N1 HA IgG (A), IgM (B) and IgA (C) production in adenotonsillar MNC cultures. The values represent anti-sH1N1 HA IgG, IgM and IgA titre mean  $\pm$  SEM from five individual tonsils.

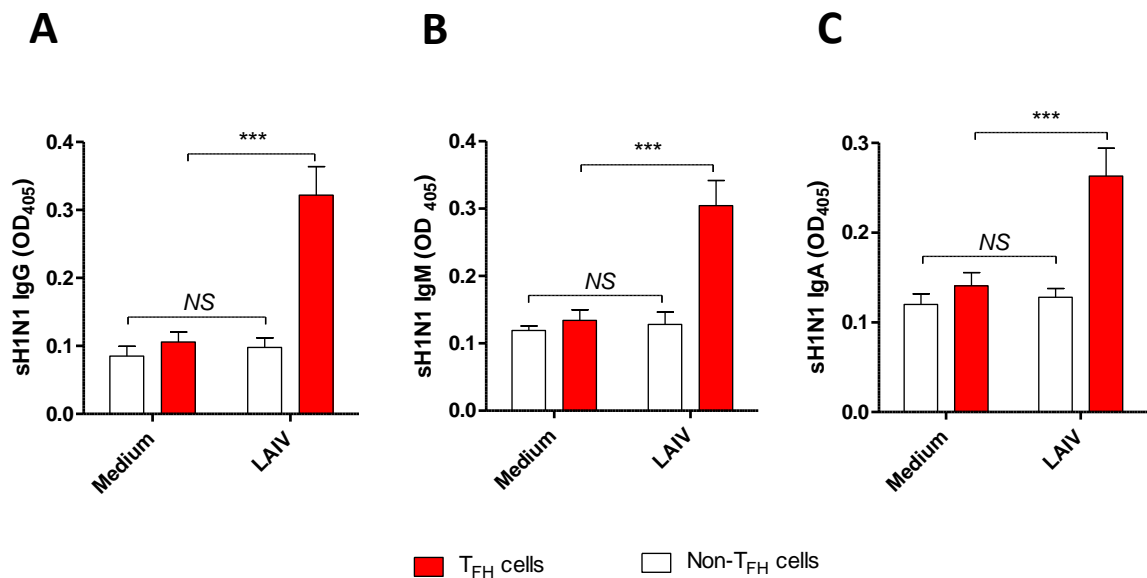
To investigate the effect of intranasal vaccine on B cell antibody production in association with T<sub>FH</sub> cells, influenza HA-specific antibody response was studied following LAIV stimulation. Adenotonsillar MNC were stimulated with LAIV for 14 days. Cell culture supernatants were collected and analyzed by ELISA assay for the detection of specific sH1N1 HA antibodies. As shown in figure 5.7.A, significant influenza HA-specific IgG titres were detected in the MNC culture supernatants after stimulation (mean  $\pm$  SEM:  $0.75 \pm 0.13$ ) compared with unstimulated medium control, ( $p < 0.001$ ). LAIV stimulation also induced HA-specific IgM production (mean  $\pm$  SEM:  $0.31 \pm 0.10$ ) compared with unstimulated medium control ( $p < 0.001$ ) (Figure 5.7.B). HA-specific IgA antibodies were also induced in cell culture supernatants (mean  $\pm$  SEM:  $0.22 \pm 0.02$ ) compared with unstimulated medium control ( $p < 0.001$ ) (Figure 5.7.C).



**Figure 5.7. LAIV induces HA-specific anti-sH1N1 IgG, IgM and IgA in adenotonsillar MNC**

Adenotonsillar MNC were stimulated with LAIV. After 14 days, Cell culture supernatants were collected and analysed for anti-sH1N1 HA IgG (A), IgM (B) and IgA (C) antibody using ELISA. The values represent the mean  $\pm$  SEM from 16 individual tonsils ( $p$ -value derived from Paired two-tailed  $t$  test, \*\*\* $P < 0.001$ ).

To ascertain whether T<sub>FH</sub> are required for the antibody induction induced by LAIV, T<sub>FH</sub> (CD4<sup>+</sup> CXCR5<sup>hi</sup>) cells were purified, co-cultured with purified B cells in the presence of LAIV. As shown in figure 5.8, the co-cultured cells stimulated by LAIV induced significant antibody production only in the presence of CD4<sup>+</sup> CXCR5<sup>hi</sup> (T<sub>FH</sub>) cells, but not in the presence of CD4<sup>+</sup> CXCR5<sup>-</sup> (non-T<sub>FH</sub>) cells; i.e. T<sub>FH</sub> but not non-T<sub>FH</sub> cells were able to help B cells for the production of HA-specific IgG (Figure.5.8.A), IgM (Figure.5.8.B) and IgA (Figure.5.8.C) antibodies.

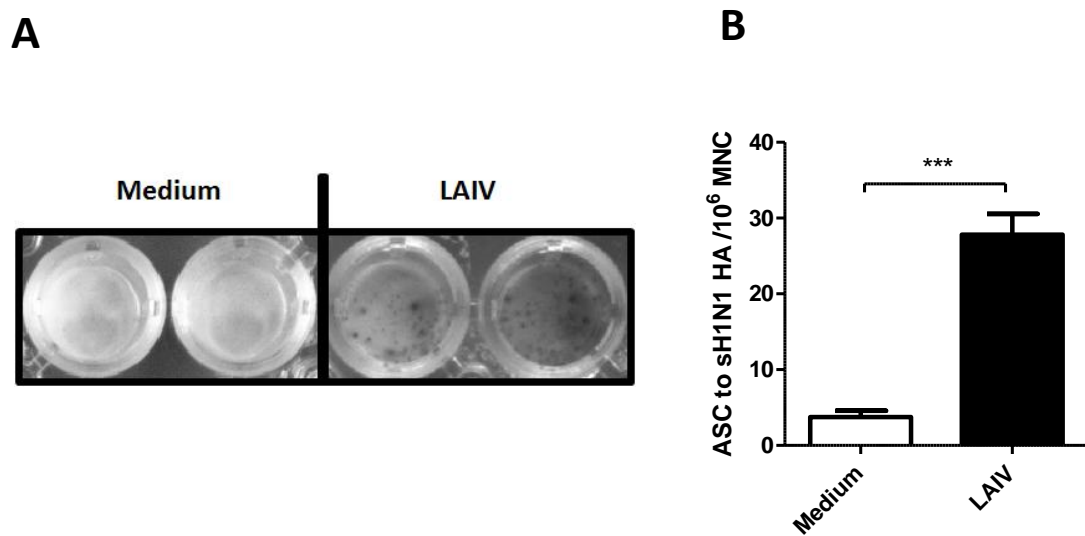


**Figure 5.8. LAIV induces HA-specific sH1N1 IgG, IgM and IgA from B cells co-cultured with purified T<sub>FH</sub> cells but not with non-T<sub>FH</sub> cells**

Purified B cells were co-cultured with either purified T<sub>FH</sub> cells (Red bar) or non-T<sub>FH</sub> cells (open bar) (at a ratio of 1:1) and stimulated with LAIV. After 10 days, cell culture supernatants were collected and analysed for anti-sH1N1 HA IgG (A) IgM (B) and IgA (C) antibody production using ELISA. The value represents the mean  $\pm$  SEM from 16 individual tonsils (*p*-value derived from Paired two-tailed *t* test, NS: Not Significant, \*\*\**P* < 0.001).

#### 5.4.6 Detection of ASC following stimulation by LAIV in adenotonsillar cells

To confirm antibody production measured by ELISA, ELISpot assay was performed to enumerate the numbers of anti-sH1N1 HA B cell responses following stimulation by LAIV. It is shown in figure 5.9 that LAIV induced significant numbers of HA-specific sH1N1 IgG ASC compared to unstimulated medium control (mean  $\pm$  SEM,  $27.8 \pm 2.7$  versus  $4.0 \pm 0.65$ ) in human adenotonsillar tissues.

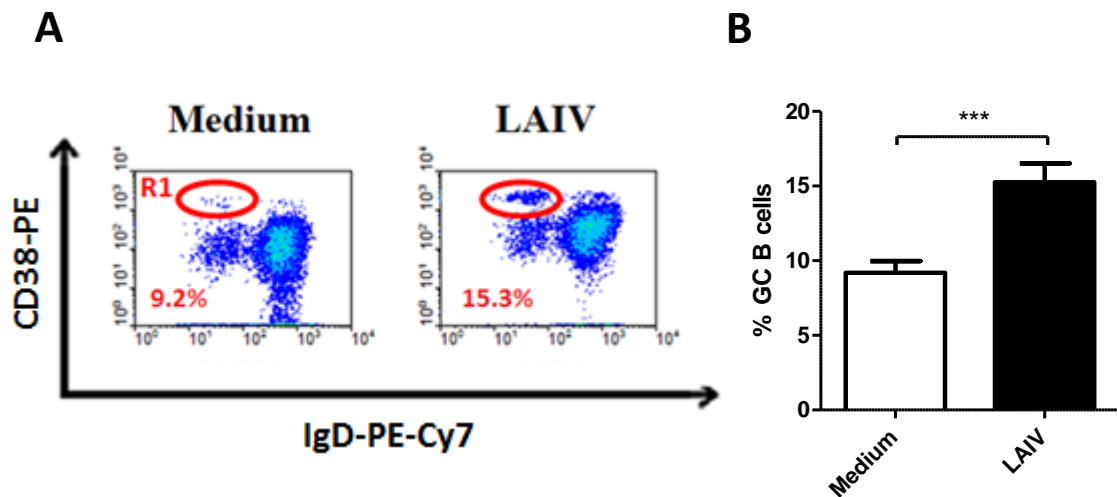


**Figure 5.9. ELISpot assay to enumerate IgG ASC to sH1N1-HA**

Adenotonsillar MNC were stimulated with LAIV for 5 days. **(A)** A representative sample showing the number of HA-specific ASC induced by LAIV. **(B)** Summary of the number of HA-specific ASC from MNC stimulated with LAIV. The values represent the mean  $\pm$  SEM from 8 samples (paired two-tailed *t* test, \*\*\**P* < 0.001).

#### 5.4.7 Effect of LAIV on Human adenotonsillar GC B cells

B cell subsets in adenotonsillar MNC was analysed with a combination of CD19, CD38 and IgD fluorescence-labelled anti-human antibodies. After gating on CD19<sup>+</sup> B cells, GC B cells were identified as (CD19<sup>+</sup> CD38<sup>hi</sup> IgD<sup>-</sup>) (R1, Red). In order to study the effect of LAIV on GC B cells *in vitro*, freshly isolated adenotonsillar MNC were stimulated for 72 hours with LAIV intranasal vaccine. As shown in figure 5.10.A, LAIV significantly increased the number of GC B cells compared to unstimulated medium control (mean, 15.3% versus 9.2%).

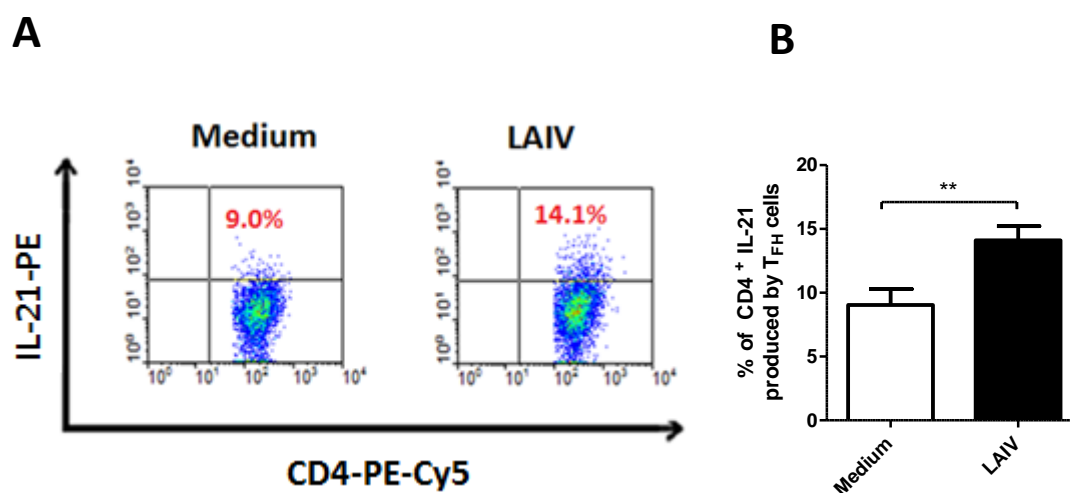


**Figure 5.10. Effect of LAIV on adenotonsillar GC B cells**

(A) Representative flowcytometry dotplot showing B cell subsets including GC B cells (CD19<sup>+</sup> CD38<sup>hi</sup> IgD<sup>-</sup>) in adenotonsillar MNC after stimulation with LAIV. The values represent the mean percentage of GC B cells. (B) Summary of GC B cells from adenotonsillar MNC stimulated with LAIV. The values represent the mean  $\pm$  SEM from 13 individual tonsils (Paired two-tailed *t* test, \*\*\**P* < 0.001).

#### 5.4.8 Induction of CD4<sup>+</sup> IL-21<sup>+</sup> cells in adenotonsillar cells by LAIV

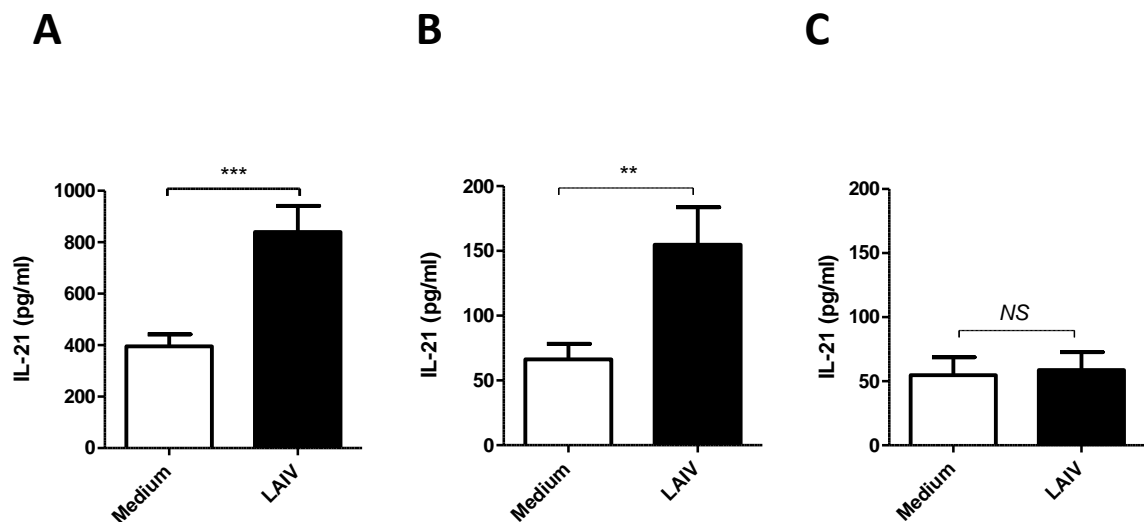
In order to study the effect of LAIV on IL-21 producing cells, freshly isolated adenotonsillar MNC were stimulated for 24 hours with LAIV (2 $\mu$ l/ml). These cells were then harvested and stained for IL-21 following a protocol described earlier. Compared to unstimulated medium control, figure 5.11 shows that LAIV induced a significant increase in the number of IL-21 producing cells (9.0% versus 14.1%) within T<sub>FH</sub> cells in human adenotonsillar tissues.



**Figure 5.11. Induction of CD4<sup>+</sup> IL-21<sup>+</sup> cells in adenotonsillar cells by LAIV**

(A) Representative density plot flowcytometry analysis of MNC stimulated for 24 hours with LAIV. The value shown is the percentage of CD4<sup>+</sup> IL-21<sup>+</sup> gated from T<sub>FH</sub> cells. (B) Summary percentage of CD4<sup>+</sup> IL-21<sup>+</sup> cells (gated from T<sub>FH</sub> cells) from MNC stimulated with LAIV. The values represent the mean  $\pm$  SEM from 12 individual tonsils (*p*-value derived from Paired two-tailed *t* test, \*\**P* < 0.01).

Further, the induction of IL-21 was also analysed in unfractionated adenotonsillar MNC culture supernatants 24 hours after stimulation with LAIV. Production of IL-21 was shown to be significantly higher with LAIV stimulation compared to unstimulated medium control (mean  $\pm$  SEM,  $846.5 \pm 89.2$  versus  $389.7 \pm 48.2$ ) (Figure.5.12.A). To ascertain the effect of LAIV intranasal vaccine on  $T_{FH}$  production of IL-21, purified CXCR5<sup>hi</sup> CD4<sup>+</sup> T cells ( $T_{FH}$ ) were co-cultured with purified B cells in the presence of LAIV for 24 hours. IL-21 concentration was shown to increase significantly following LAIV stimulation in the presence of  $T_{FH}$  cells (Figure.5.12.B) but not in the presence of CXCR5<sup>-</sup> CD4<sup>+</sup> T cells (non- $T_{FH}$ ) (Figure.5.12.C).

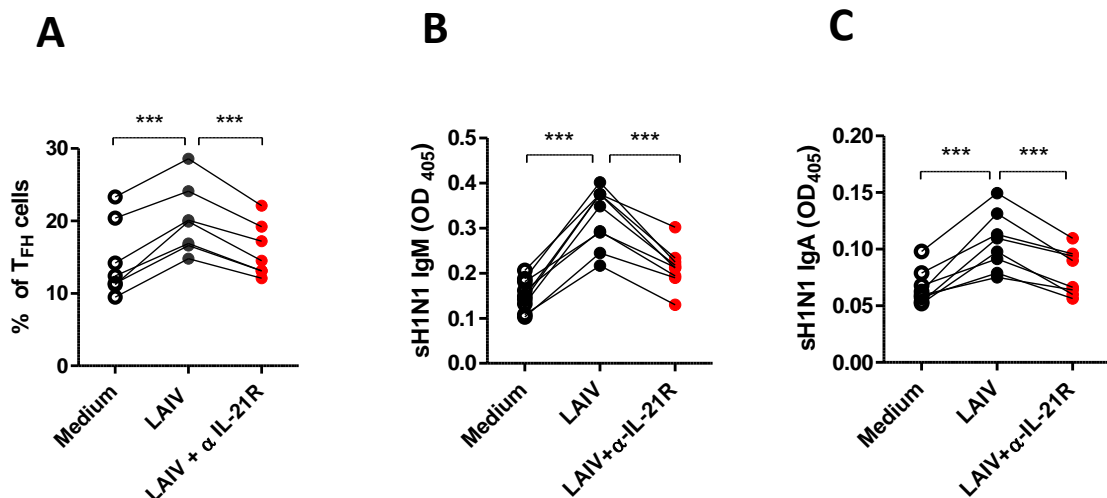


**Figure 5.12. IL-21 concentration increases in the presence of  $T_{FH}$  cells, but not with non- $T_{FH}$  cells following stimulation with LAIV**

Unfractionated adenotonsillar MNC were stimulated with LAIV (A) and purified  $T_{FH}$  cells (B) or non- $T_{FH}$  cells (C) were co-cultured with B cells in the presence of LAIV intranasal vaccine. After 24 hours, cell culture supernatants were collected and analysed for IL-21 using ELISA. The value represents the mean  $\pm$  SEM (A, n=22; B, n=17; C, n=17) (Paired two-tailed *t* test, \*\**P* < 0.01, \*\*\**P* < 0.001, NS: not significant).

#### 5.4.9 IL-21 is important in T<sub>FH</sub> cell-mediated B cell antibody production in adenotonsillar cells

In order to study the importance of IL-21 in T<sub>FH</sub> cell-mediated B cell antibody production, anti-IL-21R blocking antibody ( $\alpha$ -IL-21 R) was added to adenotonsillar MNC and incubated for 1 hour at 37 °C, then stimulated with LAIV for 3 days (for T<sub>FH</sub> cells analysis) and 10 days (for antibody production). As shown in figure 5.13.A blocking IL-21 receptor significantly reduced the percentage of T<sub>FH</sub> cells compared to un-blocked samples, (mean, 20.1% versus 15.9%). Further, the antibody level (sH1N1 IgM and sH1N1 IgA) was also significantly reduced when IL-21 receptor was blocked as shown in figures 5.13.B and 5.13.C respectively.

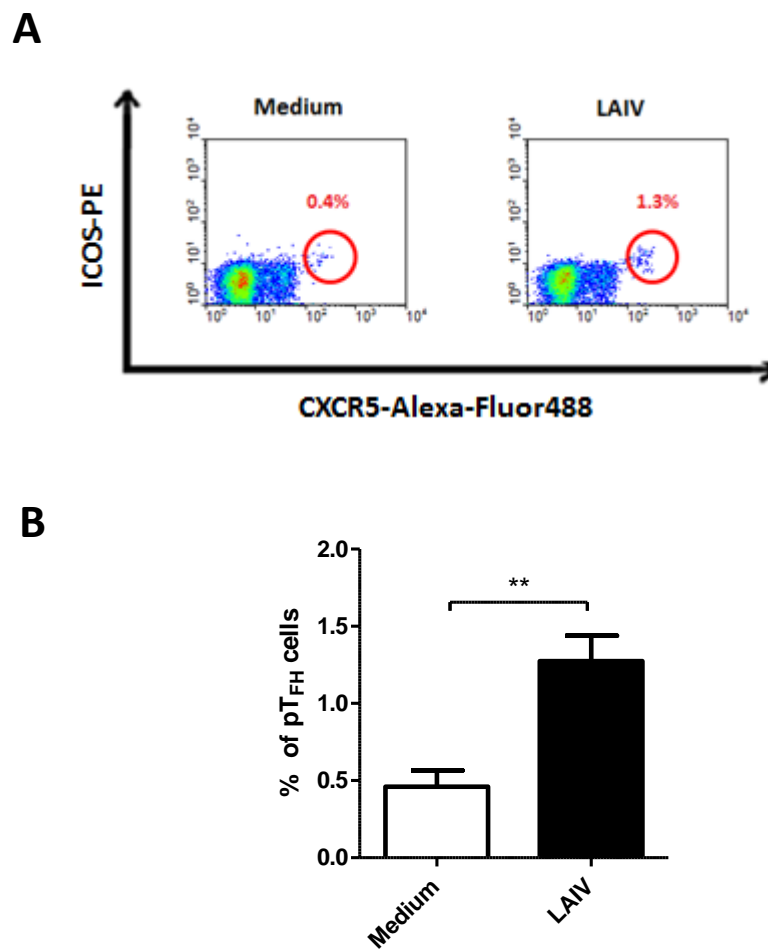


**Figure 5.13. Blocking IL-21R leads to a significant reduction in percentage of T<sub>FH</sub> cells and antibody production in human adenotonsillar tissue**

$\alpha$ -IL-21R was added to MNC and incubated for 1 hour/37 °C, then stimulated with LAIV. (A) Shows the percentage of T<sub>FH</sub> cells with and without  $\alpha$ -IL-21R. The specific sH1N1 IgM (B) and IgA (C) OD levels from MNC stimulated with LAIV with and without  $\alpha$ -IL-21R after 10 days measured by ELISA. The values represent the mean  $\pm$  SEM from eight individual tonsils (*p*-value derived from Paired two-tailed t test, \*\*\**P* < 0.001).

#### 5.4.10 LAIV can promote pT<sub>FH</sub> cells, anti-sH1N1-HA antibodies and IL-21 in PBMC

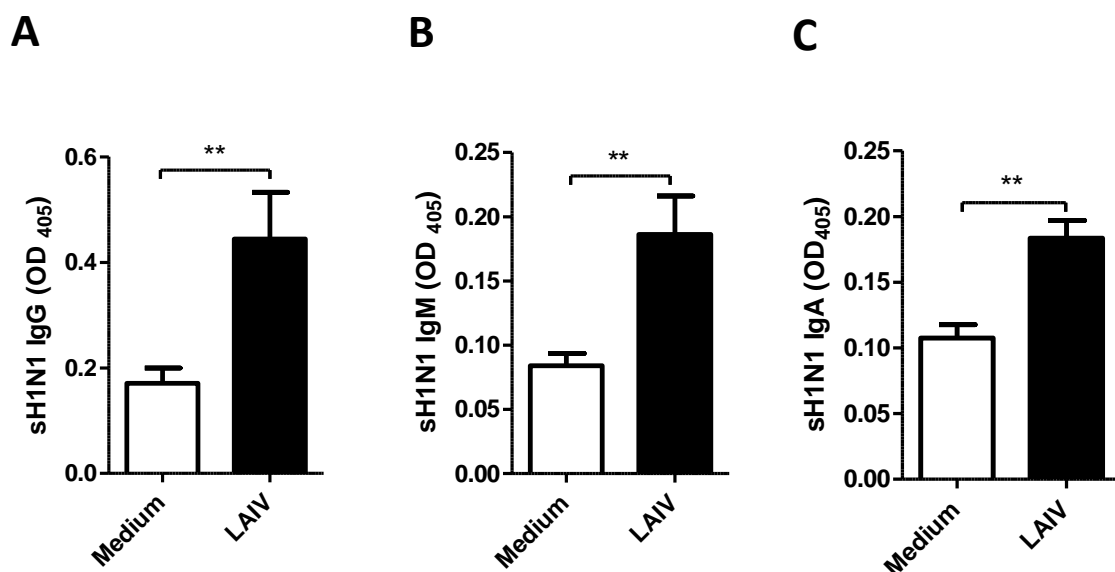
In order to study the effect of LAIV on pT<sub>FH</sub> cells *in vitro*, freshly isolated PBMC were stimulated for 72 hours with LAIV (2 $\mu$ l/ml). As was shown in figure 5.14.A that LAIV induced a significant increase in pT<sub>FH</sub> cells compared to unstimulated medium control (mean, 1.3% versus 0.4%) in human PBMC.



**Figure 5.14. LAIV can promote pT<sub>FH</sub> cells in PBMC**

(A) Representative density plot flowcytometry analysis of PBMC stimulated for 72 hours with LAIV. The value shown is the mean percentage of pT<sub>FH</sub> cells in CD4<sup>+</sup> T cells. (B) Summary percentage of pT<sub>FH</sub> cells from PBMC stimulated with LAIV (2 $\mu$ l/ml). The values represent the mean  $\pm$  SEM from 10 individual patients (*p*-value derived from Paired two-tailed *t* test, \*\**P* < 0.01).

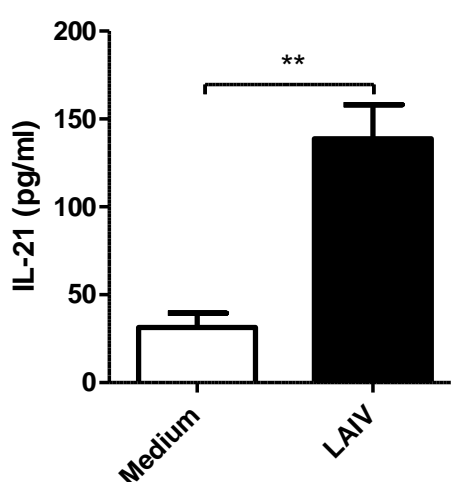
Further, PBMC were isolated and co-cultured with LAIV for 14 days. Cell culture supernatants were collected and analyzed by ELISA assay for the detection of specific sH1N1 HA antibodies. As seen in figure 5.15.A, significant specific influenza HA-IgG antibody titres were detected in the PBMC culture supernatants after stimulation with LAIV (mean  $\pm$  SEM:  $0.44 \pm 0.08$  compared with unstimulated medium control,  $p < 0.001$ ). (Figure 5.15.B) LAIV stimulation also induced specific influenza HA-IgM antibody production in PBMC, (mean  $\pm$  SEM:  $0.18 \pm 0.03$ ) compared with unstimulated medium control,  $p < 0.001$ . IgA antibodies to sH1N1 HA were also induced in PBMC culture supernatants (Figure 5.15.C), (mean  $\pm$  SEM:  $0.18 \pm 0.01$ ) compared with unstimulated medium control,  $p < 0.001$ .



**Figure 5.15. LAIV induces HA-specific anti-sH1N1 IgG, IgM and IgA in PBMC**

PBMC stimulated with LAIV. After 14 days, cell culture supernatants were collected and analysed for anti-sH1N1 HA IgG (A), IgM (B) and IgA (C) antibody using ELISA. The values represent the mean  $\pm$  SEM from 10 individual donors ( $p$ -value derived from Paired two-tailed  $t$  test,  $**P < 0.01$ ).

Induction of IL-21 was also analysed in PBMC culture supernatants, 24 hours after stimulation with LAIV. Production of IL-21 was shown to be significantly higher with LAIV stimulation compared to unstimulated medium control (mean  $\pm$  SEM,  $138.8 \pm 19.3$  versus  $39.9 \pm 3.8$ ) (Figure 5.16).



**Figure 5. 16. IL-21 Levels increases after stimulation with LAIV in PBMC**

Human PBMC were stimulated with LAIV. After 24 hours, cell culture supernatants were collected and analysed for IL-21 levels using ELISA. LAIV induced high levels of IL-21 compared to unstimulated medium control. The value represents the mean  $\pm$  SEM from seven individual samples (*p-value* derived from Paired two-tailed *t* test, \*\**P* < 0.01).

## 5.5 Discussion

As LAIV intranasal vaccines were previously shown to induce strong immune response including mucosal IgA and IgG, and serum IgG and enhanced local cytokine responses (De Filette et al. 2006; Vajdy et al. 2007), we asked whether T<sub>FH</sub> cells contribute to the potent induction of antibodies in NALT B cells by LAIV. In this study, numbers of T<sub>FH</sub> cells and GC B cells and antibody responses in NALT to HA-specific sH1N1 were investigated following stimulation of adenotonsillar MNC with LAIV vaccine which contains an A/Brisbane/59/2007 (H1N1), A/Brisbane/10/2007 (H3N2) and B influenza strains.

We found that the percentage of T<sub>FH</sub> cells was increased significantly following stimulation by LAIV in a dose dependent manner. Galli et al. previously reported that an early post-vaccination increase in the number of vaccine-specific CD4<sup>+</sup> T cells is correlated with the rise and long-term maintenance of protective antibody titres to avian influenza (Galli et al. 2009). More recently, Spensieri and colleagues showed in a clinical trial that blood-derived influenza-specific ICOS<sup>+</sup> IL-21<sup>+</sup> CD4<sup>+</sup> T cells help the *in vitro* differentiation of autologous B cells into cells secreting influenza-specific antibody in an antigen, IL-21 and ICOS dependent manner (Spensieri et al. 2013). In line with these findings, Bentebibel et al. reported, using a non-adjuvanted trivalent split seasonal influenza vaccine, a significant induction of ICOS<sup>+</sup> CXCR5<sup>+</sup> CD4<sup>+</sup> T cells representing a crucial immunological response associated with highly protective antibody levels after seasonal influenza vaccination. These ICOS<sup>+</sup> CXCR5<sup>+</sup> CD4<sup>+</sup> T cells were seen in blood 7 days after vaccination and correlated with the increase of antibody responses (Bentebibel et al. 2013). Furthermore, an *in vitro* induction of ICOS<sup>+</sup> CXCR5<sup>+</sup> CD4<sup>+</sup> T cells by split

influenza vaccines was found to support B cells to differentiate into plasma cells (Bentebibel et al. 2013).

In this study, significant increases in  $CD4^+$  BCL-6 $^+$  cells were also seen after LAIV stimulation. Consistently, the BCL-6 expression was significantly increased by LAIV, and that was correlated with the increase in number of  $T_{FH}$  cells. BCL-6 is highly expressed by  $T_{FH}$  cells and is required for the  $T_{FH}$  cell differentiation program (Johnston et al. 2009; Nurieva et al. 2009; Yu et al. 2009b).

We also showed the LAIV intranasal vaccine induces the  $T_{FH}$  cell proliferation in NALT. Similar finding was seen by Morita et al. showing the induction of blood CXCR5 $^+$  CD4 $^+$  T cells to proliferate after stimulation with CMV or an inactivated influenza virus (Morita et al. 2011). A study by Rasheed and colleagues reported that a polyclonal stimulation of human tonsillar CD4 $^+$  T cells with anti-CD3/CD28 antibodies induced the  $T_{FH}$  cell proliferation (Rasheed et al. 2006).

As expected, a significant increase in CD19 $^+$  CD38 $^{hi}$  IgD $^-$  (GC B cells) was seen after LAIV stimulation. As shown in chapter 3, numbers of  $T_{FH}$  cells strongly correlates with numbers of GC B cells. This is consistent with previous studies in both mice and humans (Baumjohann et al. 2013; Renand et al. 2013). A strong enhancement in number of GC B cells which was also shown to be correlated with the appearance of  $T_{FH}$  cells after an influenza virus infection (Elsner, Ernst & Baumgarth 2012). Fernandez and colleagues using an intranasal vaccination with a recombinant SEB vaccine in a mouse model, reported that elevated GC B cells within NALT were accompanied by high levels of IgA $^+$  and IgG $^+$  B cells (Fernandez et al. 2011). A study by Bessa et al. reported a strong specific IgG and IgA responses in serum and lungs using an intranasal virus-like particles vaccine. Also An efficient

induction of B cell responses was also seen characterized by the presence of large numbers of GC B cells as well as plasma cells in the bone marrow and memory B cells in the spleen (Bessa et al. 2008).

Significant antibody responses of all three isotypes (IgG, IgM and IgA) to the HA of seasonal H1N1 virus were observed in adenotonsillar MNC following LAIV stimulation. As we showed in this study (Figure 5.8), co-culture of purified T<sub>FH</sub> cells, but not non-T<sub>FH</sub> cells, with purified B cells was able to induce antigen specific IgG, IgM and IgA antibodies. This finding supports the assumption that T<sub>FH</sub> help to B cells is needed for the production of these antibodies in NALT compartments. Using a similar *in vitro* system, Bryant and colleagues co-cultured tonsillar CD4<sup>+</sup> CXCR5<sup>+</sup> cells (T<sub>FH</sub> cells) and CD4<sup>+</sup> CXCR5<sup>-</sup> (non-T<sub>FH</sub> cells) with autologous B cells and showed similar results following stimulation by PHA and IL-2. (Bryant et al. 2007). Similar finding was also shown by Rasheed et al. reporting that CXCR5<sup>-</sup> ICOS<sup>-</sup> CD4<sup>+</sup> T cells (non-T<sub>FH</sub> cells) did not influence B cell production of IgG, and in contrast, CXCR5<sup>hi</sup> ICOS<sup>hi</sup> CD4<sup>+</sup> T cells (T<sub>FH</sub> cells) promoted B cell secretion of IgG (Rasheed et al. 2006). In addition, Rodríguez-Bayona et al. reported similar finding by co-culturing either the whole fraction of tonsil total CD4<sup>+</sup> T cells or purified T<sub>FH</sub> cells with purified tonsil plasma cells. The purified T<sub>FH</sub> cell co-culture showed an increase of tonsillar plasma cell antibody production but not total CD4<sup>+</sup> T cells co-cultures (Rodríguez-Bayona et al. 2012). Using an ELISpot assay, we also detected significant numbers of IgG ASC upon stimulation by the LAIV intranasal vaccine. This confirms the results of antibody induction measured by ELISA.

IL-21 plays an important role in T<sub>FH</sub> cell differentiation and the development of B cell immunity (Eto et al. 2011). Our results showed that stimulation with the LAIV

intranasal vaccine significantly enhances IL-21 production. Mainly T<sub>FH</sub> cells but not non-T<sub>FH</sub> cells stimulated with LAIV produced IL-21. In line with this finding, a significant increase in CD4<sup>+</sup> IL-21<sup>+</sup> cells was seen after LAIV stimulation. Other groups also showed elevated IL-21 levels after Influenza vaccination. Pallikkuth and colleagues reported an increased level of serum IL-21 in individuals vaccinated with an inactivated monovalent A/California/07/2009 H1N1 vaccine (Pallikkuth et al. 2011). An elevated IL-21 in plasma samples was also seen in individuals vaccinated with the 2011-2012 recommended influenza vaccine, containing the following strains: A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2) and B/Brisbane/60/2008 (Parmigiani et al. 2013). A recent study using a trivalent split seasonal influenza vaccine, reported that circulating CXCR5<sup>+</sup> ICOS<sup>+</sup> CD4<sup>+</sup> T cells were found to co-express several cytokines, including IL-21. In contrast, very few CXCR5<sup>+</sup> ICOS<sup>-</sup> CD4<sup>+</sup> T cells expressed IL-21 (Bentebibel et al. 2013).

As shown in chapters 3 and 4, T<sub>FH</sub> express high levels of IL-21 and that were important in T<sub>FH</sub> cell-mediated B cell antibody production in adenotonsillar cells. Here we show that blocking the IL-21R by a recombinant neutralizing antibody significantly reduced the numbers of T<sub>FH</sub> cells. The reduction of T<sub>FH</sub> cells was correlated with the reduction of specific-HA IgM and IgA. Similar to other groups finding, the specific-HA IgG could not be evaluated in these cultures because of the cross-reactivity of the Fc portion of IL-21R-Fc in the IgG ELISA (Bryant et al. 2007; Rodríguez-Bayona et al. 2012). The importance of IL-21/IL-21R was discussed in more details in results chapter 4.

In accordance with these findings, several studies reported that IL-21 deficient mice exhibited a marked reduction in T<sub>FH</sub> cells, GC formation and antibody production

compared with wild type mice after immunization (Nurieva et al. 2008; Vogelzang et al. 2008). Using purified T<sub>FH</sub> cells and B cells from human tonsillar tissues, Berglund et al. have reported a major reduction in IgM production when IL-21 was blocked by neutralizing antibody (IL-21R-Fc) compared to unblocked controls (Berglund et al. 2013). Rodríguez-Bayona et al. reported purified tonsillar T<sub>FH</sub> cells and plasma cells co cultured in the presence or absence of neutralizing antibody (IL-21R-Fc). This study showed that secretion of IgA by plasma cells was significantly reduced when IL-21R was neutralized as a result of blocking T<sub>FH</sub> cell-derived IL-21 (Rodríguez-Bayona et al. 2012).

Our results support that human NALT are likely to be a major induction site of immune response against influenza following LAIV immunization. It is also suggested that the *in vitro* model of human NALT using adenotonsillar cell culture could be used to study the LAIV-induced immune responses which may predict the immunogenicity and efficacy of candidate LAIV vaccines in humans. It has been shown previously that LAIV intranasal vaccination induces an immune response that more closely resembles natural immunity than that elicited by inactivated vaccine by injection and more likely to induce broader immunity (Cox & Subbarao 1999).

In summary, stimulation with LAIV induced potent HA-specific antibody production against influenza virus in NALT that was correlated with the significant increase in the number of T<sub>FH</sub> cells. The findings that co-culture of T<sub>FH</sub> cells, but not of non-T<sub>FH</sub> cells with B cells induced significant antibody production together with IL-21 production support that T<sub>FH</sub> cells provide critical support to B cell-mediated humoral immunity following LAIV intranasal vaccination.

# **Chapter 6**

## **General Discussion and Conclusion**

## 6.1 Discussion

T<sub>FH</sub> cells have been identified as a new T helper subset specialized to regulate the development of antigen-specific B cells and long-lived plasma cells. As they play a critical role in mediating B cell antibody response, promoting T<sub>FH</sub> cell number and function may be a good vaccination strategy. Research into intranasal vaccination strategy against respiratory infection has attracted a great interest in recent years, especially following the successful use of live-attenuated influenza vaccines. Intranasal vaccination relies on local mucosal immune tissue, ie. NALT, to mount a local as well as a systemic immune responses. Considering the crucial role of T<sub>FH</sub> help for B cell immunity, it is valuable to determine whether T<sub>FH</sub> plays a role in intranasal vaccine-induced immunity. In this study, we have characterised T<sub>FH</sub> cells in NALT in children and adults and investigated the possible effect of candidate adjuvant CpG-DNA on T<sub>FH</sub> cells in NALT and in mediating the B cell antibody response to influenza antigens.

### 6.1.1 Characterisation of T<sub>FH</sub> cells in human NALT

Firstly, T<sub>FH</sub> cells were characterised using a combination of surface markers and CD4<sup>+</sup> CXCR5<sup>high</sup> ICOS<sup>high</sup> were identified as T<sub>FH</sub> cells as reported previously (Bentebibel et al. 2011; Rasheed et al. 2006; Yu & Vinuesa 2010a). The frequencies of T<sub>FH</sub> expressed as a proportion in CD4<sup>+</sup> T cells range from 12% to 36% (in children) and from 5% to 20% (in adults). These are in general consistent with previous reports (Bentebibel et al. 2011; Rasheed et al. 2006; Yu & Vinuesa 2010a).

In addition to the use of CXCR5 and ICOS markers, additional markers for T<sub>FH</sub> cells including PD-1, BCL-6 and CCR7 were also used to confirm the identification of T<sub>FH</sub> in this study (Ma et al. 2009). Several cytokines are expressed by T<sub>FH</sub> cells that

have major roles in B cell function and antibody production, including IL-4, IL-10 and IL-21 (Kim et al. 2001; King 2009). We showed that T<sub>FH</sub> cells in human NALT expressed higher levels of IL-4, IL-10 and IL-21 as compared to non-T<sub>FH</sub> cell populations. These findings are consistent with the previous report that CD4<sup>+</sup> CXCR5<sup>hi</sup> cells express higher amounts of IL-4, IL-10 and IL-21 compared to CD4<sup>+</sup> CXCR5<sup>low</sup> T cells (Kim et al. 2001; Ma et al. 2009).

There was a prominent percentage of T<sub>FH</sub> cells in adenotonsillar tissues from both children and adults, especially the former. The mean T<sub>FH</sub> percentage in adenotonsillar MNC was shown to be significantly higher in children than in adults. In general, there appeared to be an age-associated decrease in T<sub>FH</sub> percentage. The finding that the percentage of T<sub>FH</sub> cells in NALT was already at a relatively high level in young children at the age 1.5-2 years suggests that T<sub>FH</sub> cells were primed fairly early and rapidly in young childhood. It is possible that immediately after birth, the intensive exposure to foreign agents (eg. microbial infection) during infancy rapidly primed T<sub>FH</sub> cells during which GC in secondary lymphoid tissues form intensively.

### **6.1.2 CpG-DNA promotes T<sub>FH</sub> cells in human NALT**

Secondly, to study whether T<sub>FH</sub> could be promoted by immunological adjuvant, CpG-DNA was used with or without an influenza antigen to stimulate adenotonsillar MNC, followed by analysis of antigen-specific B cell antibody response. We found that the number of T<sub>FH</sub> cells, defined as CXCR5<sup>hi</sup> ICOS<sup>hi</sup> cells (Figure 4.1) or as CXCR5<sup>hi</sup> BCL-6<sup>+</sup> cells (Figure 4.2) was increased by CpG-DNA stimulation in a dose dependent manner. We also showed that co-incubation of CpG-DNA with an influenza virus antigen induced a marked increase in the number of T<sub>FH</sub> cells (Figure

4.6) and that was correlated with the enhancement in HA-specific antibody production (Figure 4.4). Further analysis using co-culture of purified CD4<sup>+</sup> CXCR5<sup>hi</sup> (T<sub>FH</sub>) cells, but not non-T<sub>FH</sub> cells, with B cells in the presence of CpG-DNA and influenza antigen, induced an enhanced antigen-specific antibody response. These results suggest that the enhanced antibody production by CpG-DNA is mediated, at least in part, by T<sub>FH</sub> cells.

These results are also corroborated by several recent studies. Using a murine model Mastelic et al reported that CpG-DNA increased the T<sub>FH</sub> and GC B cell responses which was also correlated with an increase in antigen-specific antibody production (Mastelic et al. 2012). More recently, Chakarov and Fazilleau reported that mice vaccinated with a peptide in IFA combined with CpG-DNA induced a significant increase in antigen-specific T<sub>FH</sub> cells and GC B cells compared to mice vaccinated with IFA only. This CpG-DNA effect on T<sub>FH</sub> cells was dose dependent and was seen only in CpG-DNA type B and not type A or C. The increment of antigen specific GC B cells and plasma cells due to CpG-DNA was correlated with a rise in serum antigen-specific IgG antibody (Chakarov & Fazilleau 2014). An expansion of antigen-specific T<sub>FH</sub> cells, GC B cells and high titre of antigen specific antibodies in mice vaccinated with an antigen adjuvanted with CpG-DNA was also reported by Rookhuizen and DeFranco (Rookhuizen & DeFranco 2014).

### **6.1.3 LAIV promotes T<sub>FH</sub> cells in human NALT**

Thirdly, we found that the number of tonsillar T<sub>FH</sub> cells increased significantly following stimulation by LAIV in a dose dependent manner. Galli et al reported that an early post-vaccination increase in the number of vaccine-specific CD4<sup>+</sup> T cells is correlated with the rise and long-term maintenance of protective antibody titres to

avian influenza (Galli et al. 2009). More recently, Spensieri and colleagues showed in a clinical trial that blood-derived influenza-specific ICOS<sup>+</sup> IL-21<sup>+</sup> CD4<sup>+</sup> T cells help the *in vitro* differentiation of autologous B cells into cells secreting influenza-specific antibody in an antigen, IL-21 and ICOS dependent manner (Spensieri et al. 2013). In line with these findings, Bentebibel et al. reported, using a non-adjuvanted trivalent split seasonal influenza vaccine, a significant induction of ICOS<sup>+</sup> CXCR5<sup>+</sup> CD4<sup>+</sup> T cells representing a crucial immunological response associated with highly protective antibody levels after seasonal influenza vaccination. These ICOS<sup>+</sup> CXCR5<sup>+</sup> CD4<sup>+</sup> T cells were seen in blood 7 days after vaccination and correlated with the increase of antibody responses (Bentebibel et al. 2013). Furthermore, an *in vitro* induction of ICOS<sup>+</sup> CXCR5<sup>+</sup> CD4<sup>+</sup> T cells by split influenza vaccines was found to support B cells to differentiate into plasma cells (Bentebibel et al. 2013).

#### **6.1.4 Effect of CpG-DNA and LAIV on T<sub>FH</sub> cell proliferation in human NALT**

We showed in this study that tonsillar T<sub>FH</sub> cells were able to proliferate following stimulation with LAIV or with CpG-DNA combined to sH1N1 virus antigen. This may suggest that there are antigen-specific T<sub>FH</sub> (eg. influenza antigen-specific T<sub>FH</sub>) which proliferate upon antigenic stimulation. The fact CpG-DNA enhances T<sub>FH</sub> proliferation would indicate that it is possible to use CpG-DNA to modulate T<sub>FH</sub> function to enhance B cell response. Studies by Morita et al. showed the induction of blood CXCR5<sup>+</sup> CD4<sup>+</sup> T cells to proliferate after stimulation with CMV or an inactivated influenza virus (Morita et al. 2011). A study by Rasheed and colleagues reported that a polyclonal stimulation of tonsillar CD4<sup>+</sup> T cells with anti-CD3/CD28 antibodies induced T<sub>FH</sub> cell proliferation (Rasheed et al. 2006). Bendigs et al. showed that the addition of CpG-DNA to anti-CD3-stimulated T cells induced a proliferative responses in the absence of APC and suggested that CpG-DNA could

directly co-stimulate T cells (Bendigs et al. 1999). Lacroix-Lamandé *et al.* reported in a mouse model, CpG-DNA administered orally induced a significant increase in numbers of CD4<sup>+</sup> T cell observed in the intestinal mucosa and the draining LN (Lacroix-Lamandé et al. 2009).

#### **6.1.5 GC B cells strongly correlate with T<sub>FH</sub> cells**

In this study, we reported a significant increase in CD19<sup>+</sup> CD38<sup>hi</sup> IgD<sup>-</sup> (GC B cells) after LAIV stimulation. It is now known that T<sub>FH</sub> cell works closely together with GC B cells for optimal B cell response. As shown in chapter 3 (Figure 3.11), numbers of T<sub>FH</sub> cell strongly correlates with numbers of GC B cell. This is consistent with previous studies in both mice and humans (Baumjohann et al. 2013; Renand et al. 2013). A strong enhancement in number of GC B cells was shown to be correlated with the appearance of T<sub>FH</sub> cells after an influenza virus infection (Elsner, Ernst & Baumgarth 2012).

#### **6.1.6 Effect of CpG-DNA and LAIV on B cell antibody production in relation to T<sub>FH</sub> cells**

Significant antibody responses of IgG, IgM and IgA to HA of seasonal H1N1 virus were observed in adenotonsillar MNC following CpG-DNA combined with sH1N1 virus antigen or LAIV stimulation. As we showed in this study (Figures 4.5 and 5.10), co-culture of purified T<sub>FH</sub> cells, but not non-T<sub>FH</sub> cells, with purified B cells was able to induce antigen specific IgG, IgM and IgA antibodies. This finding supports the assumption that T<sub>FH</sub> help to B cells is needed for the production of these antibodies in NALT. Using a similar *in vitro* system, Bryant and colleagues co-cultured tonsillar CD4<sup>+</sup> CXCR5<sup>+</sup> cells (T<sub>FH</sub> cells) and CD4<sup>+</sup> CXCR5<sup>-</sup> (non-T<sub>FH</sub> cells) with autologous B cells and showed similar results following stimulation by PHA

and IL-2. (Bryant et al. 2007). Similar finding was also shown by Rasheed et al. reporting that CXCR5<sup>-</sup> ICOS<sup>-</sup> CD4<sup>+</sup> T cells (non-T<sub>FH</sub> cells) did not influence B cell production of IgG, and in contrast, CXCR5<sup>hi</sup> ICOS<sup>hi</sup> CD4<sup>+</sup> T cells (T<sub>FH</sub> cells) promoted B cell secretion of IgG (Rasheed et al. 2006). In addition, Rodríguez-Bayona et al. reported similar finding by co-culturing either tonsillar CD4<sup>+</sup> T cells or purified T<sub>FH</sub> cells with purified tonsil plasma cells. The purified T<sub>FH</sub> cell co-culture showed a increase of tonsillar plasma cell antibody production but not non-T<sub>FH</sub> cells co-cultures (Rodríguez-Bayona et al. 2012). Using an ELISpot assay, we also detected significant numbers of IgG ASC upon stimulation by CpG-DNA combined with sH1N1 virus antigen or the LAIV intranasal vaccine. This confirms the results of antibody induction measured by ELISA.

#### **6.1.7 Effect of pDC on T<sub>FH</sub>-mediated help for B cell antibody production**

To determine whether pDC cells contribute to the adjuvant effect of CpG-DNA to antigen-specific T cell-dependent B cell response, purified pDC were added to T<sub>FH</sub> cells co-cultured with B cells. We found that the addition of pDC to T<sub>FH</sub>-B cell co-culture significantly enhances influenza HA-specific antibody response. This suggests that pDC may contribute significantly to CpG-DNA-mediated T<sub>FH</sub> help to B cell antibody response. There has been no data available on any potential effect of pDC on T<sub>FH</sub> in the published literature. However, it has been shown that antigen presentation by myeloid DC is necessary to initiate T<sub>FH</sub> cell development (Choi et al. 2011; Goenka et al. 2011), despite that in most cases, antigen presentation by B cells is responsible for promoting the full differentiation program of T<sub>FH</sub> cells (Ballesteros-Tato & Randall 2014; Johnston et al. 2009; Nurieva et al. 2009).

As it is known that pDC express high levels of TLR-9, and we showed there is a prominent number of pDC in adenotonsillar tissue, pDC may play an important part in the T<sub>FH</sub>-mediated B cell help for antibody response in human NALT. Recently, Chakarov and Fazilleau reported a significant increase in T<sub>FH</sub> cell development upon the addition of CpG-DNA to other vaccine adjuvant and that was depended on TLR-9 signalling in CD11c<sup>+</sup> DC. They also demonstrated an important role played by monocyte-derived DCs in the T<sub>FH</sub> cell response after CpG-DNA vaccination (Chakarov & Fazilleau 2014).

#### **6.1.8 TLR-9 expression in T<sub>FH</sub>, non-T<sub>FH</sub>, B cells and pDC**

In this study, we found that TLR-9 was detectable only at a low level on T<sub>FH</sub> cells. Although it may be possible that CpG-DNA promotes T<sub>FH</sub> cells through a direct effect on T<sub>FH</sub> cells via TLR-9, it is likely that CpG-DNA may also work through B cells and or pDC, as it is known that these latter two cell types express higher levels of TLR-9. TLR expression in T cells remains inconclusive, despite several studies have reported the expression of TLR-9 on CD4<sup>+</sup> T cells and the direct co-stimulatory effects of CpG-DNA on primary T cell activation (Kabelitz 2007). A previous study by Bendigs et al. showed that CpG-DNA was able to co-stimulate T cell proliferation and cytokine production in the absence of APCs (Bendigs et al. 1999). Another study demonstrated in murine purified CD4<sup>+</sup> T cells that CpG-DNA directly enhances CD4<sup>+</sup> T cell proliferation and IL-2 production (Gelman et al. 2006). Treatment of highly purified activated mouse CD4<sup>+</sup> T cells with CpG-DNA upregulated the expression of TLR-9 (Gelman et al. 2004). TLR-9 was also expressed in tonsillar B cells. The TLR-9 expression by B cells is well documented (Bourke et al. 2003; Jiang et al. 2007). pDC was also found to express TLR-9. In humans, the expression of TLR-9 by pDC is unique in the DC system and other DC types including

conventional DC were not found to express TLR-9 (Fuchsberger, Hochrein & O'Keeffe 2005; Hornung et al. 2002).

#### **6.1.9 IL-21 is important in T<sub>FH</sub>-mediated B cell antibody production in NALT**

It is known that IL-21 plays an important role in T<sub>FH</sub> cell differentiation and B cell immunity (Eto et al. 2011). In this study, we found a rise in number of T<sub>FH</sub>-IL-21<sup>+</sup> cells after stimulation with CpG-DNA or LAIV that was correlated with an enhanced IL-21 production in cell culture supernatant. We further showed that mainly T<sub>FH</sub> cells but not non-T<sub>FH</sub> cells produced IL-21. Elevated IL-21 levels after influenza vaccination was reported by other groups (Pallikkuth et al. 2011; Parmigiani et al. 2013). A recent study using a trivalent split seasonal influenza vaccine, reported that circulating CXCR5<sup>+</sup> ICOS<sup>+</sup> CD4<sup>+</sup> T cells were found to co-express several cytokines, including IL-21. In contrast, very few CXCR5<sup>+</sup> ICOS<sup>-</sup> CD4<sup>+</sup> T cells expressed IL-21 (Bentebibel et al. 2013).

As shown in chapters 3 and 4 and 5, T<sub>FH</sub> express high levels of IL-21 that were important in T<sub>FH</sub> cell-mediated B cell antibody production in adenotonsillar cells. T<sub>FH</sub> cells also express IL-21 receptor (IL-21R), and this coexpression reflects an autocrine loop (Nurieva et al. 2008; Silver & Hunter 2008). Here we show that blocking the IL-21R by a recombinant neutralizing antibody significantly reduced the numbers of T<sub>FH</sub> cells. The reduction of T<sub>FH</sub> cells was correlated with the reduction of specific-HA IgM and IgA. These results support the importance of IL-21 in T<sub>FH</sub> cell-mediated B cell antibody production in adenotonsillar cells.

In accordance with these findings, several studies reported that IL-21 deficient mice exhibited a marked reduction in T<sub>FH</sub> cells, GC formation and antibody production compared with wild type mice after immunization (Nurieva et al. 2008; Vogelzang et

al. 2008). Using purified  $T_{FH}$  cells and B cells from human tonsillar tissues, Berglund et al. have reported a major reduction in IgM production when IL-21 was blocked by neutralizing antibody (IL-21R-Fc) compared to unblocked controls (Berglund et al. 2013). Rodríguez-Bayona et al. reported purified tonsillar  $T_{FH}$  cells and plasma cells co cultured in the presence or absence of neutralizing antibody (IL-21R-Fc). This study showed that secretion of IgA by plasma cells was significantly reduced when IL-21R was neutralized as a result of blocking  $T_{FH}$  cell-derived IL-21 (Rodríguez-Bayona et al. 2012).

#### **6.1.10 $pT_{FH}$ found in PBMC**

Recent studies suggest there may be  $T_{FH}$  equivalent in the circulation i.e. peripheral blood ( $pT_{FH}$ ) that express CXCR5 and ICOS markers and have similar B cell-help functions (Bentebibel et al. 2013; Morita et al. 2011; Pallikkuth et al. 2012; Simpson et al. 2010; Vinuesa & Cook 2011). Using these markers, we demonstrated the presence of such  $pT_{FH}$  cells in PBMC. Like NALT  $T_{FH}$  cells, an age-associated difference in the number of  $pT_{FH}$  cells was also seen and numbers of  $pT_{FH}$  cell were much lower in adults than in children PBMC. The similar correlation of these  $T_{FH}$  cell numbers found in NALT and PBMC with age may suggest that these  $pT_{FH}$  cells in the circulation are related to  $T_{FH}$  cells found in secondary lymphoid tissues. In addition, the increment of IL-21 found in PBMC culture supernatant after stimulation with CpG-DNA combined with sH1N1 influenza virus or with LAIV and its correlation with elevated numbers of  $pT_{FH}$  cells may also suggest that these  $pT_{FH}$  cells have a same origin with  $T_{FH}$  cells found in secondary lymphoid tissues.

A number of reports offer support to the hypothesis that these peripheral blood  $CD4^{+}$  CXCR5<sup>+</sup> T cells are related to  $T_{FH}$  cells found in NALT (Bossaller et al. 2006).

Recently, Morita et al. report that pT<sub>FH</sub> cells (CXCR5<sup>+</sup> CD4<sup>+</sup> T cells) from human peripheral blood provide better help to B cells than CXCR5<sup>-</sup> CD4 T cells (Morita et al. 2011). Similar to tonsillar T<sub>FH</sub> cells, a recent study reported that blood CXCR5<sup>+</sup> CD4<sup>+</sup> T cells secreted IL-21 upon contact with B cells, whereas CXCR5<sup>-</sup> CD4 T cells barely secreted any IL-21 (Morita et al. 2011). B cells were able to produce IgG, IgM and IgA only when co-cultured with CXCR5<sup>+</sup> cells in an IL-21- and ICOS-dependent manner. In contrast, CXCR5<sup>-</sup> CD4 T cells were unable to induce any switched immunoglobulin and only small amounts of IgM were produced (Morita et al. 2011).

## 6.2 Conclusion

In summary, we have shown the presence of a prominent percentage of T<sub>FH</sub> in human NALT and the percentages in children were higher than in adults, which may correlate with the enhanced exposure to microbial colonisation in children especially in early childhood. The fact that young children at 1.5-2 years already developed a high percentage of T<sub>FH</sub> suggests that T<sub>FH</sub> cell priming by antigens during early childhood is fairly efficient. Since T<sub>FH</sub> provides crucial help for B cell immune response, novel vaccine adjuvants or intranasal vaccines to boost T<sub>FH</sub> number or function may be an attractive vaccination strategy to enhance vaccine efficacy in young children. The use of CpG-DNA as an adjuvant enhanced B cell antibody response that was correlated with induction of T<sub>FH</sub> cells in human NALT, suggesting that CpG-DNA may be able to enhance vaccine immunogenicity through modulating T<sub>FH</sub> function. Stimulation with LAIV induced potent HA-specific antibody production against influenza virus in NALT that was correlated with the significant increase in the number of T<sub>FH</sub> cells. The findings that co-culture of T<sub>FH</sub> cells, but not non-T<sub>FH</sub> cells with B cells induced significant antibody production together with IL-21 production support that T<sub>FH</sub> cells provide critical support to B cell-mediated humoral immunity following stimulation with CpG-DNA and LAIV intranasal vaccine. IL-21 is crucial in T<sub>FH</sub> cell-mediated B cell antibody production in adenotonsillar cells. We also showed an important role of pDC in enhancing the antibody levels in our tonsillar T<sub>FH</sub> - B cells co-cultures. Enhancing vaccine immunogenicity through modulation of T<sub>FH</sub> cells function in human NALT using immunological adjuvants such as CpG-DNA or intranasal vaccines may be an effective vaccination strategy against respiratory pathogens.

### 6.3 Future directions

Although the present findings show novel insights of the role of T<sub>FH</sub> cells, more research is required before any definitive conclusions can be drawn on the exact role and contribution of human T<sub>FH</sub> cells in humoral immune responses.

Finding the percentage of T<sub>FH</sub> cell and their B cell helper functional capabilities from other secondary lymphoid tissue such as LN will be interesting to look at in comparison to T<sub>FH</sub> cells found in adenotonsillar tissues. This may not only support current data but also help provide a better insight into the role of T<sub>FH</sub> cells in humoral immune response.

Showing the significant age-dependent decrease in percentage of T<sub>FH</sub> cells in this study, investigating the colonization status for several pathogens in different age groups will be interesting to study to see if there may have any correlation with percentage of T<sub>FH</sub> cells.

Future studies would also involve investigation of other TLR ligands as mucosal adjuvants, including, flagellin (TLR-5 ligand), LPS (TLR-4 ligands) and other mucosal adjuvants to study their effect on T<sub>FH</sub> cell percentage and function. Other mucosal vaccines such as Rotavirus vaccines and oral Polio vaccines could be studied to investigate the role of T<sub>FH</sub> cells in promoting B cell antibody production.

Ideas for future *in vitro* co-culture experimental work would include blocking of other T<sub>FH</sub> main markers including CXCR5 and ICOS to reveal their importance in T<sub>FH</sub> cells for enhancing B cell helper capability. This would also involve investigating crosstalk between T<sub>FH</sub> cells and B cells in regulating cytokine production and B cell responses (i.e. antibody production and isotype switching),

which would be carried out under several different culture conditions.

Purification of pT<sub>FH</sub> cells found in PBMC and studying their role in promoting B cells for better antibody production and the adhesion molecules involved will be an important future study to support the hypothesis that pT<sub>FH</sub> found in PBMC and T<sub>FH</sub> found in NALT share the same origin.

Finally, *in vivo* studies using pDC deficient mice will be able to show more details in the importance of pDC in T<sub>FH</sub> cell-mediated B cell antibody production.

# **Chapter 7**

## **References**

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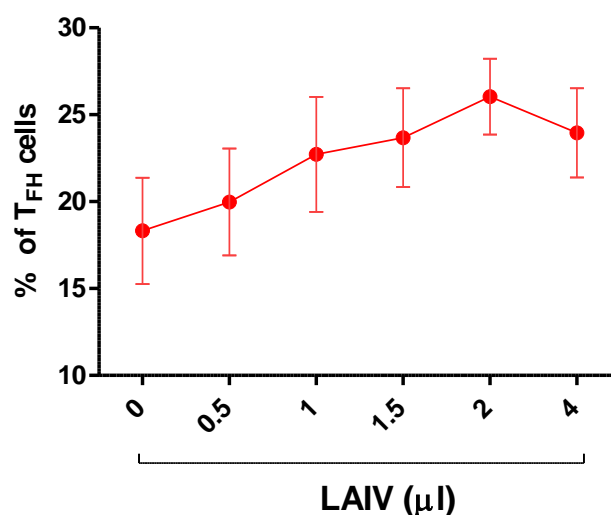
# **Chapter 8**

## **Appendices**

## Appendix-I

### *Optimisation of LAIV for $T_{FH}$ cell stimulation in adenotonsillar MNC*

The optimal concentration of LAIV required for  $T_{FH}$  cells stimulation in unfractionated adenotonsillar MNC was determined by stimulation of 5 samples with different concentrations (0, 0.5, 1.0, 1.5, 2 and 4  $\mu\text{l/ml}$ ) of LAIV. As shown in figure 8.1, 2 $\mu\text{l/ml}$  of LAIV showed to give the highest stimulatory effect compared to the unstimulated controls.



**Figure 8.1. Dose-response curve for  $T_{FH}$  cell induction by stimulation with LAIV**

Summary percentage of  $T_{FH}$  cells from unfractionated adenotonsillar MNC stimulated with different concentrations of LAIV. The values represent the mean  $\pm$  SEM from five individual tonsils.

## Appendix-II

### Preparation of different media and buffers

- **10x Phosphate Buffered Saline (PBS) pH 7.4**

1. One litre of 10x PBS was prepared in a sterile 1 litre glass bottle, using following recipe:

Na <sub>2</sub> HPO <sub>4</sub>	14.4 gm
KH <sub>2</sub> PO <sub>4</sub>	2.4 gm
NaCl	80 gm
KCl	2 gm
Deionized H <sub>2</sub> O	900 ml

2. Then pH was adjusted to 7.4 by adding concentrated HCl drop by drop, with gentle shaking of the bottle and simultaneous measurement with a pH meter. When the pH is adjusted then the bottle was topped-up to 1 litre by adding deionized water.

- **ELISA/ELISpot Washing Buffer (PBS- Tween-20)**

1. One litre of ELISA/ELISpot washing buffer (1xPBS with 0.05% Tween 20) was prepared in a sterile glass bottle, using following recipe:

10x PBS	100 ml
Deionized H <sub>2</sub> O	900 ml
Tween-20	0.5 ml

- **P-Nitrophenyl Phosphate (PNPP) Substrate for ELISA**

1. One litre of p-nitrophenyl phosphate (PNPP) substrate (1M diethanolamine) buffer (pH 9.8) was prepared in a sterile glass bottle, using following recipe:

Diethanolamine	97 ml
Deionized H <sub>2</sub> O	800 ml
MgCl <sub>2</sub>	100mg

2. Then the bottle was placed on a magnetic stirrer with a magnetic flea inside. Whilst stirring the pH was measured and adjusted to 9.8 by adding 10M hydrochloric acid.

3. Once the pH is correctly adjusted, the buffer was transferred to a measuring cylinder and distilled water was added to give a final volume of 1000 ml.
4. Thereafter, the buffer was transferred back to the bottle and stored at 4°C.
5. Finally, the substrate was prepared by dissolving p-nitrophenyl phosphate disodium salt (5mg) tablet into 5 ml of substrate buffer to give a PNPP concentration of 1 mg/ml.

- ***Acetate buffer for AEC Substrate***

1. One litre of 50 mM acetate buffer (pH 5.0) was prepared in a sterile glass bottle, using following recipe:
2. 0.2M Acetic acid solution was prepared by adding 1.16 ml glacial acetic acid into 100 ml deionized H<sub>2</sub>O.
3. 0.2M Sodium acetate solution was prepared by adding 6.8 gm sodium acetate into 250 ml deionized H<sub>2</sub>O.
4. Then, the acetate buffer was prepared by adding 74 ml of 0.2M acetic acid solution, 176 ml of 0.2M sodium acetate solution and 700 ml of deionized H<sub>2</sub>O.
5. Thereafter, the bottle was placed on a magnetic stirrer with a magnetic flea inside. Whilst stirring the pH was measured and adjusted to 5.0 by adding glacial acetic acid.
6. Once the pH is correctly adjusted, the buffer was transferred to a measuring cylinder and distilled water was added to give a final volume of 1000 ml.
7. Finally, the buffer was transferred back to the bottle and stored at 4°C.

- ***3-Amino-9-ethylcarbazole (AEC) Substrate for ELISpot***

1. AEC Substrate was prepared using following recipe:
2. 2.5 gm of 3-Amino-9-ethylcarbazole was added into 250 ml of dimethyl formamide (DMF) solution and kept in dark at 4°C (refrigerator).
3. Finally, the working substrate solution for the ELISpot assay was prepared by adding 0.5 ml of AEC substrate into 9.5 ml of acetate buffer; and 25 µl of H<sub>2</sub>O<sub>2</sub> immediately before dispensing into the plate.

## Appendix-III

### Accepted abstracts

**A. N. Aljurayyan**, N. Upile, C. Vaughan, C. Xie, R. Sharma, H. Beer, M.S. McCormick, S. Gordon & Q. Zhang (2014) A study on T follicular helper cells (T<sub>FH</sub>) in human NALT and effect of CpG-DNA on T<sub>FH</sub> -mediated antibody production. *Abstracts of the 9th European Mucosal Immunology Group Meeting, 9-12 October 2014, Glasgow, Scotland. Immunology*, 143 (Suppl. 1), 12-42.

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**A. N. Aljurayyan**, C. Loh, P. McNamara, M. McCormick, S. Gordon & Q. Zhang (2011) Effect of T follicular helper cells on regulation of mucosal immunity to upper respiratory tract pathogens by novel immunological adjuvants. *Abstracts of the Annual Congress of the British Society for Immunology, 5-8 December 2011, Liverpool, UK. Immunology*, 2011, 135, (Suppl. 1), 52-212.