ABSTRACT

Determination of Functionally Distinct Subsets of Human Tonsillar and Blood T Follicular Helper (Tfh) Cells

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T follicular helper (Tfh) cells represent a $CD4^+$ T cell subset specialized for B-cell help. While our knowledge on the biology of Tfh cells has dramatically increased during the past decade, a vast majority of observations were made in mouse models. Our knowledge on the biology of human Tfh cells has been limited compared to mouse Tfh cells. Therefore, we aimed to determine the biology of Tfh cells in human tonsils. We hypothesized that human Tfh cells are composed of functionally distinct subsets. We also determined the type of Tfh responses induced by influenza vaccination in healthy adults. First, we identified a Tfh subset localized outside germinal centers (GCs) in human tonsils. This subset expressed low-levels of CXCR5 and ICOS ($CXCR5^{lo}ICOS^{lo}$) and helped naïve B cells more efficiently than GC-Tfh cells through high IL-21 production. Notably, $CXCR5^{lo}ICOS^{lo}$ Tfh cells lacked the capacity to help GC-B cells due to FAS-L expression. This study shows that $CXCR5^{lo}ICOS^{lo}$ Tfh cells are specialized for providing

help to naïve and memory B cells outside GCs. Second, we found that influenza vaccination induces exclusive activation of $CXCR3^+$ blood Tfh subset at day 7. The emergence of activated $CXCR3^+$ blood Tfh cells at day 7 correlated with the generation of protective antibody responses. Mechanistically, we found that activated $CXCR3^+$ blood Tfh cells efficiently help memory B cells, but not naïve B cells, to differentiate into plasma cells. Thus, our study has identified a Tfh subset responsible for the induction of antibody responses in influenza vaccinations. Last, we found that $CXCR3^+$ Tfh cells localized in tonsillar GCs suppress the activity of GC-B cells and GC-Tfh cells. $CXCR3^+$ GC-Tfh cells displayed three features distinct from GC-Tfh cells: expression of surface FAS-L, secretion of large amount of IFN-γ and the lack of CD40L expression. These three features contributed the suppression of survival, growth, and activation of GC-B cells and GC-Tfh cells. $CXCR3^+$ GC-Tfh cells are clearly distinct from $Foxp3^+$ Tregs, and therefore likely represent a previously undefined regulatory T cell subset in human GCs.

Determination of Functionally Distinct Subsets of Human Tonsillar and Blood T Follicular Helper (Tfh) Cells

by

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A Dissertation

Approved by the Institute of Biomedical Studies

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Doctor of Philosophy

Approved by the Dissertation Committee

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December 2014

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LIST OF ABBREVIATIONS

ANOVA……Analysis of variance

APC……..……..Allophycocyanin

β-ME……β-merceptoethanol

CCRCC…….Chemokine receptor

CD……..Cluster of differentiation

cDNA…….Complementary Deoxyribonucleic acid

CFDA-SE……Carboxyfluorescein diacetate, succinimidyl ester

cRNA……Complementary ribonucleic acid

CTLA-4……Cytotoxic T-lymphocyte associated protein 4

CXCL…….CXC Chemokine ligand

CXCR…….CXC Chemokine receptor

DC……Dendritic cell

DMSO……Dimethyl Sulfoxide

DNA…….Deoxyribonucleotide

DNase…….Deoxyribonuclease

DNTP……..Deoxyribonucleotide phosphate

ELISA……Enzyme linked immunosorbent assay

FACS…….Fluorescent activated cell sorting

FCS……...Fetal calf serum

FDC……..Follicular dendritic cell

FITC……..Fluorescence isothiocyanate

GAPDH……...Glyceraldehyde 3-phosphate dehydrogenase

GC……Germinal center

HCl……Hydrochloric acid

ICOS……Inducible Co-stimulator

IFN-γ……...Interferon-γ

Ig……Immunoglobulin

IL……..Interleukin

LCDA……Light cycler data analysis

MACS……..Magnetic cell sorting

MHC……Major histocompatibility complex

mRNA……messenger ribonucleic acid

NaCl……Sodium chloride

NaOH……...Sodium hydroxide

NP40…….Nonidet P-40

PBMC……Peripheral blood mononuclear cells

PBS……..Phosphate buffered saline

PBST……..Phosphate buffered saline tween

PCA…….Principal component analysis

PCR……..Polymerase chain reaction

PD-1……...Programmed cell death 1

PE……Phycoerythrin

PerCP……...Peridinin chlorophyll

pH……Power of hydrogen

RNA……Ribonucleic acid

RNase……...Ribonuclease

RPM……..Revolutions per minute

RPMI…….Rosewell park memorial institute

RT……...Reverse Transcriptase

RT-PCR……Reverse transcription polymerase chain reaction

SDS……..Sodium dodecyl sulfate

SHM…….Somatic hypermutation

SLE……Systemic lupus erythematosus

STAT……..Signal transducer and activator of transcription

Tfh…….T Follicular helper

Th1……T helper 1

Th2……T helper 2

cRPMI…….Complete Roswell Park Memorial Institute Medium

HEPES……4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ICS……..Intracellular Cytokines Staining

IONO……Ionomycin

PMA……Phorbol 12-myristate 13-acetate

SEB……Staphylococcal Enterotoxin

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Hideki Ueno for his support, guidance, encouragement, and providing me freedom to attend courses and meetings. I would like to thank him also for training me during these years on how to examine the experimental observations very carefully in order to determine the underlying mechanistic basis and to ask the next set of questions. I would like to thank Dr. Jacques Banchereau for giving me the opportunity to join Baylor Institute for Immunology Research. I would like to thank Dr. Luis Vence, who has taught me basic techniques in the beginning of my studies. I would like to thank my thesis committee: Dr. Karolina Palucka, Dr. Virginia Pascual, Dr. George Cobb and Dr. Bob Kane. I would like to thank Dr. Chris Kearney and Rhonda Bellert from the Biomedical Studies program at Baylor University for their support of my journey towards this degree. I would also like to thank the core facilities for their expertise including, Elizabeth Trahan, Sebastian Coquery, Kay Kayembe, Nicolas Loof, Emily Ruchaud, Elizabeth Lavecchio in the Flow Core; Sandra Clayton in the Microscope Core; Sandy Zurawski, Amy O'Bar, and Olivier Agouna-Deciat, in the Luminex Core; Esperanza Anguiano, Indira Munagala, and Benjamin Lemoine, in the genomic core. Many thanks to my present and former colleagues Dr. Nathalie Schmitt, Dr. Rimpei Morita, Dr. Yang Liu, Dr. Radu Marches, Dr. Daisuke Chujo, Dr. Clement Jacquemin, Emile Foucat, Samir Akrour, Laure Bourdery, Parvathi Kurup, Cindy Mueller, Jonathan Provot for their daily support and their contribution to this work.

Finally, I would like to thank the administrative staff from Baylor Institute for Immunology Research including, Dr. Carson Harrod, Cindy Samuelsen, David Schackmann, Angela Plata, Pamela W. Jennings, and Dorcus Simmons for their assistance in helping to coordinate coursework, presentations, and publications.

DEDICATION

To my wife and family

# CHAPTER ONE

## Introduction

The first line of defense toward an infecting agent is called innate immunity which is characterized by broad specificity, lack of memory responses and limited repertoire of molecule recognizing antigens. On the other hand, adaptive immunity refers to antigen specific responses, which are long lasting and generated after complex interactions between various immune cells. Adaptive immunity can be classified into humoral and cell mediated immunity. The humoral component of immune system is essential for the generation of high affinity antigen specific antibodies through B cells and the cell-mediated immunity involves in the generation of effector cells, which are mainly composed of $CD4^+$ T cells and $CD8^+$ T cells. It has been known for decades that $CD4^+$ T cells are crucial in supporting and regulating the efficacy, and longevity of humoral immune responses (Goodnow et al., 2010; Miller et al., 1965). In the secondary lymphoid organs, T cell help to B cells (T dependent antibody response) is provided to naïve B cells that have captured protein antigen and present the processed peptides in the context of major histocompatibility complex (MHC) class II to cognate $CD4^+$ T cells at the border of the T cell zone and the B cell follicle (Garside et al., 1998; Okada et al., 2005). Once naïve B cells are activated, they can adopt one of the two B cells differentiation pathways: (1) movement into extrafollicular area and then proliferation and differentiation into short-lived plasma cells that secrete antibodies with low affinity which are important for immediate defense against infection; (2) movement into B cell

follicle and then proliferation and establishment of germinal centers (GCs), a structure where GC-B cells differentiate into either long-lived plasma cells or memory B cells (Jacob et al., 1991; MacLennan and Gray, 1986; MacLennan et al., 2003; McHeyzer-Williams et al., 1993). A GC gathers at least three types of cells: GC-B cells, follicular dendritic cells (FDCs), and T follicular helper (Tfh) cells (Figure 1).

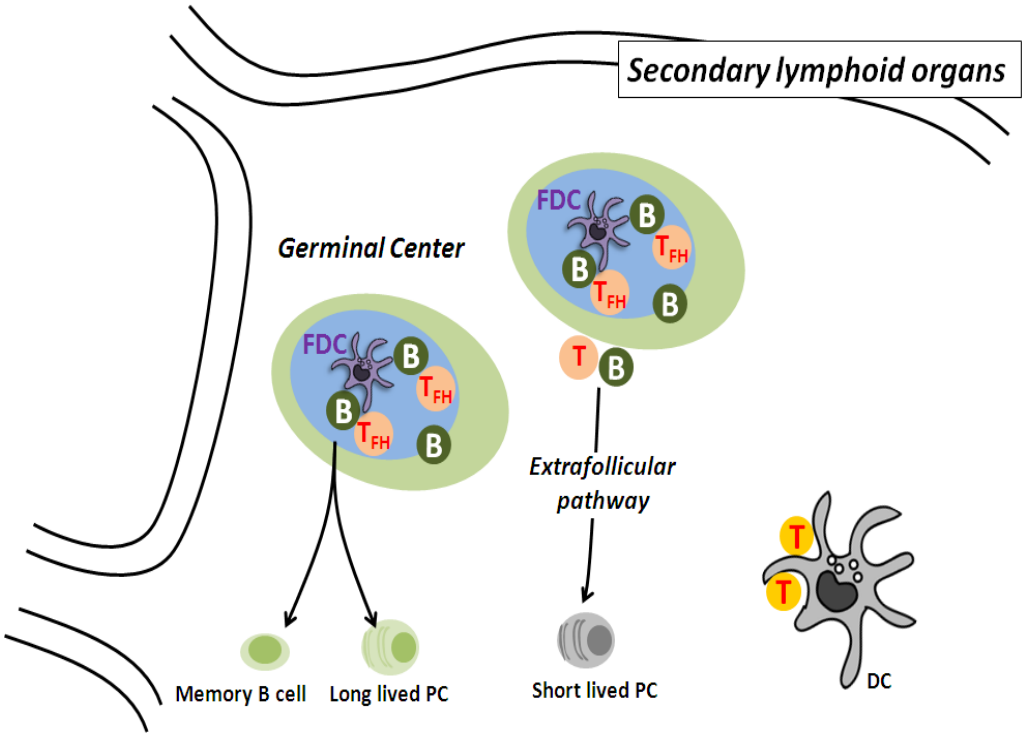


Figure 1: T dependent antibody response. Dendritic Cell (DC) internalizes antigen, processes into peptides, and presents peptides together with MHC molecules to T cells which result in their activation and migration toward B cell follicle, and interact with B cells. T and B cell interaction initiate the B cell differentiation process toward two different paths: extracellular plasma cells and cells forming GCs.

GCs are highly organized and specialized structures found within the B cell follicle of secondary lymphoid tissues, such as tonsils, lymph nodes, and spleen. GCs are the sites of B cell expansion, somatic hypermutation (SHM), isotype switching, affinity maturation, plasma cell and memory B cell generation. B cells that have received T-cell help migrate into the B cell follicle, initiate robust proliferation, and differentiate into

highly proliferating B cells named centroblasts. The part of GCs that contained intense concentration of centroblasts is called “dark zone” (Cozine et al., 2005). In contrast to dividing centroblasts, centroblasts that have lost the capacity to divide become centrocytes, and migrate out of the “dark zone” to the other half of GCs known as “light zone”. Isotype switching or class switch recombination (CSR) and somatic hypermutation primarily takes place in the dark zone, whereas affinity maturation primarily occurs in the light zone. As GCs extends, resting naïve B cells within primary follicles are displaced towards the outer edge of the follicle and form distinctive ring surrounding GCs named “follicular mantle” (Liu et al., 1992)(Figure 2).

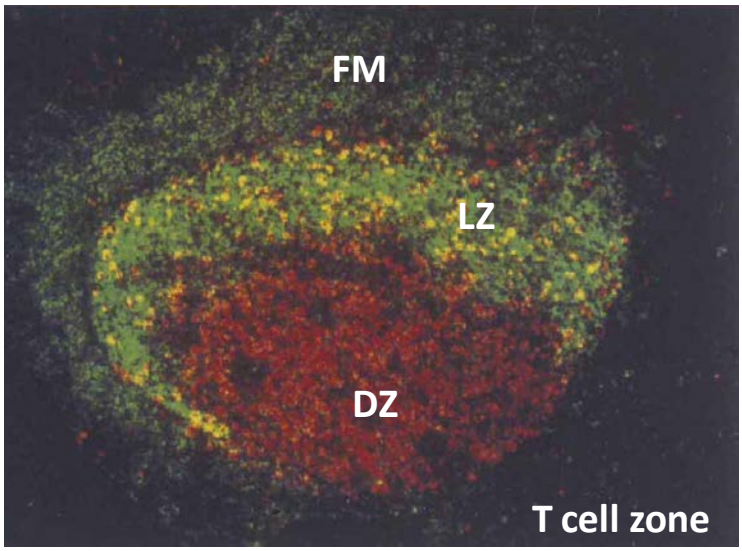


Figure 2: Germinal center (GC) compartments in human tonsil. Modified figure From Liu et al., Immunology Today 13, 17-21 (1992) GCs are organized into dark zone, light zone, outer zone, and mantle zone. Green immunofluorescence identifies weak CD23 expression by B cells in the follicular mantle (FM) and strong CD23 expression by follicular dendritic cells in the light zone (LZ). Red immunofluorescence shows the heavy concentration of cells in cell cycle in the dark zone (DZ) identified by the Ki67 monoclonal antibody.

Naïve mature B cells synthesize immunoglobulins (Igs) IgM and IgD. However, after exposure to antigen, they switch to other class of (Igs) such as IgG, IgA or IgE to increase the functional diversity of Igs molecules. Isotype switching or class switch

recombination (CSR) involves the replacement of the μ (for IgM) constant region with one of the downstream γ (for IgG), α (for IgA) or ε (for IgE) constant regions. The constant region of the heavy chain determines the isotype of the antibody. The variable region (VDJ) specific for the antigen will be retained along with the newly formed heavy chain. Several decades ago, it was observed that after immunization with a foreign antigen, the average affinity of serum antibody for the foreign antigen increases over time (Eisen and Siskind, 1964). This process was later named affinity maturation and shown to be due to the somatic mutation (SHM), a process leading to point mutations in the variable regions of heavy and light chain genes to substitute new amino acids (Jacob et al., 1991). A high frequency of somatic mutation was evident in GC-B cells, suggesting that a hypermutation mechanism is activated in GC (Berek et al., 1991). Somatic mutation enables B cells to generate post GC clones with high affinity for the antigen. High affinity GC-B cells receive stimuli from Tfh cells and FDCs cells give rise to memory B cells and long lived plasma cells. On the other hand low affinity and self-reactive clones do not receive survival signals undergo apoptosis.

### *$CD4^+$ T Helper Subsets*

$CD4^+$ T cells, beside their effector activity, play an essential role in regulating the function of other immune cells. $CD4^+$ T cells consist of several different T helper (Th) subsets, including Th1, Th2, Th17 and regulatory T cells (Tregs) (Figure 3).

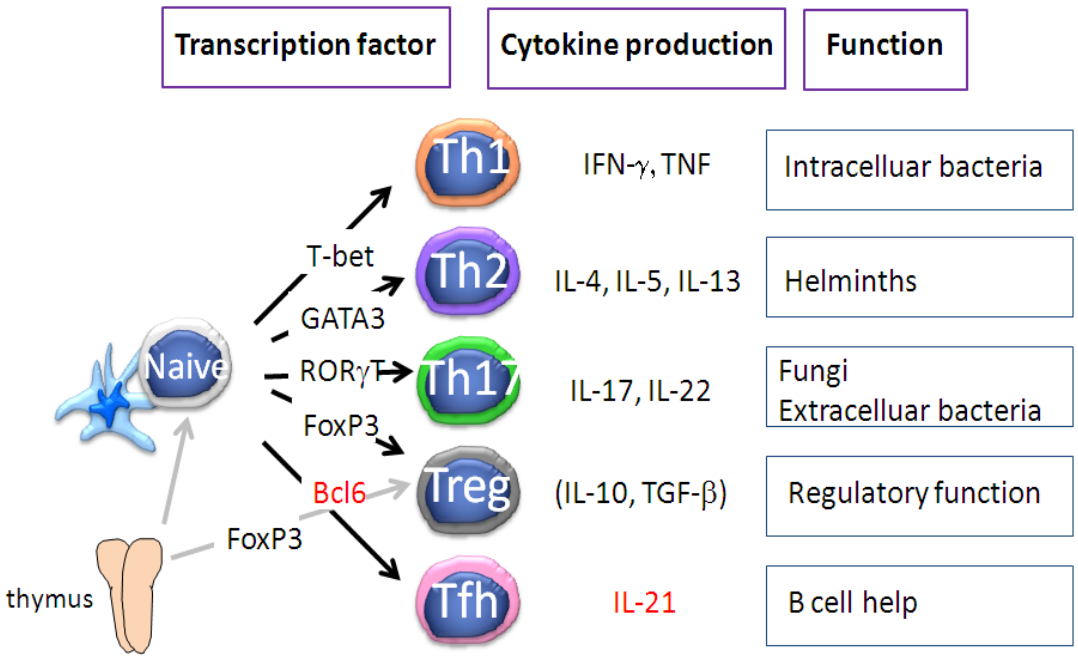


Figure 3: CD4$^+$ T helper cell lineages. When a naïve T cells encounters a DCs bearing its cognate antigen, it is activated and in the presence of the right environmental signals, it can polarize into different T helper cells subsets including Th1, Th2, Th17, T regulatory (Tregs), and T follicular helper (Tfh) cells. Each of them exhibits a unique phenotype, production of a selective set of cytokines, transcriptional profile and exerts different functions in immune response.

These subsets differentially control immune responses through the type of cytokines they secrete. Th1 cells express the lineage-specific transcription factor T-bet and secrete IFN-γ which is necessary for protection from intracellular microbes (Szabo et al., 2000). Th2 cells are characterized by the expression of GATA-3, a transcription factor that drive production of the cytokines IL-4, IL-5, and IL-13. These cytokines are essential for clearance of helminthes (Zheng and Flavell, 1997). Th2 cells have been linked to humoral immune responses and IgG1 and IgE class switching (Coffman and Carty, 1986). On the other hand an overabundance of Th2 cells leads to pathogenic conditions, such as atopic airway hypersensitivity and asthma (Kay, 1991; Robinson et al., 1992). More recently, distinct subsets of regulatory T cells (Tregs) including naturally occurring Treg (nTreg) and inducible (iTreg) have been described as negative regulators

of immune responsiveness to inhibit self-reactivity or guard against over-reactivity to pathogens (Fehervari and Sakaguchi, 2004). The lineage commitment of Treg cells is controlled at the transcriptional level by specific transcription factors, most notably by FoxP3 (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). FoxP3 expressing cells are very important in maintaining peripheral tolerance as mice deficient in this transcription factor develop a fatal lympho-proliferative autoimmune syndrome that affects multiple organs (Brunkow et al., 2001). Similarly, in humans mutations in the FoxP3 gene results in the immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), inflammatory conditions that can manifest as diabetes-mellitus and psoriasis-like dermatitis (Bennett et al., 2001). In addition, reduction in number or reduced activity of Tregs cells has been reported in various autoimmune diseases, such as diabetes, SLE and multiple sclerosis (MS) (Lindley et al., 2005; Venken et al., 2008). In 2005, other subsets of $CD4^+$ T cells producing IL-17 were identified and named Th17 cells (Harrington et al., 2005; Park et al., 2005). The Th17 cells subset add an additional layer to the this complex system of immune regulation, Th17 cells express the retinoid-related orphan receptor (ROR)-γt and (ROR)-α and secrete IL-17A and IL-17F, two cytokine that act on different cell types and induce the production of IL-6, IL-8, GM-CSF, G-CSF, CXCL1, and CCL20 and in this way attract neutrophils and activate them (Dubin and Kolls, 2008). Indeed, these two cytokines were shown to be essential for the clearance of extracellular bacteria and fungi. Apart from IL-17, Th17 express, IL-6, TNF, GM-CSF, IL-21, IL-22 and in humans also IL-26 (Langrish et al., 2005; Liang et al., 2006; Nurieva et al., 2007; Wilson et al., 2007). The major function of the cytokines produced by these cells is to chemo-attract other cells through the induction of other

cytokines and chemokines (Romagnani, 2008). Th17 cells can also produce IL-22 (Liang et al., 2006), a cytokine required for regulation of host defenses by cell populations at epithelial barriers (Eyerich et al., 2009). Recently, a subset of $CD4^+$ T cells present in the B cell follicle, named T follicular helper cells (Tfh) cells have been established as the $CD4^+$ T cells that provide help to B cells to induce antibody response (Crotty, 2011; King et al., 2008).

### *Function of Tfh Cells*

Tfh cells have been established as Th subset specialized for providing help to GC-B cells (Fazilleau et al., 2009; King et al., 2008). Several sets of molecules distinguish them from other Th subsets. The C-X-C chemokine receptor 5 (CXCR5) (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000), which guides them to migrate into B cell follicle through the recognition of its ligand, the chemokine CXCL13, which is secreted by follicular dendritic cells (FDCs) (Ansel et al., 2000; Cyster et al., 2000) as well as Tfh cells that have migrated into GCs (Lim et al., 2004). Expression of CXCR5, together with a down-regulation of the T cell zone homing receptor C-C chemokine receptor 7 (CCR7), allows them to relocate from the T cell zone of the lymphoid tissue to the B cell follicles, where they are positioned to directly support B cell activation, expansion and differentiation (Ansel et al., 1999).

Tfh cells secrete large amount of IL-21 (Bryant et al., 2007; Chtanova et al., 2004), a type I cytokine that shares the common receptor γ-chain with IL-2, IL-4, IL-7, IL-9, and IL-15 (Parrish-Novak et al., 2000). IL-21 is critical cytokine for the growth and differentiation, and class switching of B cells (Kuchen et al., 2007; Ozaki et al., 2002; Spolski and Leonard, 2008). The predominant mechanism underlying IL-21 induced B

cell differentiation is STAT3 (signal transducer and activator of transcription 3) mediated induction of blimp-1, a transcriptional repressor critical for the generation of plasma cells (Ozaki et al., 2004). In the absence of STAT3, blimp-1 is not induced and plasma cells are not generated (Fornek et al., 2006). Indeed, patients with STAT3 mutation show impaired functional antibody responses (Avery et al., 2010). IL-21 is important also for Tfh cells differentiation and survival in an autocrine manner (Johnston et al., 2009; Vogelzang et al., 2008).

Tfh cells express high levels of programmed Death-1 (PD-1)(Haynes et al., 2007), a potent inhibitory receptor that belongs to the CD28/CTLA-4 family (Keir et al., 2008). PD1 is expressed by activated $CD4^+$ and $CD8^+$ T cells, and associated with the exhaustion of $CD8^+$ T cells during chronic virus infection and cancer, thus playing a negative role in immune responses (Haymaker et al., 2012; Kaufmann and Walker, 2009). However, little is known about its function in Tfh cells. It has been shown that PD1 deficient Tfh cells have altered cytokine secretion with decreased in IL-21 production (Good-Jacobson et al., 2010), and mice deficient in PD1 have dysfunctional Tfh cells that cannot appropriately select IgA precursor cells in the GC of Peyer's patches (Kawamoto et al., 2012). Furthermore, PD1 blockade was shown to expand Tfh number in several studies (Good-Jacobson et al., 2010; Hamel et al., 2010; Hams et al., 2011). However, these studies yielded controversial results about GC magnitude. For instance, Good-Jacobson et al. reported impaired plasma cells and GC responses to alum/protein T cell dependent immunization. In contrast, Hams et al. found increased germinal center responses in PD-L1 deficient mice after infection with the parasite Schistosoma mansoni or immunization with keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant

(CFA). These controversial results might arise from the different immunization protocols and may lie in the genetic differences of mice strains used in these studies.

Tfh cells also express multiple surface molecules required for the interaction with B cells, including CD40-ligand (CD40L), Inducible co-stimulator (ICOS), and signaling lymphocyte activation molecule-associated protein (SAP) (King et al., 2008). CD40L is a crucial factor that ligates CD40 at the surface of B cells to induce their activation, proliferation, survival, and differentiation in vitro and in vivo. Of particular interest, CD40 signaling is critical for the maintenance of GC-B cells (Takahashi et al., 1998). CD40 signaling for maintenance of GC reaction was shown by the finding that established GC can be disturbed using blocking antibody specific for CD40L (Han et al., 1995) as well as the finding that GC are absent in mice and human with inactivating mutation in their CD40L gene (Gulino and Notarangelo, 2003). Indeed, human CD40L deficient patient have a severe lack of memory B cells (Agematsu et al., 1998; Kawabe et al., 1994; Longo et al., 2009).

The efficient help of Tfh cells to GC-B cannot occurs without the formation of stable conjugate between the two partners. Therefore, adhesion molecules are important in the establishment of this conjugates, and among them, the family of the signaling lymphocyte activation molecule (SLAM) receptors. CD84 and ly108 have emerged as adhesion molecules expressed by Tfh and they are necessary for stable interaction between T and B lymphocyte. It has been shown that CD84 is critical for late Tfh cells differentiation and GC maintenance (Cannons et al., 2010). Both CD84 and ly108 signal via the adaptor SLAM-associated protein (SAP). The absence of SAP expression in Tfh cells leads to an absence of long lived plasma cells and memory B cells, and a severe

impairment in GC formation (Crotty et al., 2003). Indeed, SAP deficient Tfh cells are unable to stably interact with B cells, and as result to induce their normal proliferation (Qi et al., 2008).

ICOS is another critical molecule for Tfh cells. ICOS is fundamental for the helper function (Hutloff et al., 1999) as well as the development of Tfh cells both in mice and humans (Akiba et al., 2005; Bossaller et al., 2006). ICOS-deficient mice showed substantially impaired development of Tfh cells and GC B cells in response to primary or secondary immunization and have profound defects in B cell maturation and immunoglobulin (Ig) isotype switching (Dong et al., 2001). A similar phenotype was observed in ICOS-L deficient mice (Mak et al., 2003; Wong et al., 2003). In human, ICOS deficiency is characterized by a severe alteration of GCs formation, and significantly reduced numbers of circulating Tfh cells, indicating an essential role of ICOS in the differentiation of Tfh cells (Bossaller et al., 2006). Human tonsillar Tfh cells found in GCs express ICOS at the highest levels among tonsillar Th cells (Rasheed et al., 2006). Studies using a mouse model of systemic lupus erythematosus (SLE) demonstrated that overexpression of ICOS leads to the substantial increase of GC formation associated with exaggerated Tfh responses (Vinuesa et al., 2005), which lead to the development of autoimmunity (Linterman et al., 2009). Indeed, Blockade of ICOS signaling has a therapeutic benefit in lupus prone mice (Iwai et al., 2003). Gene array analysis showed that Tfh cells have a distinct transcriptional profile from other Th subsets (Chtanova et al., 2004). These analyses reported that Tfh cells express high levels of B-cell lymphoma-6 (Bcl-6), a transcription factor essential for the development of GC B cells (Dent et al., 1997; Ye et al., 1997). Bcl-6 is necessary for the development of Tfh

cells in vivo as its deficiency in $CD4^+$ T cells results in impaired Tfh cells generation and GC formation in vivo, whereas its overexpression promotes Tfh development. Bcl-6 acts as a transcription repressor by binding to the promoter region of T-bet, GATA3, and RORγT, thus suppressing Th1, Th2, and Th17 differentiation (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009) and it dominantly directs T cells toward Tfh cells. Bcl-6 also downregulates expression of molecules required for the T cell zone localization and promoting the Tfh migration cells toward the B cell follicle (Poholek et al., 2010). Thus, Bcl-6 represents a master transcriptional factor controlling Tfh generation. In contrast, blimp-1 a transcription factor that represses the function of Bcl-6, inhibits the generation of Tfh cells. Indeed, $CD4^+$ T cells that over-express blimp-1 suppress the expression of Bcl-6 and fail to differentiate to Tfh cells (Johnston et al., 2009). More importantly, the expression of blimp-1 does not inhibit the differentiation of non Tfh $CD4^+$ T cells. Taken together, the balance between Bcl-6 and blimp-1 expression in T cells is critical for the fate of Tfh cells differentiation.

### *Development of Tfh Cells*

Considerable attention in recent years has focused on the pathways involved in the generation and development of Tfh cells. Several signals seems to be involved in their generation, including cytokines, T cell receptor (TCR) signaling, and specific receptor ligands on antigen presenting cells (APCs) with receptors on $CD4^+$ T cells. It has been shown in several mouse studies that IL-6, IL-21, induce IL-21 production by naïve $CD4^+$ T cells in vitro in a STAT3-dependent manner (Eto et al., 2011; Lu et al., 2011; Nurieva et al., 2008). In contrast to mice, IL-12 was found to be more potent than IL-6 and IL-21 at inducing IL-21 production by human naïve $CD4^+$ T cells (Ma et al., 2009; Schmitt et

al., 2009). Stimulation of $CD4^+$ T cells with IL-6 or IL-12 cytokine induce them to up-regulate CXCR5, ICOS, and Bcl-6 expression (Ma et al., 2009; Schmitt et al., 2009). However, the in vitro generated Tfh cells expressed lower levels of CXCR5, ICOS, and bcl-6 than GC Tfh cells indicated that additional signals are required for full Tfh cells differentiation. In addition to cytokines, T cell activation requires engagement of the receptor on T cells such TCR, CD28, CD40L, and ICOS with multiple ligands expressed on antigen presenting cells (APCs) such as peptide/MHC class II, CD80/CD86, CD40, and ICOS-L, respectively. For instance, $CD4^+$ T cells fail to up-regulate CXCR5 when stimulated in the absence of signaling though CD28, ICOS/ICOS-L or CD40/CD40L (Akiba et al., 2005; Bossaller et al., 2006). Furthermore, both CD28 and CD86 deficient mice exhibited profound alteration of Tfh cells development (Salek-Ardakani et al., 2011). Tfh cells generation requires also interactions between activated $CD4^+$ T cells and B cells. Mice lacking B cells have decreased numbers of Tfh cells after immunization or infection (Deenick et al., 2010; Haynes et al., 2007; Johnston et al., 2009; Poholek et al., 2010; Salek-Ardakani et al., 2011). Indeed, ICOS-L expression on B cells is critical to induce IL-21, Bcl-6, and c-Maf on activated $CD4^+$ T cells (Bauquet et al., 2009; Chevalier et al., 2011; Choi et al., 2011; Gigoux et al., 2009). SAP is another important molecule required for Tfh cells maintenance and the formation of stable conjugates with B cells (Cannons et al., 2010; Deenick et al., 2010; Qi et al., 2008).

### *Tfh in Immunity and Disease*

It has become now clear that the control of Tfh cells generation and function is essential to human health as their overabundance is associated with autoimmunity, whereas a reduction in their number is associated with immunodeficiency. Furthermore,

an understanding of the regulation of Tfh cells may provide a significant insight in the development of novel vaccine and improvement of the vaccines efficacy that are currently in use. For instance, influenza vaccines provide protection mainly by generating high-affinity antibodies against hemagglutinin thereby preventing virus entry (Nichol et al., 1994; Osterholm et al., 2012). However, early immunological events that lead to the development of protective immunity following vaccinations remain largely unknown (Crotty, 2011; King et al., 2008).

Tfh cells are also found in human blood, and share functional properties with Tfh cells in secondary lymphoid organs (Chevalier et al., 2011; Morita et al., 2011). We have also shown that human blood Tfh cells are composed of subsets which differentially express the chemokine receptors CXCR3 and CCR6, and display different functions (Morita et al., 2011). For example, $CXCR3^{+}CCR6^{-}$ cells produce IFN-γ, while the $CXCR3^{-}CCR6^{+}$ cells produce IL-17A (Morita et al., 2011). At variance with Tfh cells in secondary lymphoid organs, blood Tfh cells are in a resting state, and do not express ICOS (Simpson et al., 2010). It has been shown that quantifying the frequency of circulating Tfh cells can be used as biomarkers of successful vaccination. Pallikkuth and colleagues demonstrated in two separate studies that the ability of the H1N1 vaccine to induce protective antibody response correlated with the induction of detectable feature of Tfh cells mediated immunity (Pallikkuth et al., 2012; Pallikkuth et al., 2011). In contrast to their important role in inducing protective antibody response, emerging evidence suggests that altered Tfh response contributes to pathogenesis of various diseases. The first demonstration was overrepresentation of Tfh cells in several murine model of SLE (Subramanian et al., 2006; Vinuesa et al., 2005). Consistently, circulating $ICOS^{+}$ Tfh

cells are also found at increased frequencies in the blood of patients with SLE and Sjorgen's syndrome (Simpson et al., 2010), which are autoimmune condition characterized by the production of auto-antibodies. In contrast to autoimmune diseases, Tfh cells are found at decreased frequencies in several primary immunodeficiency disorders. For instance, patients with deficiency in CD40L have a severe reduction in peripheral blood Tfh cells (Bossaller et al., 2006). Similar to CD40L deficient patients, ICOS deficient patients have a reduced number of Tfh cells in the blood (Bossaller et al., 2006). Tfh cells are also reduced in patients with mutations in STAT3 gene, a transcription factor, which is a downstream of numerous cytokines signaling pathway, including those involved in the differentiation of Tfh cells, specifically, IL-6, IL-21, and IL-12. Furthermore, patients with Mendelian susceptibility to mycobacterial due to mutation in the beta chain of IL-12 receptor (IL-12Rβ1) have fewer circulating Tfh cells, reduced number of memory B cells and impaired GC formation (Schmitt et al., 2013).

Studies in humans and rhesus macaques have recently demonstrated an increase of Tfh cells in the lymph nodes when infected with HIV and SIV, respectively (Lindqvist et al., 2012; Perreau et al., 2013; Petrovas et al., 2012). An increase of Tfh cell frequency positively correlated with the number of GC B cells and plasma cells in the lymph nodes in these studies. However, the quality of the generated antibodies appears to be poor in HIV-infected subjects (Lindqvist et al., 2012). Furthermore, Tfh cells from HIV-infected subjects were found to have a poor capacity to help B cells in vitro due to high expression of PDL1 by GC-B cells in HIV patients (Cubas et al., 2013). Thus, the diversion of Tfh cell function appears as an important mechanism of HIV escape from humoral immunity

*Regulation of Tfh Cells and GCs Formation*

The eradication of infectious diseases and the development of effective vaccines require Tfh cell mediated antibody secretion. In contrast, the alteration of their function could trigger autoimmunity. However, mechanisms that regulate Tfh cells activity were largely unknown until the emergence of follicular regulatory T (Tfr) cells as a novel subset that controls normal GC response. It has been shown that Tfr cell deficient mice showed increased numbers of Tfh cells, GC-B cells and increased antibody production (Chung et al., 2011; Wollenberg et al., 2011). Furthermore, mice lacking functional Tfr cells have an increased number of non-antigen-specific B cells indicating that Tfr cells may be specialized in suppressing self-reactive GC-B cells. Similar to Tfh cells, Tfr cells required Bcl-6 and SAP signaling for their development (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011). Furthermore, Tfr cells share features with both Tfh cells (expression of Bcl-6, CXCR5, PD1, and ICOS) and Treg cells (expression of GITR, CTLA4, CD25, and Blimp-1). However, they differ from Tfh cells in that they lack CD40L, IL-4, and IL-21 (Linterman et al., 2011) and instead appear to play a regulatory role as Tfr cell deficient mice exhibit increased numbers of Tfh cells, GC-B cells and increased antibody production (Chung et al., 2011; Wollenberg et al., 2011). The effects of Tfr cells may be complemented by the actions of regulatory $CD8^+$ T cells. These cells have had a long history in immunology. The concept of $CD8^+$ T cells capable of suppressing other immune system cells was introduced by Gershon and Kondo in the 1970s (Gershon and Kondo, 1970) . However, the major problem facing scientists today is the lack of a specific marker. Furthermore, these regulatory cells were not extensively studied particularly after the emergence of $CD4^+$ Tregs (Sakaguchi et al., 1995). Many

subpopulations of $CD8^+$ T cells capable of suppressing the activation of other immune system cells have been identified, with various mechanisms of suppression. Recently, a subset of $CD8^+$ T cells restricted by the nonclassical MHC molecule Qa1 was established as a subset, and suppress the function of Tfh cells in the B cell follicles (Kim et al., 2010). In contrast to conventional Treg cells, regulatory $CD8^+$ T cells lack expression of FoxP3 and CTLA4, and their suppressive capacity required their expression of perforin and a source of IL-15 (Kim et al., 2010). Thus, the emergence of $CD8^+$ T regulatory cells in the GC highlights a new mechanism for intracellular cross talk between T cells of the GC that is fundamentally critical for shaping protective and pathological immunoglobulin production.

### *Aims of Dissertation*

The main goal of my dissertation is to investigate the phenotypic and functional heterogeneity within human tonsillar and blood Tfh cells. We hypothesized that human tonsillar Tfh cells are composed of subsets that differ in their phenotype and the functions, as we observed in human blood Tfh cells. Some subset might play a regulatory role. We also hypothesized that a particular subset of Tfh cells might be responsible for the generation of antibody responses in influenza vaccination.

My research focus is on these three topics.

First, we aim at identifying $CD4^+$ T cells interacting with B cells outside GCs in human tonsils. While Tfh cells are considered to help the selection and differentiation of B cells in GCs, what type of $CD4^+$ T cells interact with B cell outside GCs is unknown. These $CD4^+$ T cells could be precursors of Tfh cells. Alternatively, $CD4^+$ T cells

interacting with B cells outside GCs might be largely similar to conventional effector $CD4^+$ T cells.

Second, we aim at determining the type of blood Tfh cell response after seasonal influenza vaccination. We have previously shown that human blood Tfh cells are composed of subsets including Tfh1 ($CXCR3^+CCR6^-$), Tfh2 ($CXCR3^-CCR6^-$), and Tfh17 ($CXCR3^-CCR6^+$) cells. While, Tfh2 and Tfh17 cells are efficient B cell helper, Tfh1 cells are not. Thus, it is possible that activation of blood Tfh2 or Tfh17 cells might correlate with antibody responses in influenza vaccination.

Last, we aim at determining the functions of $CXCR3^+$ Tfh cells in GCs in human tonsils. We were intrigued by the finding that blood $CXCR3^+$ Tfh cells lack the capacity to help naïve B and memory B cells to produce immunoglobulins. The phenotype and function of $CXCR3^+$ Tfh cells in the secondary lymphoid organs are unknown. It is possible that tonsillar $CXCR3^+$ Tfh cells act as regulatory cells in GCs.

# CHAPTER TWO

## Materials and Methods

### *Primary Human Material*

#### *Tonsils and Spleen*

Tonsils specimen were obtained from patients undergoing tonsillectomy at Baylor University Medical Center and single cells were collected by mechanical disruption of tonsil samples. Spleen samples were obtained from a cadaveric organ donor, or from the tissue bank at Baylor University Medical Center.

#### *Peripheral Blood*

Blood samples were obtained from healthy control subjects before and after the administration of a single intramuscular dose of a non-adjuvant trivalent split seasonal influenza vaccine (Fluzone®, Sanofi Pasteur). A cohort of 12 healthy adults (M 6, F 6; Age 34 ± 9) received vaccination in 2009/2010 winter, another cohort of 37 healthy adults received vaccination in 2011/2012 winter, and a cohort of 20 healthy children (M 12, F 8; Age 11 ± 3) received vaccination in 2010/2011 winter. 2009/2010 vaccine contained A/Brisbane/59/2007 (H1N1)-like, A/Brisbane/10/2007 (H3N2)-like, and B/Brisbane/60/2008-like, while 2010/2011 and 2011/12 vaccines contained A/California/7/2009 (H1N1)-like A/Perth/16/2009 (H3N2)-like, and B/Brisbane/60/2008-like. Blood apheresis was performed from healthy adults at 7 d after administration of influenza vaccines. Whole blood samples were used for phenotypic analysis of $CD4^+$ T cells by flow cytometry. PBMCs were isolated by centrifugation on a gradient

(Ficoll-Paque, Stemcell), frozen and stored in liquid nitrogen. The study was approved by the Institutional Review Boards (IRBs) of Baylor Health Care System and Nationwide Children's Hospital. Informed consent was obtained from subjects, parents, or legal guardians.

### *Tonsillar $CD4^+$ T and B Cell Isolation*

All of the studies described here were approved by the Institutional Review Board of Baylor Research Institute. For tonsillar experiment, the entire tissue was wrapped in a nylon membrane and mechanically disrupted using backside of a 60 ml syringe plunger in a cell culture plate. The cell suspension was collected in a conical tube and diluted up to 50 ml. B cells were first positively selected with CD19 MACS Microbeads (Miltenyi Biotech). For the isolation of Th subpopulations, CD19-negative fraction was stained with biotin-conjugated anti-ICOS (ISA-3; eBioscience)/Streptoavidin PE-Cy7 (BD pharmigen), anti-CD20 FITC (2H7; eBioscience), anti-CXCR5 PE (51505.111; R&D Systems), anti-CD4 Pacific Blue (RPA-T4; BD pharmigen), anti-CD8 APC (RPA-8; eBioscience), and anti-CD56 APC (MEM 188; eBioscience). The four Th subpopulations were sorted with FACSAria (BD Biosciences) according to the expression of CXCR5 and ICOS within $CD4^+CD20^-CD8\text{-}CD56^-$ cell population. For the isolation of B cell subsets, $CD19^+$ B cells were stained with anti-CD20 FITC (2H7; eBioscience), anti-CD3 PE-Cy5 (UCHTI; eBioscience), anti-IgD PE (IA6-2; BD pharmigen) and anti-CD38 APC (HIT2; eBioscience). Naïve B cells ($CD20^+IgD^+CD38^-$), memory B cells as ($CD20^+IgD^-CD38^-$), and GC B cells ($CD20^+$ $IgD^-CD38^+$) were sorted within a CD3 negative cell population.

*Phenotype Analysis of Tonsillar $CD4^+$ T Cells*

Phenotype of tonsillar Th and B cell subsets was analyzed with the following antibodies: Anti-PD1 PE (J116; eBioscience), CD57 PE (NK-1; SouthernBiotech), CD200 PE (MRC OX104; BD pharmigen), CD84 PE (CD84.1.21; Biolegend), BTLA PE (MIH25; Biolegend), CD69 PE (FN50; eBioscience), CD45RO PE-Cy5 (UCHL1; eBioscience), CD127 PE (hIL-7R-M21; BD pharmigen), and CD95 Pacific blue (DX2; ebiosciences), after gating to each population as described above.

*FACS Analysis of Transcription Factors Expression*

For the analysis of Bcl6, T-bet and Foxp3 protein expression, tonsillar CD19-negative fraction was first stained with anti-CD4 (BD Biosciences), anti-CD3 (UCHT1; BD Biosciences), anti-CXCR5 (RF8B2; BD Biosciences), anti-ICOS (C398.4A; Biolegend) anti-CXCR3 (1C6/CXCR3; BD Biosciences) or anti-CXCR3 (G025H7; Biolegend). After fixation and permeabilization using transcription factor buffer set (BD pharmigen), cells were incubated with anti-Bcl6 Alexa Fluor 647 (K112-91; BD Biosciences), anti-T-bet PE (04-46; BD pharmigen), and anti-Foxp3 PE (206D; Biolegend) for 30 min at room temperature. The stained cells were acquired on BD LSRII.

*Immunohistochemistry and Immunofluerescence*

Tonsils were placed in Tissue-Tek OCT (Sakura), frozen in liquid nitrogen and stored at -80°C. For immunohistochemistry, 6 μm cryostat sections were fixed in acetone and air dried. After treatment with FcReceptor Block and CytoQ Background Buster (Innovex Biosciences) for 20 min each, slides were incubated with the primary antibody against CD127 (eBioRDR5, purified; eBioscience) diluted in Cyto Q Immuno Diluent for

40 min at room temperature. The Stat-Q Peroxidase Staining System with AEC (Innovex Biosciences) was used according to manufacturer's directions. Sections were counterstained with Aqua Hematoxylin (Innovex Biosciences), rinsed in tap water, and coverslipped using Advantage Mounting media from Innovex. Slides were imaged using a Nikon DXM1200C digital camera and Olympus BX60 microscope with Plan4x/0.13 objective. For immunofluerescence, the following antibodies were used: anti-CD4 (RPA-T4, purified; BD pharmigen) conjugated with Molecular Probes Alexa 568 monoclonal antibody kit, anti-CD127 Alexa647 (eBioRDR5; eBioscience) and anti-CD45RO FITC (UCHL1; eBioscience). Anti-FITC Alexa 488, goat IgG fraction (Molecular Probes) was used as a secondary to enhance the FITC signal. Sections were blocked with both FcReceptor Block and Background Buster in succession, and then incubated for 1 hour in the antibodies diluted in CytoQ immunodiluent, rinsed and incubated in anti-FITC. DAPI (Molecular Probes) was used to stain the nuclei. Slides were mounted with Fluoromount-G (Southern Biotech), observed using Metamorph software version 6.2 (Universal Imaging Corporation, UK), Roper coolsnap HQ camera and Olympus BX51 microscope with Plan10x/0.40 objective. Slides were also observed under a Leica SP5 confocal microscope with a 40x/1.25 Planapo objective.

*Tonsillar $CD4^+$ T and B Cell cultures*

Sorted Th subpopulations were co-cultured with B cell subsets ($2 \times 10^4$ cells/well each for 8 d for Ig measurement, and $5 \times 10^4$ cells/well each for 2 d for cytokine measurement) in RPMI 1640 medium (GIBCO) supplemented with 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma), 1% sodium pyruvate (Sigma), 1% non-essential amino acids (Sigma), 50 μM β-mercaptoethanol (Sigma), 50 μg/ml gentamycin

(GIBCO) and 10% heat-inactivated FCS (ATCC) in the presence of SEB (1 μg/ml, Sigma-Aldrich) in 96 well U bottomed plates. The levels of Igs (IgM, IgG and IgA) were determined by ELISA. Indicated cytokines were measured with Luminex. The levels of CXCL13, soluble FAS-L (TNFSF6) were determined with ELISA (both R&D Systems). Sorted Th subpopulations ($5x10^4$ cells/well) were also stimulated for 2 d with CD3/CD28 beads (0.2 μl per well) for cytokine measurement. In some experiments, anti-ICOS blocking mAb (10 μg/ml; Ancell), IL-21R/Fc (20 μg/ml; R&D Systems), anti-IL-10 blocking mAb (25 μg/ml, developed and validated at the Institute) plus anti-IL-10R mAb (20 μg/ml; R&D Systems), anti-Fas blocking antibody (20 μg/ml; Millipore) or isotype-matched controls were added to the co-cultures. For the analysis of plasma cell differentiation, naïve B cells labelled with CFSE (Molecular probes) were cultured for 8 d with Th subpopulations. The cultured cells were stained with anti-CD3 PE-Cy5, anti-CD4 Pacific Blue, and anti-CD38 APC, and the frequency of $CFSE^-CD38^{hi}$ plasmablast cells within $CD3^-CD4^-$ cells were analyzed by FACS Canto II. In some experiments $CD4^+$ T cell subsets were cultured with naïve B cells for 5 days in the presence of SEB and then assessed for their expression of ICOS and PD1.

*Immunoglobulin Measurement*

Flat-bottomed microtiter plates (Nunc) were coated with goat anti-human IgM, IgG, IgA Abs (5 μg/ml; all from SouthernBiotech, Inc.) in carbonate buffer (pH 9.5). After blocking with PBS containing 10% FBS or 2% BSA for 1 h, the plates were incubated with diluted supernatants at room temperature for 2 h (IgM, IgG, and IgA). After washing, plates were incubated at room temperature for 1 h with alkaline phosphatase-conjugated goat anti-human IgM, IgG, or IgA Abs (at a final dilution of

1/2000, 1/2500, or 1/2500, respectively. all from SouthernBiotech, Inc.). For IgM, IgG, or IgA measurement, the plates were incubated with p-nitrophenyl phosphate (Sigma) after extensive wash. The reaction was stopped with 2 N H2SO4. The optical density was read by SpectraMax plate reader (Molecular Devices). The lower limits of the detection levels are 20 ng/ml, 3 ng/ml, 2 ng/ml for IgM, IgG, and IgA, respectively.

*Cytokine Measurement by Luminex Technique*

The concentrations of IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, IL-21, IFN-γ and TNF-α were determined by bead-based multi-cytokine assay (LuminexTM). Mouse monoclonal antibodies specific for IL-2, IL-5, IL-10, IL-13, IL-17, IL-21, and TNF-α were raised in the Institute, and conjugated to microbeads. Antibody pairs for cytokine measurement were validated for the measurement by Luminex. Briefly, Luminex analysis incorporates the use of colored microbeads into samples that can bind to a variety of cytokines. Microbeads will fluoresce a particular color, which is read by the analyzer, which is similar to a flow cytometer, and converted to a numerical value. A minimum of 50 µl of culture supernatant is required to measure multiple cytokines. If sample volume is less than 50 µl, samples will be diluted to increase the volume but the sensitivity may be lost. The Bio-Plex suspension array system employs patented multiplexing technology that uses up to 100 color-coded bead sets, each of which can be conjugated with a specific reactant. Each reactant is specific for a different target molecule. Bio-Plex cytokine assays are designed in a capture sandwich immunoassay format. Antibody specifically directed against the cytokine of interest is covalently coupled to color-coded 5.6µm polystyrene beads. The antibody-coupled beads are allowed to react with a sample containing an unknown amount of cytokine, or with a standard solution containing a

known amount of cytokine. After performing a series of washes to remove unbound protein, a biotinylated detection antibody specific for a different epitope on the cytokine is added to the beads. The result is the formation of a sandwich of antibodies around the cytokine. The reaction mixture is detected by the addition of streptavidin-phycoerythrin (streptavidin-PE), which binds to the biotinylated detection antibodies. The constituents of each well are drawn up into the flow-based Bio-Plex suspension array system, which identifies and quantities each specific reaction based on bead color and fluorescence. The magnitude of the reaction is measured using fluorescently labeled reporter molecules associated with each target protein. Unknown cytokine concentrations are automatically calculated by Bio-Plex Manage 5.0 software (Bio Rad, CA) using a standard curve derived from a recombinant cytokine standard. By using colored beads as the solid phase instead of a coated well, up to 100 differently colored beads can be mixed and used for quantitating up to 100 different analyses simultaneously. Information on the standard curve for the experiment is plotted, and compared to the values generated for each different kind of microbead to calculate a value in pg/ml of that cytokine in the supernatant.

*Real Time PCR*

Total RNA was extracted from tonsil Th subpopulations using RNeasy mini kit (QIAGEN), and reverse-transcribed into cDNA in a 96-well plate using the High Capacity cDNA Archive kit (Applied Biosystems). The primer pairs (Integrated DNA Technology) used in this study was designed using the Roche Primer Design Program. Primer sequences were as follows: Bcl-6 **(**Accession number: NM_001706.2) forward primer: 5’ ttccgctacaagggcaac-3’, reverse primer: 5’- tgcaacgatagggtttctca-3’; Prdm1

(Accession number: NM_001198.2) forward primer: 5’ gtggtgggttaatcggtttg -3’, reverse primer: 5’- gaagctcccctctggaataga-3’; FAS-L **(**Accession number: NM_001706.2) forward primer: 5’ tggggatgtttcagctcttc-3’, reverse primer: 5’- tgtgcatctggctggtagac-3’, IL-21 (accession number NM_021803.2) forward primer: 5′-aggaaaccaccttccacaaa-3′, reverse primer: 5′-gaatcacatgaagggcatgtt-3′; CXCL13 (accession number NM_0006419.2) forward primer: 5′-tctctgcttctcatgctgct-3′, reverse primer: 5′-tcaagcttgtgtaatagacctcca-3′. Real-time PCR was set up with Roche Probes Master reagents and Universal Probe Library hydrolysis probes. PCR reaction was performed on the LightCycler 480 (Roche Applied Science) followed these conditions: step 1 (denaturation) at 95°C for 5 min, step 2 (amplification) at 60°C for 30 min, step 3 (cooling) at 40°C for 30 seconds. The expression level of each gene was normalized to the levels of housekeeping gene *HRPT1*.

*NanoString nCounter Assay*

NanoString nCounter is a gene analysis expression system which captures and counts specific mRNA transcripts directly without enzymatic reaction and the levels of each transcript can be determined within a sample by counting the number of molecule of each sequence type and calculating concentration with reference to internal standards. The nCounter system is suitable for gene profiling signatures measuring over 500 genes in a single reaction, particularly from samples with limited amounts of RNA. Briefly, freshly isolated Tonsillar $CD4^+$ T cells cells were lysed in RLT buffer and total RNA was purified using RNeasy Micro Kit (Qiagen). NanoString reactions were performed according to manufacturer's instructions (NanoString Technologies). The nCounter code set includes 100 genes of interest and 10 control genes. Samples were hybridized using

100 ng of total RNA. The expression levels of each gene were normalized to those of 20 control genes. The normalized results are expressed as the relative mRNA level.

### *Western Blotting*

Total proteins were extracted from sorted tonsil Th subpopulations using radio immunoprecipitation assay buffer (RIPPA, Sigma-Aldrich) supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich). 9 μg of protein per sample was separated on NuPAGE (Invitrogen) 4-12% Bis-Tris gradient gels and transferred on PVDF membranes (Invitrogen). Membranes were incubated with Abs against Bcl-6 (clone D8) and Blimp-1 (clone 6D3) antibody (Both from Santa Cruz Biotechnology, Inc) followed by HRP conjugated anti-mouse or anti-rat, respectively (Both from Santa Cruz Biotechnology). Equal protein loading was confirmed using goat anti actin (clone I-19; Santa Cruz Biotechnology, Inc).

### *Flow Cytometry and Gating Strategy for the Analysis of Blood Tfh Cell Subsets*

Whole blood samples (200 μl) were incubated with the indicated antibodies and LIVE/DEAD fixable Aqua (Invitrogen) for 15 minutes at room temperature. MAbs: CXCR5 (1G10), CD3 (UCHT1), CD8 (SK1), CD4 (RPA-T4), CCR6 (11A9), CXCR3 (1C6/CXCR3), from BD. ICOS (C398.4A) were from Biolegend. CD45RA (2H4) mAbs were from Beckman Coulter. CD45 (HI30) mAbs were from Invitrogen. Stained cells were acquired on a BD LSRII. Phenotype of $CD4^+$ T cells was assessed with FlowJo software (TreeStar).

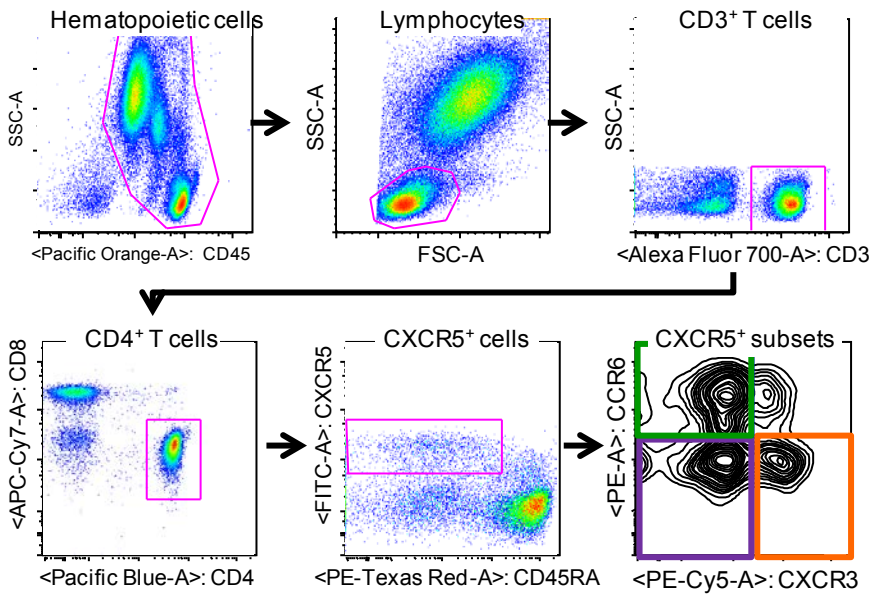


Figure 4: Gating strategy for the analysis of blood Tfh cell subsets.

*Assessment of the Proliferation of Th Subsets by Ki67 Staining*

For the assessment of Ki67 expression, enriched $CD4^+$ T cells ($2 \times 10^6$) at day 7 post vaccination were stained with anti-Ki67 A488 (B56; BD pharmigen). The phenotype of $CD4^+$ Th subsets was also analyzed for with anti-PD1 PE (J105; eBioscience).

*Antibody Assays*

Hemagglutination inhibition (HI) and virus neutralization (VN) antibody titers for each strain of influenza virus incorporated in the vaccines were determined at baseline and at day 28 postvaccination.

*Hemagglutination Inhibition (HI) Assay*

HI assays were performed by standard methods. Briefly, collected sera were first treated with receptor-destroying enzyme (RDE) to remove non specific inhibitors of hemagglutination. Each influenza virus strain was mixed with twofold dilutions of the specific RDE-treated serum in PBS in V-bottomed 96-well plates. After 30 min of

incubation at room temperature, chicken erythrocytes were added to the mixtures. The plates were kept at 4°C until a positive hemagglutination was developed in non-serum containing control wells. The HI titer was defined as the highest dilution of the serum able to inhibit hemagglutination.

*Microneutralization Assay*

Briefly, Influenza virus containing 100 PFU was incubated with the specific RDE-treated serum for 1 h at room temperature in a 96-well plate containing an MDCK cell monolayer. After the incubation, the virus-serum samples were removed from the wells. The cells were incubated at 37°C for 2 days in minimal essential medium-bovine albumin. The microneutralization titer was defined as the highest dilution of serum that neutralized 100 PFU of virus in MDCK cell cultures.

*CD154 Assay*

PBMCs at day 7 post-vaccination were stimulated for 6 h with Fluzone® or killed Flu-virus (PR8) in the presence of Brefeldin A and monensin, and expression of intracytoplasmic cytokines together with CD154 was analyzed. After the stimulation period, cells were washed twice with PBS. Cells were subsequently incubated with anti-CD3 (UCHT1), anti- CD4 (S3.5), anti-CXCR5 (RF8B2), anti-ICOS (C398.4A), and Aqua live/Dead (Invitrogen). After permeabilization, cells were stained intracellularly with the following combination of antibodies: anti-IL-2 (MQ1-17H12), anti-IL-10 (JES3-9D7), anti-IL-21 (3A3-N2.1), anti-IFN-γ (4S.B3), and CD154 (24-31) for 30 min at room temperature. The stained cells were acquired with LSR Fortessa.

*Assessment of Influenza Specific $CD4^+$ T Cells Function*

Memory $CD4^+$ T cells enriched by using memory human $CD4^+$ T cell isolation kit (Miltenyi Biotec) were stained with anti-CD4 (RPA-T4), anti-CXCR5 (RF8B2), anti-CCR6 (11A9), anti-CXCR3 (1C6/CXCR3), anti-ICOS (C398.4A), anti-CD45RA (HI100), and an APC-conjugated mAb cocktail including anti-BDCA 3 (AD5-14H12), anti-CD16 APC (3G8), anti-CD19 APC (HIB19), anti-CD20 APC (2H7), anti-BDCA-2 (AC144), anti-CD56 (B159), anti-CD8 (SK1), anti-CD11c (S-HCL-3), anti-NKp46 (9E2). $CXCR5^+$ memory $CD4^+$ Th subsets were sorted from the dump-APC negative cells. For the isolation of B cell subsets, B cells were first positively selected with CD19 MACS Microbeads (Miltenyi Biotech), then stained with anti IgD (IA6-2;), anti-CD20 (2H7), anti-CD3 (UCHT1), anti-CD27 (O323). Naïve B cells ($CD20^+IgD^+CD27^-$) and memory B cells ($CD20^+IgD^-CD27^+$) were sorted after excluding $CD3^+$ cell population. Sorted $CD4^+$ $CXCR5^+$ Th populations (2 x $10^4$ cells/well) were co-cultured with naïve B cells (2 x $10^4$ cells/well) for 12 days and with memory B (2 x $10^4$ cells/well) cells for 6 days, in RPMI 1640 medium (GIBCO) supplemented with 1% L-glutamine (Sigma), 1% penicillin/streptomycin (Sigma), 1% sodium pyruvate (Sigma), 1% non-essential amino acids (Sigma), 50 μM β-mercaptoethanol (Sigma), 50 μg/ml gentamycin (GIBCO) and 10% heatinactivated FCS (ATCC) in the presence of SEB (1 μg/ml, Sigma-Aldrich) in 96 well U bottomed plates. For the phenotype analysis of recovered B cells, recovered cells were stained with anti-CD3, anti-CD4, anti-CD38 (HB7) and anti-CD138 (MI15). The frequency of $CD38^{hi}CD138^-$ plasmablasts and $CD38^+CD138^+$ plasma cells within the $CD3^-CD4^-$ B cells were analyzed with FACS LSRII. We calculated the cell recovery per well, based on the frequency of the indicated cell population in the flow data and the

recovered viable cell numbers per well. In blocking experiments, endogenous IL-21 and IL-10 were neutralized by the addition of IL- 21R/Fc (20 μg/ml; R&D Systems), anti-IL-10 blocking mAb (20 μg/ml, developed at the Institute) plus anti-IL-10R mAb (20 μg/ml; R&D Systems). In some experiment sorted $CD4^+$ Th subsets were co-cultured with memory B cells loaded with Fluzone, and the phenotype of recovered B cells and the secreted influenza-specific IgG were analyzed at day 6 of the culture.

*Fluzone Specific IgG ELISA*

Flat-bottomed microtiter plates were coated with Fluzone diluted 1/10 in carbonate buffer (pH 9.5). After blocking with PBS containing 10% FBS for 1 h, the plates were incubated with diluted supernatants at room temperature for 2 h. After washing, plates were incubated at room temperature for 1 h with alkaline phosphatase-conjugated goat anti-human, IgG at a final dilution 1/2500 (SouthernBiotech, Inc.), the plates were incubated with p-nitrophenyl phosphate (Sigma) after extensive wash. The reaction was stopped with 2 N H2SO4. The optical density was read by SpectraMax plate reader (Molecular Devices).

*Assessment of Influenza Specific $CD4^+$ T Cells Proliferation and Cytokines Secretion*

Sorted $CD4^+$ Th subsets ($5 \times 10^4$ cells/well) were co-cultured with memory B cells ($5 \times 10^4$ cells/well) loaded with Fluzone. Culture supernatants were harvested at day 2 and cytokines were measured by Luminex. At day 6, the cultured cells were stained with anti-CD3, and anti-CD4 and the frequency of $CD4^+CD3^+$ T cells in the culture were measured using FACS LSRII.

*Suppression Assay and Transwell Experiments*

GC-Tfh cells were labeled with 1μM CFSE (Cell tracer Kit, Molecular probes) according to standard protocols. Tfh cells (2 x $10^4$ cells) (effector cells) were cultured in 96-well round bottom in the presence of varying amounts of GC-Tfh1 autologous (suppressor cells). Cell cultures were stimulated with anti-CD3/CD28 mAb coated beads (0.1 μl) in 200μl complete RPMI culture medium. After 5 days, cells were harvested, stained for surface markers CD3, CD4 and CFSE signal of gated lymphocytes was analyzed by flow cytometry. In some experiment, cytokine secretions in the supernatants were measured by Luminex. To assess whether cell to cell contact was necessary for GC-Tfh1 cells to mediate suppression, transwell experiment were performed in 96 wells transwell inserts (0.4 μm pore size, Corning) by culturing GC-Tfh cells (2 x $10^4$ cells) in the lower chamber and GC-Tfh1 (2 x $10^4$ cells) in the upper chamber in the presence of anti-CD3/CD28 mAb coated beads. After 48 hours of culture, the inserts were removed, and the cytokines were measured in the lower chamber by Luminex and after 5 days, proliferation was measured based on CFSE dilution. In blocking experiment, the GC-Tfh1 cells were put together with GC-Tfh cells before adding the anti-CD3/CD28 mAb coated beads. MAbs to FAS, and IFN-γ were added to co-culture at concentration of 20 and 10 μg/ml respectively).

# CHAPTER THREE

## Results: Human Tonsillar *BCL-6*$^+$ Tfh-Committed Cell Subsets Differentially Help B Cell Subsets

### *Introduction and Rational*

As described in chapter one, Tfh represent a Th subset specialized for the help of antibody responses and they are considered to induce the differentiation of GC B cells into long-lived plasma cells or memory B cells at GCs, however, the identity of $CD4^+$ T cells interacting with B cells outside GCs remains largely unknown. Furthermore, whether Tfh cells play a role at earlier stages of B cell response remains to be addressed. Recently, studies with MRL/FASlpr lupus-prone mouse models showed the presence of extrafollicular $CD4^+$ T cells which display similar phenotype with Tfh cells, such as upregulation of Bcl-6 and IL-21, and down-regulation of PSGL-1 (Odegard et al., 2008; Poholek et al., 2010). While these studies show that Tfh-committed cells can be generated at extrafollicular sites, whether this holds true in humans is unknown. In this chapter, we used human tonsil samples to address these questions. We found that human tonsillar Tfh cells are composed of subsets specialized for the help of distinct B cell subsets. A subset of Tfh cells, expresses low-levels of CXCR5 and ICOS ($CXCR5^{lo}ICOS^{lo}$) and IL-7R, is specialized for the help of naïve and memory B cells. In contrast, Tfh cells at GCs (GC-Tfh cells) are the only Tfh subset able to help GC-B cells. $CXCR5^{lo}ICOS^{lo}$ IL-$7R^+$ cells were found to express *BCL-6* transcripts at equivalentlevels with GC-Tfh cells. Interestingly, $CXCR5^{lo}ICOS^{lo}$ IL-$7R^+$ cells were found exclusively at

extrafollicular sites. Thus, the differentiation of human Tfh cells occurs at outside of B cell follicles, and Tfh cells at different maturation stage and locations are engaged to help distinct B cell subsets. Expression of EBI2, a factor negatively regulating cell migration into B cell follicle, by GC-Tfh cells was significantly lower than $CXCR5^{lo}ICOS^{lo}$ IL-7R□ cells, which might explain their distinct location.

*ICOS and CXCR5 Define Four Subpopulations in Tonsillar Th Cells*

Previous studies demonstrated that human tonsillar Tfh cells in GCs co-express ICOS and CXCR5 at high intensities (Rasheed et al., 2006). In our study, we obtained pediatric tonsil samples (from children ages 3-12 years old), which contained 34.8 ± 0.9 % of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells among tonsillar $CD4^+$ T cells (Figure 5A and B blue gate. Mean ± SEM, n=20). Tonsillar $CD4^+$ T cells included at least three other populations defined according to the expression of CXCR5 and ICOS: $CXCR5^-ICOS^-$ (black gate, 21.4 ± 1.1 %), $CXCR5^{lo}ICOS^{lo}$ (red gate, 19.5 ± 0.9 %), and $CXCR5^{lo}ICOS^{hi}$ (green gate, 10.4 ± 0.5 %) cells (Figure 5A, B).

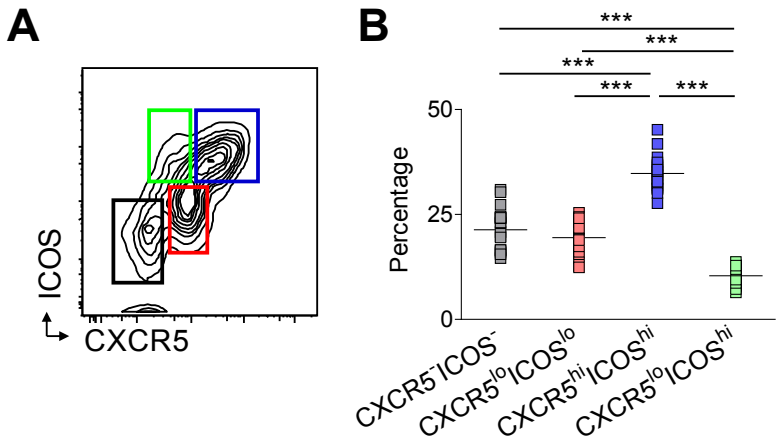


Figure 5: Four Th populations in pediatric tonsil samples (A) Four tonsillar Th populations were defined according to the expression of ICOS and CXCR5 (B) The frequency of each Th population. N=20. One-way ANOVA Bonferonni's multiple comparison test. *** $p<0.001$.

*Phenotype of Th Populations in Pediatric Tonsil Samples*

As observed earlier (Haynes et al., 2007; Ma et al., 2009), a majority of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells expressed programmed death-1 (PD-1) (Figure 6, 84 ± 2 %, Mean ± SEM, n=3). $CXCR5^{lo}ICOS^{hi}$ $CD4^+$ T cells also expressed PD-1 though at lower density than $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells (41 ± 1 %). Only a minor fraction of $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells expressed PD-1 (8 ± 3%) at very low density. CD57, a molecule expressed by a fraction of human GC-Tfh cells (Kim et al., 2001), was expressed at high density by a fraction of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells (Figure 6, 35 ± 3 %), whereas $CXCR5^{lo}ICOS^{hi}$ $CD4^+$ T cells and $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells contained less CD57-expressing cells (Figure 6, 10 ± 1 %, and 5 ± 1%, respectively). $CXCR5^-ICOS^-$ $CD4^+$ T cells did not express PD-1, CD57, or CD45RO, thus were mostly composed of naïve $CD4^+$ T cells. As reported previously (Lim and Kim, 2007; Ma et al., 2009), a large fraction of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells did not express IL-7R (CD127) (Figure 6). In contrast, $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells expressed IL-7R as much as

CXCR5$^{-}$ICOS$^{-}$ naïve CD4$^{+}$ T cells, whereas CXCR5$^{lo}$ICOS$^{hi}$ CD4$^{+}$ T cells expressed IL-7R only at low density.

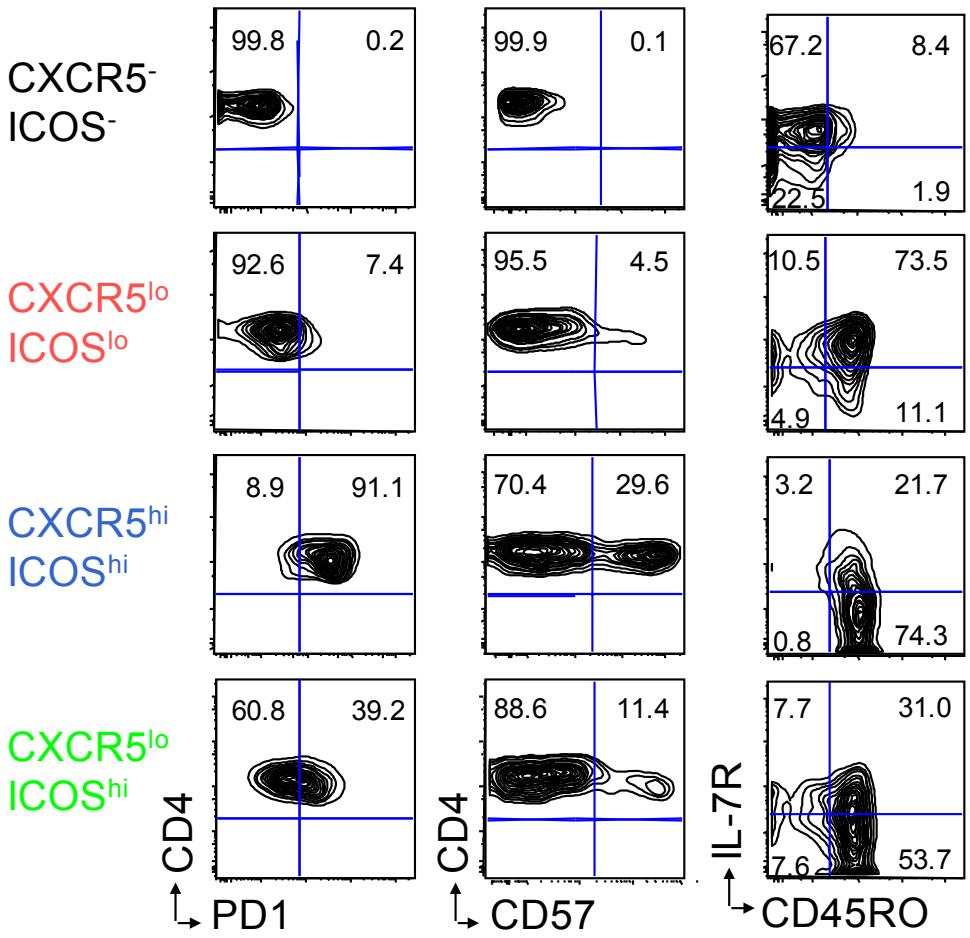


Figure 6: Phenotype of Th populations in pediatric tonsil samples. Expression of cell surface molecules (PD1, CD57, IL-7R and CD45RO). A representative from three experiments.

*CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T Cells are Localized Outside GCs*

A previous study reported the absence of IL-7R-expressing cells in GCs (Lim et al., 2007). We confirmed this observation by immunohistochemistry (Figure 7A). Whereas both CXCR5$^{-}$ICOS$^{-}$ and CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells expressed IL-7R, CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells co-expressed CD45RO (Figure 6). Thus, the localization of CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells was determined by analyzing IL-7R$^{+}$CD45RO$^{+}$CD4$^{+}$ T

cells by confocal staining. As shown in Figure 7B and Figure 7C, the cells co-expressing the three molecules (CD45RO indicated by green; CD4 indicated by red; IL-7R indicated by blue. The triple positive cells appear white in the staining) were exclusively localized outside GCs. Although we cannot exclude the possibility that the identified IL-7R$^{+}$CD45RO$^{+}$CD4$^{+}$ T cells might contain CXCR5$^{lo}$ICOS$^{hi}$ CD4$^{+}$ T cells, our observation demonstrates that CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells are exclusively localized outside, but not within, GCs.

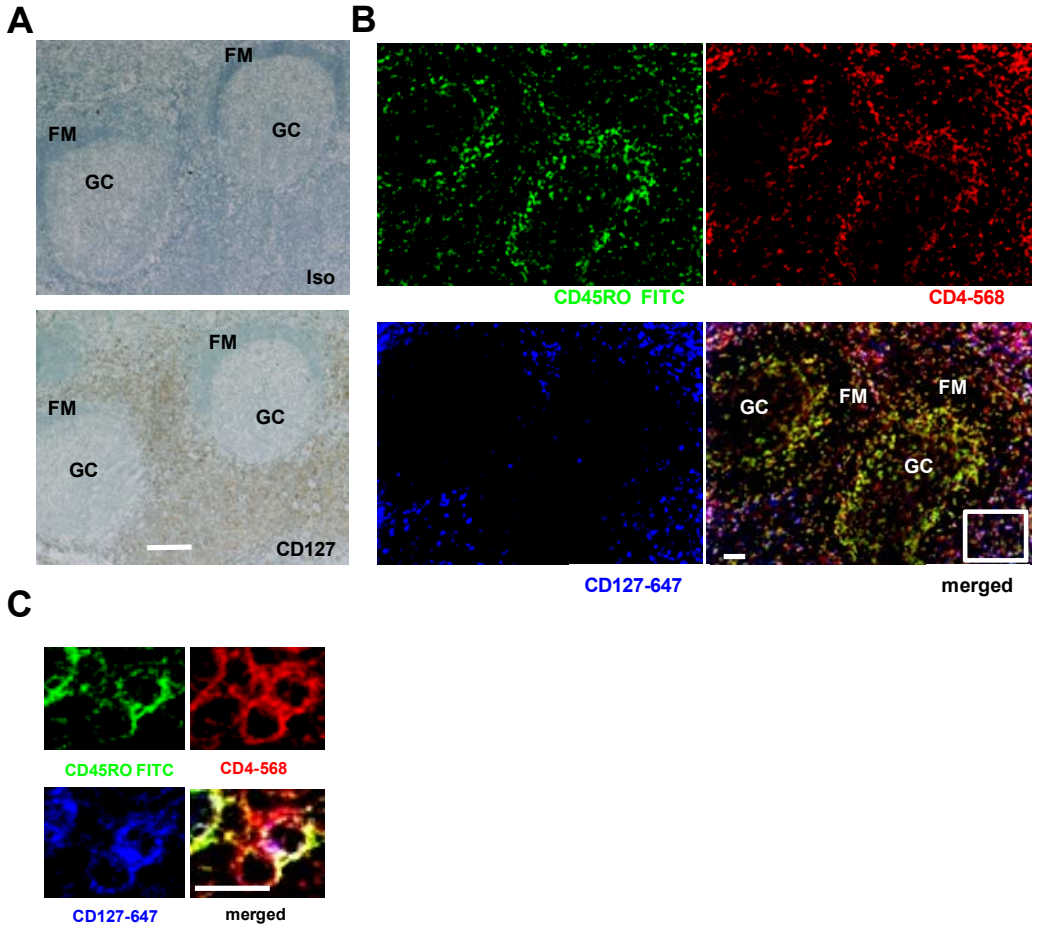


Figure 7: CXCR5$^{lo}$ICOS$^{lo}$ cells localize exclusively outside GCs (A) Localization of IL-7R$^{+}$ cells in tonsils was analyzed by immunohistochemistry using a frozen tonsil section, FM: follicular mantle Scale bar, 100 µm (B, C) Localization of CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells was analyzed by the co-expression of CD4 (red), CD45RO (green), and IL-7R (blue) by immunofluorescence microscopy, Scale bar 100 µm. Triple positive cells illustrated by the white color include CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells. A higher magnification view is shown on (C) (analyzed by a confocal microscopy) Scale bar, 15 µm.

We next analyzed which molecules explain the different localization of cells $CXCR5^{lo}ICOS^{lo}$ $CD4^{+}$ T cells and $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells. Mouse studies demonstrated that migration of Th cells is regulated by the balance of expression levels of CXCR5 and CCR7. For the entry into GCs, Th cells need to upregulate CXCR5, while they need to downregulate CCR7 (Hardtke et al., 2005; Haynes et al., 2007). While $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells do not express CCR7, $CXCR5^{lo}ICOS^{lo}$ $CD4^{+}$ T cells were found to express CCR7 though at low levels (Figure 8A). Upon co-culture with naïve B cells, $CXCR5^{lo}ICOS^{lo}$ $CD4^{+}$ T rapidly downregulated the expression of CCR7 (Figure 8B).

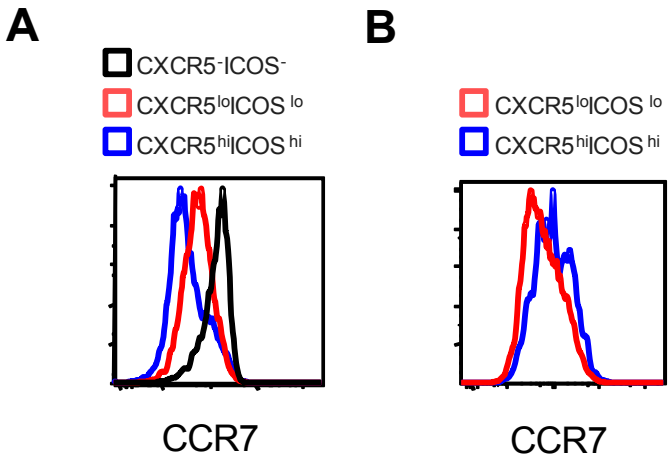


Figure 8: Differential expression of CCR7 and EBI2 by Tfh subsets (A) Expression of CCR7 protein by tonsillar Th subpopulations was analyzed by flow cytometry (B) Downregulation of CCR7 expression by $CXCR5^{lo}ICOS^{lo}$ cells after co-culture with naïve B cells.

EBI2, a Gαi-coupled orphan receptor, was recently identified a molecule in promoting B cell localization in the outer follicle (Gatto et al., 2009; Pereira et al., 2009). B cells require losing the expression of EBI2 for their entry into GCs. We found that $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells expressed very low levels of EBI2 when compared to $CXCR5^{lo}ICOS^{lo}$ $CD4^{+}$ T cells (Figure 8C). $CXCR5^{lo}ICOS^{lo}$ Th cells interacted with naïve

B cells rapidly lost the expression of EBI2 (Figure 8D). Thus, in concordance with B cells, the expression of EBI2 might control the location of Tfh cells.

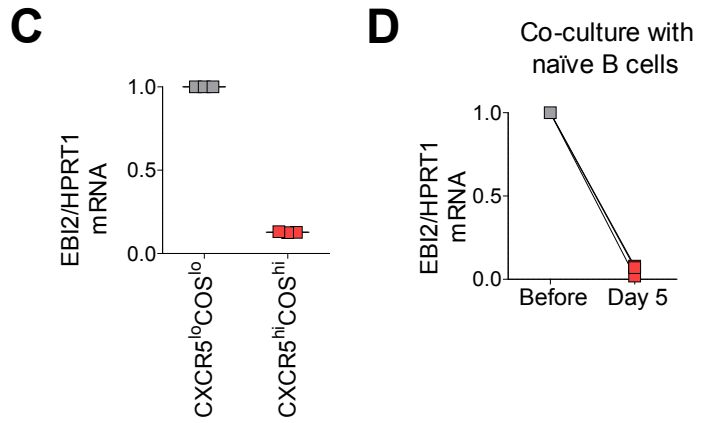


Figure 8. Differential expression of CCR7 and EBI2 by Tfh subsets (C) Expression of EBI2 mRNA by tonsillar Th subpopulations was analyzed by real-time RT-PCR. Normalized to the levels expressed by $CXCR5^{hi}ICOS^{hi}$ cells. Mean ± sem. N=3 (D) Downregulation of EBI2 expression by $CXCR5^{lo}ICOS^{lo}$ cells after co-culture with naïve B cells. The levels of EBI2 mRNA expression before and after co-culture with naïve B cells (5 d) were analyzed. A representative of two experiments.

*Tonsillar Th Populations Differentially Help B Cell Subsets*

We next assessed whether the four tonsillar Th populations were able to induce the three major tonsillar B cell subsets to produce Igs (naïve B ($IgD^{+}CD38^{-}CD19^{+}$) cells, memory B ($IgD^{-}CD38^{-}CD19^{+}$) cells, and GC-B ($IgD^{-}CD38^{+}CD19^{+}$) cells (Figure 9A). The tonsillar Th populations were carefully isolated by gating out cells expressing CD20, CD8, and CD56 during the sort. This strategy was particularly important for the isolation of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells as 3-5% of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells co-expressed CD20 (Figure 9B), likely representing conjugates of GC-Tfh and GC-B cells.

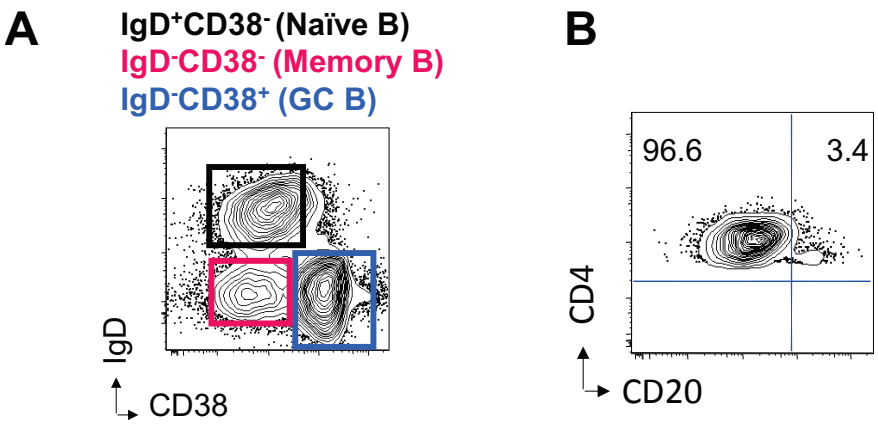


Figure 9: Isolation of tonsillar B cell subsets and CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells. (A) CD19$^{+}$ tonsillar B cells were stained with anti-CD20 FITC, anti-CD3 PE-Cy5, anti-IgD PE and anti-CD38 APC. Naïve, memory and GC-B cells were sorted as CD20$^{+}$CD3$^{-}$IgD$^{+}$CD38$^{-}$, CD20$^{+}$CD3$^{-}$IgD$^{-}$CD38$^{-}$, and CD20$^{+}$CD3$^{-}$IgD$^{-}$CD38$^{+}$ cells, respectively. (B) B cells were first removed with CD19 microbeads from tonsillar single cell suspension. The CD19-negative fraction was stained with anti-ICOS, anti-CD20, anti-CXCR5, anti-CD8, anti-CD56, and anti-CD4. Gated to CXCR5$^{hi}$ICOS$^{hi}$ CD4$^{+}$CD8$^{-}$CD56$^{-}$ T cells. The fraction of CD20$^{+}$CD4$^{+}$ cells likely represents the conjugates of GC-Tfh cells and GC-B cells.

The four tonsillar Th populations (2 x $10^4$ /well) were cultured with each autologous B cell subset (2 x $10^4$ /well) and secreted Igs were measured at day 8 (Figure 10A-C). To mimic the antigen-specific cognate interactions between B and T cells, staphylococcal enterotoxin B (SEB), a superantigen, was added to the co-cultures. Previous studies demonstrated that CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells efficiently help tonsillar B cells when compared to other tonsillar Th populations (Breitfeld et al., 2000; Rasheed et al., 2006). Consistently, CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells (blue symbols) efficiently promoted GC-B cells to produce Igs (Figure 10A. IgM 1.3 ± 0.4, IgG 1.0 ± 0.2, IgA 0.3 ± 0.1 μg/ml, Mean ± SEM n=11). Other tonsillar Th populations, CXCR5$^{-}$ ICOS$^{-}$ cells (gray symbols), CXCR5$^{lo}$ICOS$^{lo}$ cells (red symbols), and CXCR5$^{lo}$ICOS$^{hi}$ cells (green symbols) barely induced GC-B cells to produce Igs (Figure 10A).

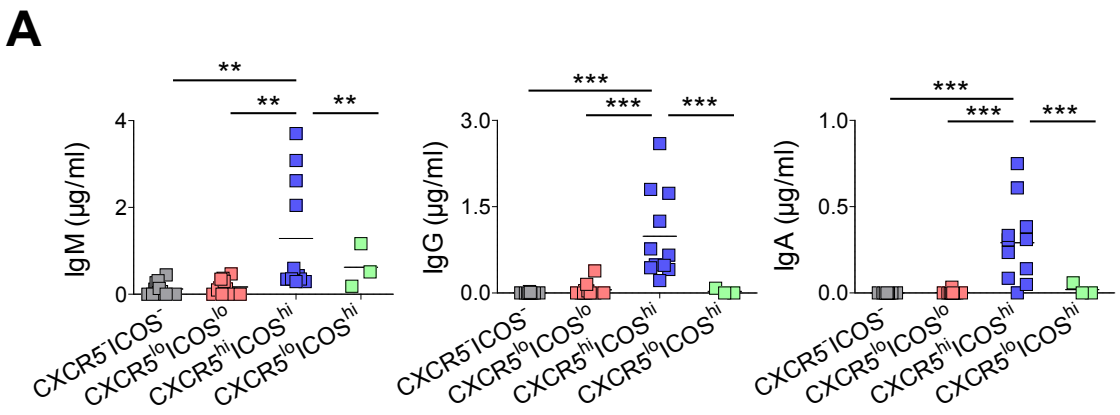


Figure 10: Tonsillar Th populations differentially help B cell subsets (A) GC-B cells, were co-cultured with each Th population in the presence of SEB for 8 d. One-way ANOVA Bonferonni's multiple comparison test. *** p<0.001, ** p<0.01, and * p<0.05.

Indeed, only CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells were able to maintain the survival of GC-B cells during the 8 d co-culture (Figure 10B), some of which expressed surface Igs (Figure 10B, right).

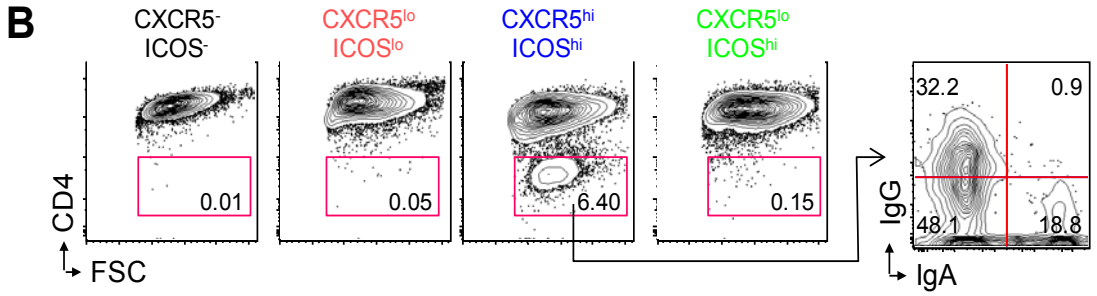


Figure 10. Tonsillar Th populations differentially help B cell subsets (B) Recovery of GC-B cells co-cultured with Th populations. GC-B cells cultured for 8 d with each Th population were stained with CD3 and CD4 mAbs. Surface IgG and IgA expression by B cells cultured with CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells is shown on the right panel.

Notably, whereas GC-Tfh cells are prone to apoptosis due to expression of FAS (Breitfeld et al., 2000; Rasheed et al., 2006), GC-B cells were able to maintain the survival of CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells and induce their proliferation (Figure 10C. 142

± 14 x $10^3$ cells/well, Mean ± SEM, n=5; 7.1 ± 0.7 fold-increase from the input). Thus, GC-Tfh cells and GC-B cells appear to reciprocally help each other.

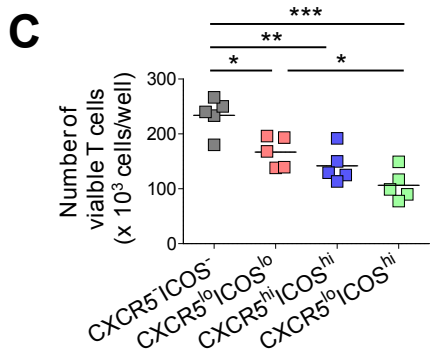


Figure 10. Tonsillar Th populations differentially help B cell subsets (C) Recovery of T cells co-cultured with GC-B cells. Absolute number of viable T cells per well (day 8). One-way ANOVA Bonferonni's multiple comparison tests.

In contrast, upon co-culture with naïve B cells, $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells were the most efficient at inducing naïve B cells to produce IgM (Figure 10D. 33.0 ± 4.9 μg/ml, Mean ± SEM n=4), as well as IgG and IgA (IgG 1.1 ± 0.2 μg/ml; IgA 0.8 ± 0.2 μg/ml).

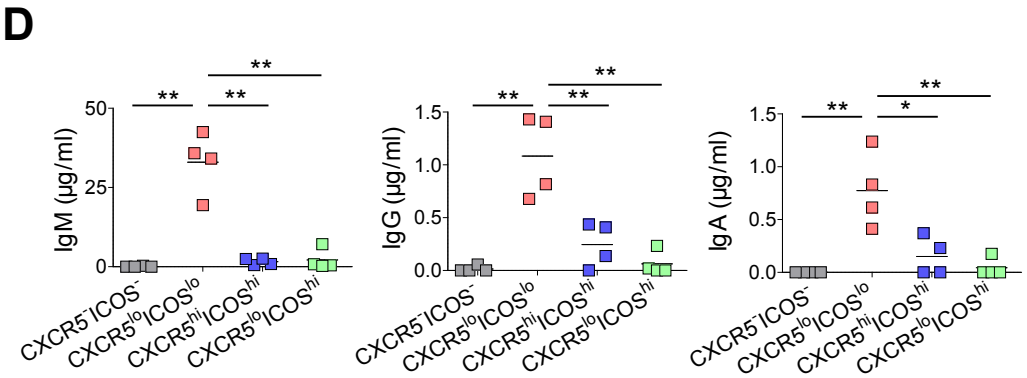


Figure 10. Tonsillar Th populations differentially help B cell subsets (D) Naïve B cells, were co-cultured with each Th population in the presence of SEB for 8 d. One-way ANOVA Bonferonni's multiple comparison test. *** $p<0.001$, ** $p<0.01$, and * $p<0.05$.

Consistently, CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells induced a robust proliferation of naïve B cells (Figure. 10E, F). Furthermore, CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells yielded more B cells expressing CD38, a marker of plasmablasts, than other tonsillar Th populations (Figure. 10G, F, right).

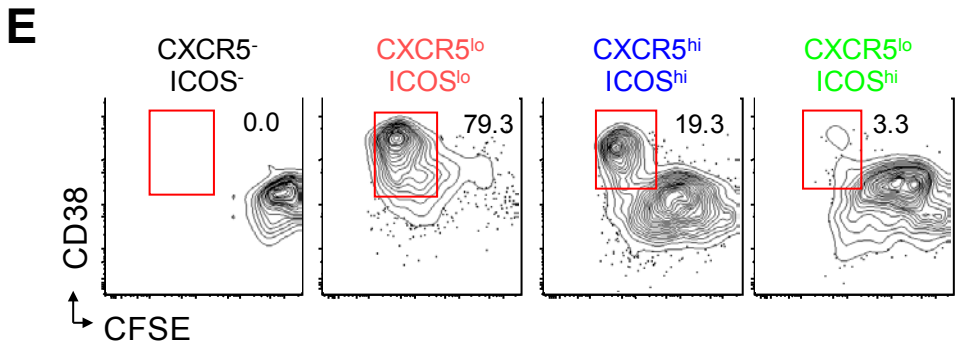


Figure 10. Tonsillar Th populations differentially help B cell subsets (E) Proliferation and CD38 expression of CFSE-labeled naïve B cells cultured for 8 d with each Th population. A representative from three experiments.

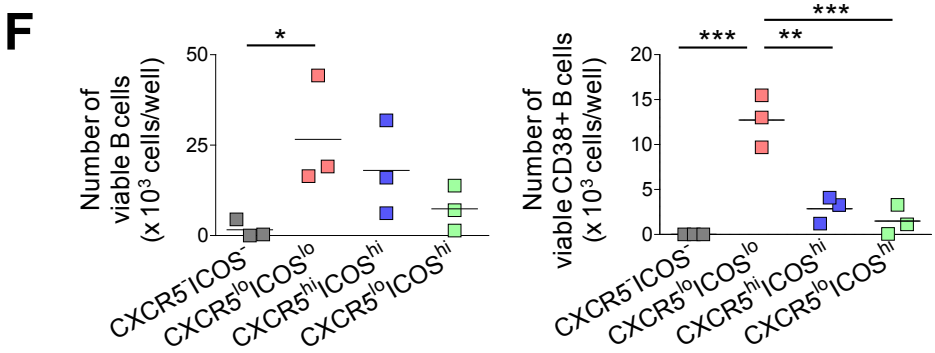


Figure 10. Tonsillar Th populations differentially help B cell subsets (F) Recovery of naïve B cells co-cultured with Th populations. Absolute number of viable B cells (left), and CD38$^{+}$ plasmablasts (right) per well (day 8). One-way ANOVA Bonferonni's multiple comparison test.

CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells were capable of inducing naïve B cells to produce Igs, but only at low amounts (Figure. 10D. IgM 1.6 ± 0.5, IgG 0.2 ± 0.1, IgA 0.1 ± 0.1 μg/ml, Mean ± SEM, n=4). This might partly be due to the poor survival of CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells, as naïve B cells did not support their survival and/or

proliferation in culture (Figure 10G. 29 ± 8 x $10^3$ cells/well, Mean ± SEM n=3; 1.5 ± 0.4 fold-increase from the input).

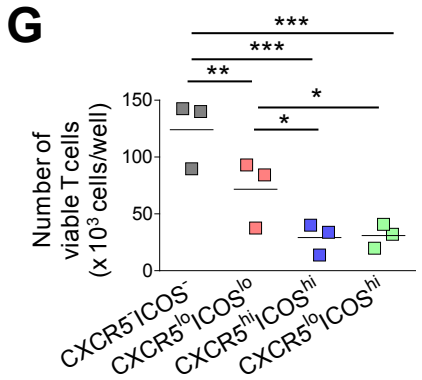


Figure 10. Tonsillar Th populations differentially help B cell subsets (G) Recovery of T cells co-cultured with naïve B cells. Absolute number of viable T cells per well (day 8). One-way ANOVA Bonferonni's multiple comparison tests.

$CXCR5^-ICOS^-$ cells and $CXCR5^{lo}ICOS^{hi}$ $CD4^+$ T cells were virtually unable to induce naïve B cells to produce Igs (Figure. 10D). Upon co-culture with memory B cells, both $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells and $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells were equally efficient at inducing their Ig production (Figure 10H). $CXCR5^-ICOS^-$ cells and $CXCR5^{lo}ICOS^{hi}$ $CD4^+$ T cells barely induced memory B cells to produce Igs. Thus, tonsillar Th populations differentially help B cell subsets. $CXCR5^-ICOS^-$ cells and $CXCR5^{lo}ICOS^{hi}$ $CD4^+$ T cells barely help any B cell subsets. Whereas $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells efficiently help GC-B cells to produce Igs, $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells were the most potent at inducing naïve B cells to differentiate into Ig-producing cells.

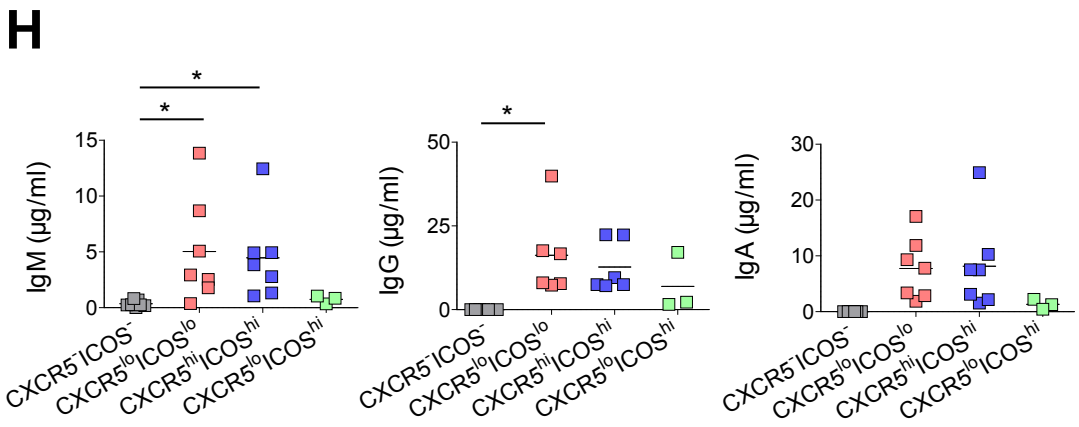


Figure 10. Tonsillar Th populations differentially help B cell subsets (H) Memory B cells, were co-cultured with each Th population in the presence of SEB for 8 d. One-way ANOVA Bonferonni's multiple comparison test. *** $p<0.001$, ** $p<0.01$, and * $p<0.05$.

*Tonsillar Th Populations Differentially Secrete Cytokines/Chemokines*

Tfh cells secrete IL-4, IL-10 and IL-21, each of which differentially regulates B cell proliferation, differentiation, and class-switching (Fazilleau et al., 2009; King et al., 2008). Thus we analyzed whether the cytokine production profiles of these tonsillar Th populations might explain their differential effects on B cell subsets. Upon co-culture with naïve B cells, $CXCR5^{lo}ICOS^{lo}$ $CD4^{+}$ T cells (Figure 11A, top, red symbols) secreted the largest amounts of IL-21 (3.3 ± 0.4 ng/ml, Mean ± SEM, n=6). This held true when tonsillar Th populations were cultured with GC-B cells (Figure 11B, top. 1.8 ± 0.4 ng/ml, n=5). $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells also secreted IL-21, but at substantially lower amounts (Figure 11A, B). Culture with naïve B cells: 0.5 ± 0.2 ng/ml, n=6; culture with GC-B cells: 0.4 ± 0.2 ng/ml). The largest amounts of IL-10 were also detected in the cultures of $CXCR5^{lo}ICOS^{lo}$ $CD4^{+}$ T cells with B cells (Figure 11A, B, middle. With naïve B cells: 1.1 ± 0.2 ng/ml, n=6; With GC-B cells: 1.4 ± 0.4 ng/ml, n=5). In contrast, consistent with previous studies (Chtanova et al., 2004; Rasheed et al., 2006),

$CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells produced the largest amounts of CXCL13, a chemokine secreted by Tfh cells, upon culture with B cells (Figure 11C. With naïve B cells: 2.7 ± 0.3 ng/ml, n=4; With GC-B cells: 2.5 ± 0.2 ng/ml, n=3). IL-4 was detected only at low concentrations (< 0.3 ng/ml) in any Th cultures with B cells (Figure 11A, B, bottom). Of note, $CXCR5^{lo}ICOS^{hi}$ $CD4^+$ T cells (green symbols) barely secreted IL-21 and IL-10 upon encounter with B cells (Figure 11A, B. With naïve B cells: 0.2 ± 0.0 ng/ml, and 0.2 ± 0.0 ng/ml, respectively. n=6; With GC-B cells: 0.4 ± 0.2 ng/ml, and 0.2 ± 0.0 ng/ml, respectively. n=5), but secreted the largest amounts of IL-17A (With naïve B cells: 4.1 ± 1.3 ng/ml; With GC-B cells: 4.3 ± 1.6 ng/ml). Thus, the four tonsillar Th populations display distinct cytokine/chemokine secretion profiles. Upon interaction with B cells, $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells secrete largest amounts of IL-10 and IL-21, whereas $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells secrete largest amounts of CXCL13. $CXCR5^{lo}ICOS^{hi}$ $CD4^+$ T cells secrete the largest amounts of IL-17A.

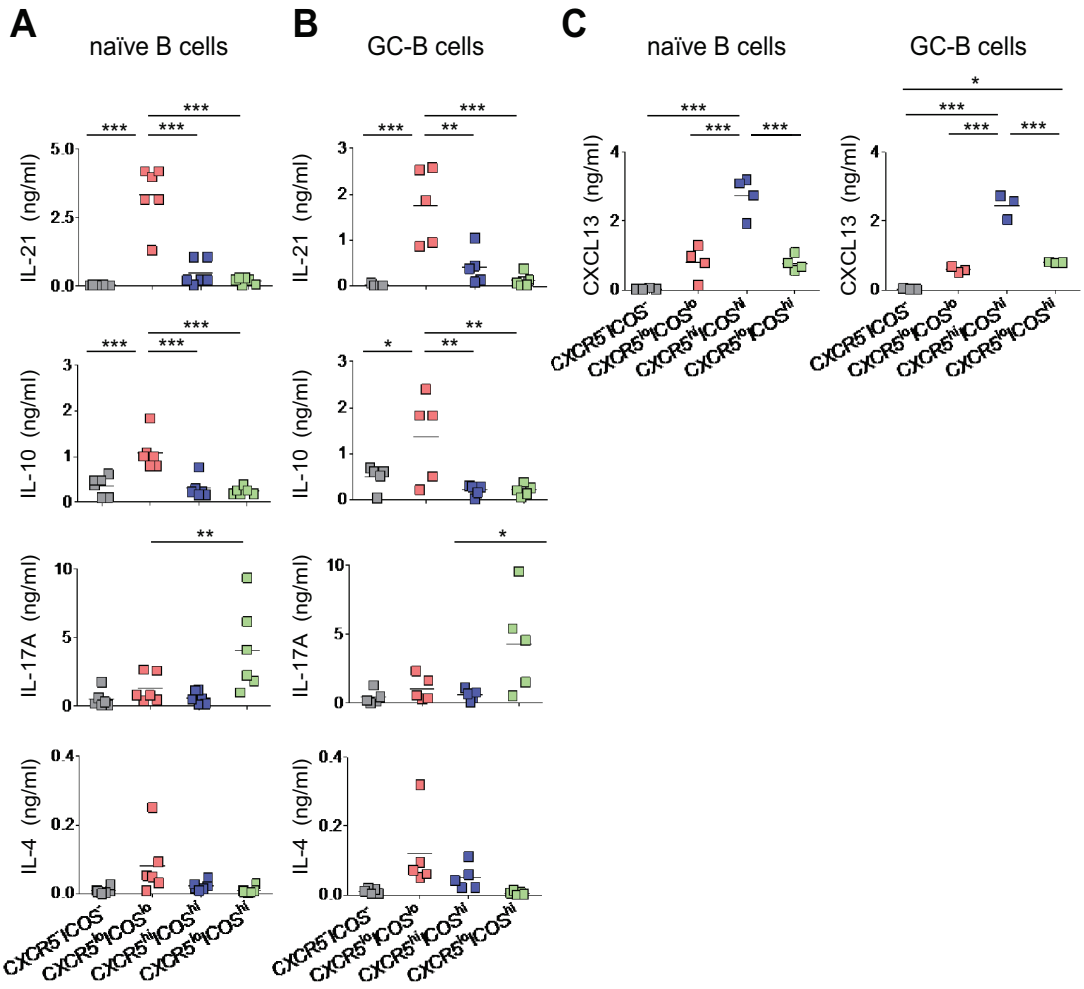


Figure 11: Cytokine secretion profiles of tonsillar Th populations (A-B) Cytokine secretion upon interaction with B cell subsets. The four Th populations were cultured with either naïve B cells (A, n=6) or GC-B cells (B, n=5), and the secretion of IL-10, IL-21, IL-17, and IL-4 was analyzed at day 2. One-way ANOVA test. *** $p<0.001$, ** $p<0.01$, and * $p<0.05$. (C) CXCL13 secretion upon interaction with naïve B cells (left, n=4) or GC-B cells (right, n=3). One-way ANOVA test.

### *Factors Involved in the Help of Naïve B cells by CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T Cells*

We next analyzed the molecules that were involved in the help of B cell subsets by tonsillar Th populations (Figures 12A-D). First, to test the role of IL-21 secreted by CD4$^{+}$ T cells, a neutralizing IL-21R-Fc chimeric protein was added to the T and B cell co-cultures. This resulted in the complete inhibition of Ig production in all the cultures of tonsillar Th populations and B cell subsets, including those of CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T

cells and naïve B cells; and $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells and GC-B cells (Figure 12A). Second, the role of ICOS was tested by blocking the ICOS/ICOS-ligand (ICOS-L) interaction with an ICOS-blocking mAb. Inhibition of the ICOS/ICOS-L interaction completely abrogated Ig production by both naïve B cells and GC-B cells (Figure 12B) as well as cytokine secretion by $CD4^+$ T cells (Figure 12E). Such complete blocking was further confirmed in multiple experiments using different anti-ICOS clones or with anti-ICOS-L (data not shown). Third, blocking IL-10 by using a combination of blocking mAbs (i.e., anti-IL-10 and anti-IL-10 receptor) resulted in a partial inhibition of Ig production in the cultures of $CXCR5^{lo}ICOS^{lo}CD4^+$ T cells with naïve B cells and $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells with GC-B cells (Figure 12C). Lastly, blocking CD40L, a fundamental molecule for B cell help, also resulted in a partial inhibition of Ig production (Figure 12D). At variance with blocking the ICOS/ICOS-L interaction, blocking the CD40/CD40L interaction did not inhibit the cytokine secretion from $CD4^+$ T cells (Figure 12E). Thus, both $CXCR5^{lo}ICOS^{lo}$ $CD4^+$ T cells and $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells help B cell subsets in a fashion dependent on IL-21, ICOS, IL-10, and CD40L. Whereas the ICOS/ICOS-L interaction was essential for T cell activation, the CD40/CD40L interaction was important for B cell differentiation.

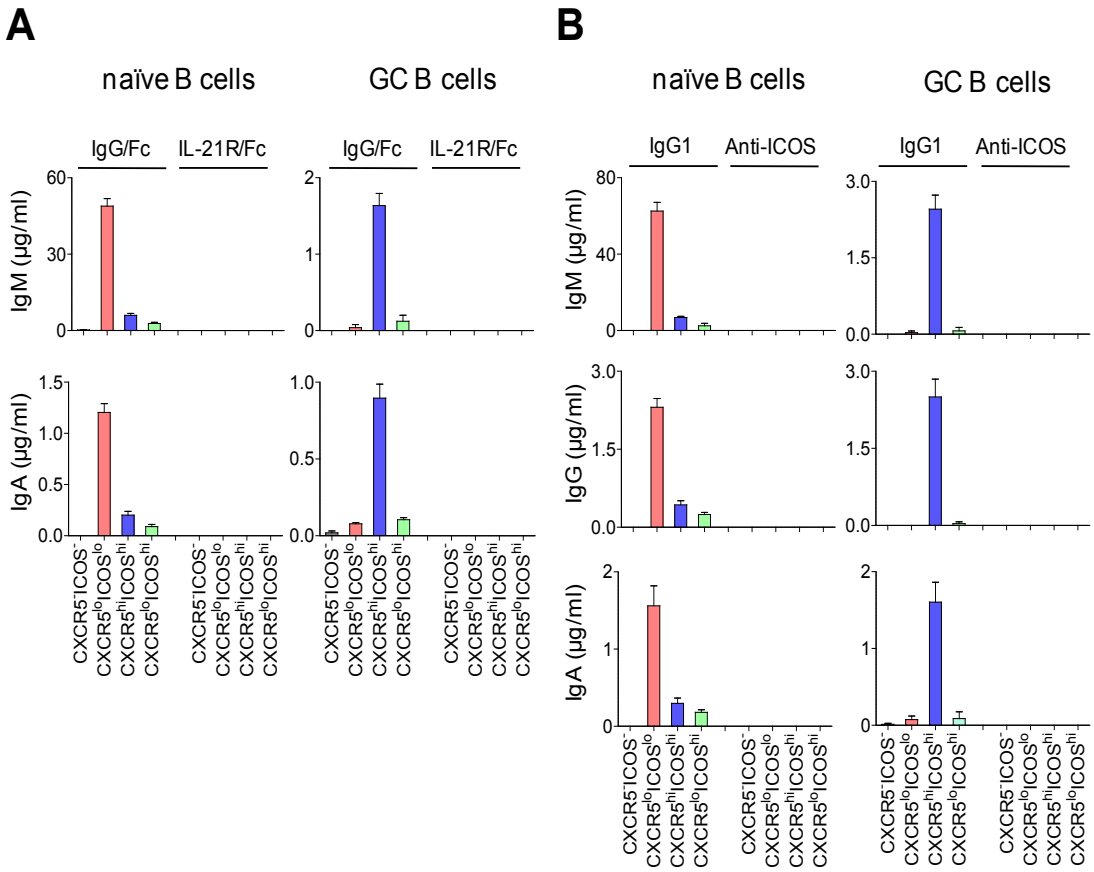


Figure 12: Molecules associated with helper activity of tonsillar Th populations (A) IL-21R/Fc chimera protein was added to the cultures of Th population and the indicated B cell subsets (B) ICOS mAb was added to the cultures of Th population and the indicated B cells.

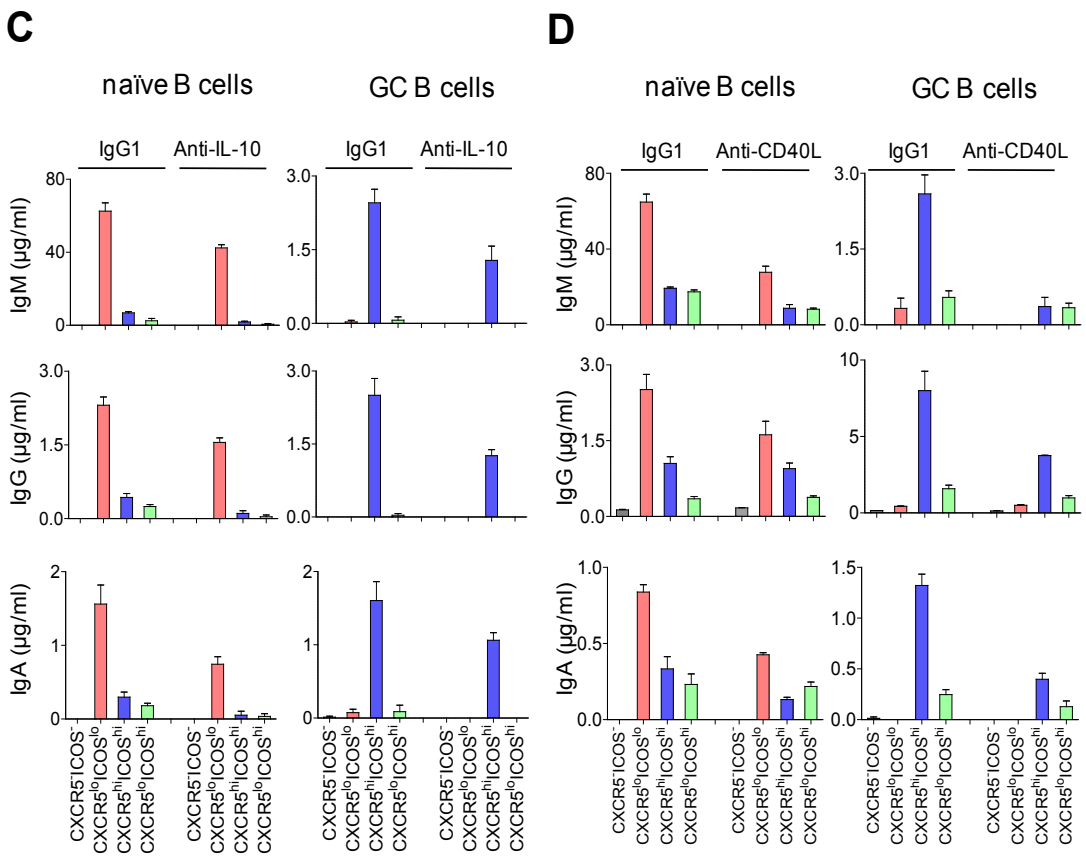


Figure 12. Molecules associated with helper activity of tonsillar Th populations (C) IL-10 and IL-10R mAbs were added to the cultures of Th population and the indicated B cells. Ig concentrations at day 8. A representative from four experiments. (D) CD40L mAb was added to the cultures of Th population and the indicated B cells. Ig concentrations at day 8. A representative from three experiments.

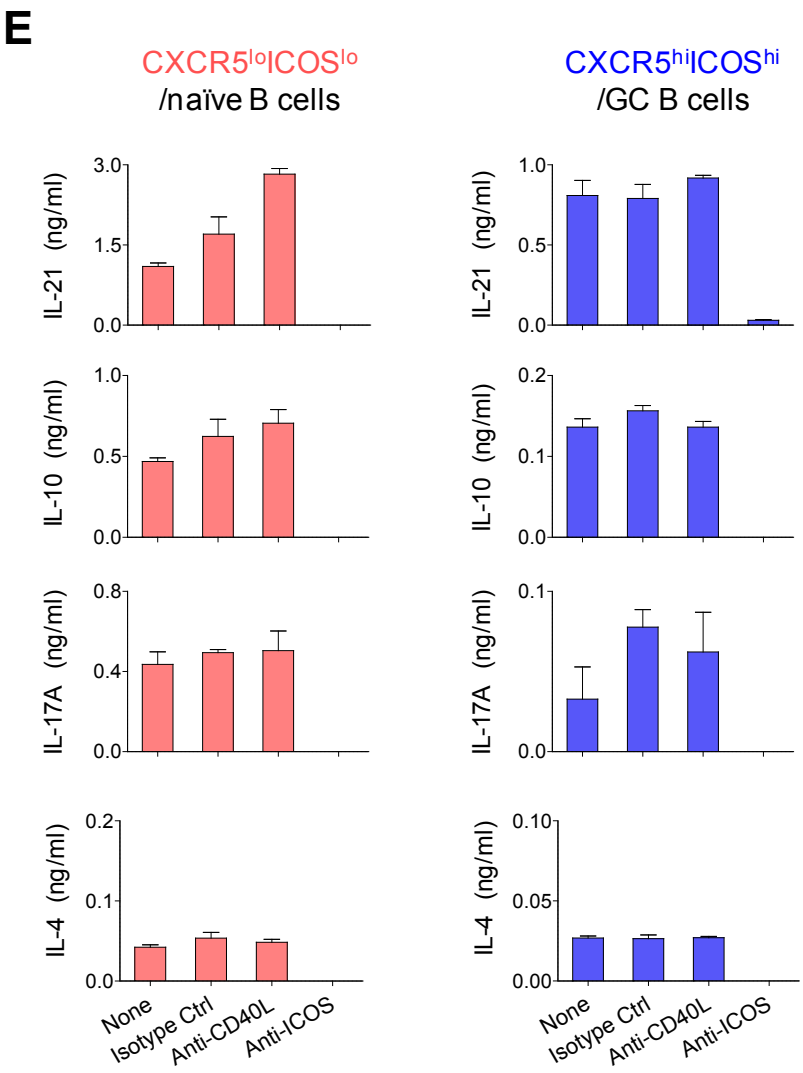


Figure 12. Molecules associated with helper activity of tonsillar Th populations (E) Cytokine secretion. CD40L mAb, ICOS mAb, or an isotype control was added to the cultures of Th population and the indicated B cells. Cytokine concentrations at day 2. A representative from three experiments.

### *CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T Cells Induce Apoptosis of GC-B Cells Via FAS/FAS-L Interaction*

We were intrigued by the ability of CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells to help both naïve and memory B cells, whereas being unable to help GC-B cells. Given that the recovery of viable T cells was similar between CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells and CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells cultured with GC-B cells (Figure 10C), the lack of GC-B cell help by CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells was unlikely due to total consumption of

nutrients in cultures. Furthermore, titrating down the number of CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells did not result in better GC-B cell help (Figure 13A). Considering that co-cultures of GC-B cells and CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells resulted in few remaining viable B cells (Figure 10B), we hypothesized that these T cells might induce the apoptosis of GC-B cells, which express high amounts of FAS (Martinez-Valdez et al., 1996; Smith et al., 1995; Watanabe et al., 1995) (Figure 13B).

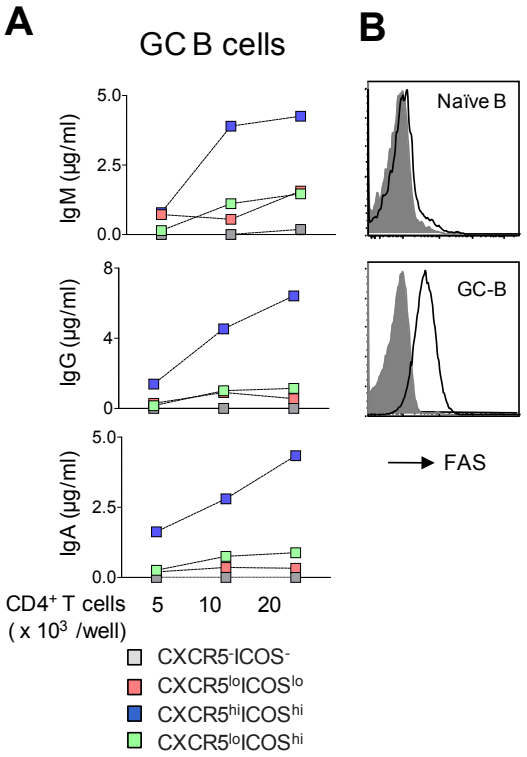


Figure 13: CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells induce apoptosis of GC-B cells via the FAS/FAS-L interaction (A) GC-B cells (20 x 10$^{3}$ cells/well) were cultured with graded numbers of Th populations as indicated (for 8 days), and produced Igs were analyzed (B) Expression of surface FAS by GC-B cells.

Indeed, soluble FAS-L was detected in co-cultures of GC-B cells with CXCR5$^{-}$ ICOS$^{-}$, CXCR5$^{lo}$ICOS$^{lo}$, and CXCR5$^{lo}$ICOS$^{hi}$ CD4$^{+}$ T cells, but not with CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells (Figure 13C). Consistently, *FASLG* transcript (encoding FAS-L) was

expressed by the former three tonsillar Th populations but very little by CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells (Figure 13D).

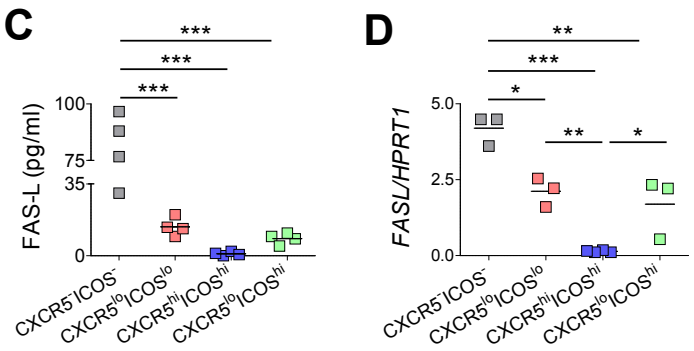


Figure 13. CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells induce apoptosis of GC-B cells via the FAS/FAS-L interaction (C) Soluble FAS-L production by Th populations co-cultured with GC-B cells. FAS-L concentrations at day 2. N=4 (D) FASLG mRNA expression by tonsillar Th populations analyzed by real-time RT-PCR. Expression of FASLG transcript was normalized to that of HPRT1 transcript. N=3.

Upon blocking the FAS/FAS-L interaction with a neutralizing anti-FAS antibody, CXCR5$^{lo}$ICOS$^{lo}$ and CXCR5$^{lo}$ICOS$^{hi}$ CD4$^{+}$ T cells were able to maintain the survival of GC-B cells (data not shown) and induced them to produce Igs (Figure 13E). Thus, FAS-L is expressed by CXCR5$^{-}$ICOS$^{-}$, CXCR5$^{lo}$ICOS$^{lo}$ and CXCR5$^{lo}$ICOS$^{hi}$ CD4$^{+}$ T cells, but not by CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells, and induces the apoptosis of FAS-expressing GC-B cells.

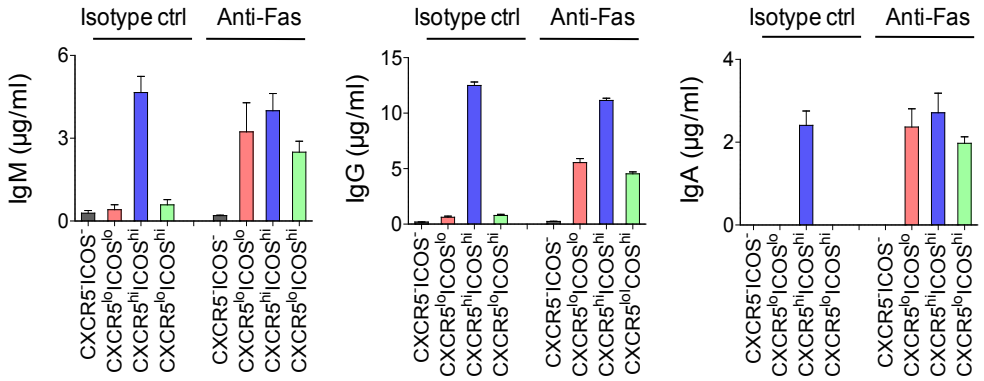


Figure 13. CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells induce apoptosis of GC-B cells via the FAS/FAS-L interaction (E) Ig secretion by GC-B cells co-cultured with tonsillar Th populations in the presence of FAS mAb. Ig concentrations were measured at day 8. A representative from four experiments

*CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T Cells Express Large Amounts of BCL6 Transcript*

Recent studies in mice indicate that development of Tfh cells is reciprocally regulated by the two transcription repressors, Bcl-6 and Blimp-1 (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009). To determine the extent of Tfh lineage commitment, the expression of Bcl-6 and Blimp-1 (encoded by *BCL6* and *PRDM1* genes, respectively) in tonsillar Th populations was analyzed by real-time RT-PCR. As shown in Figure 14A, *BCL6* transcript was abundantly expressed by CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells when compared to CXCR5$^{-}$ICOS$^{-}$ naïve CD4$^{+}$ T cells, and the expression was equivalent to that of CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells. The expression of *PRDM1* transcript was equally low between CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells and CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells. In contrast, CXCR5$^{lo}$ICOS$^{hi}$ expressed very high amounts of *PRDM1* transcript, but little *BCL6* transcript.

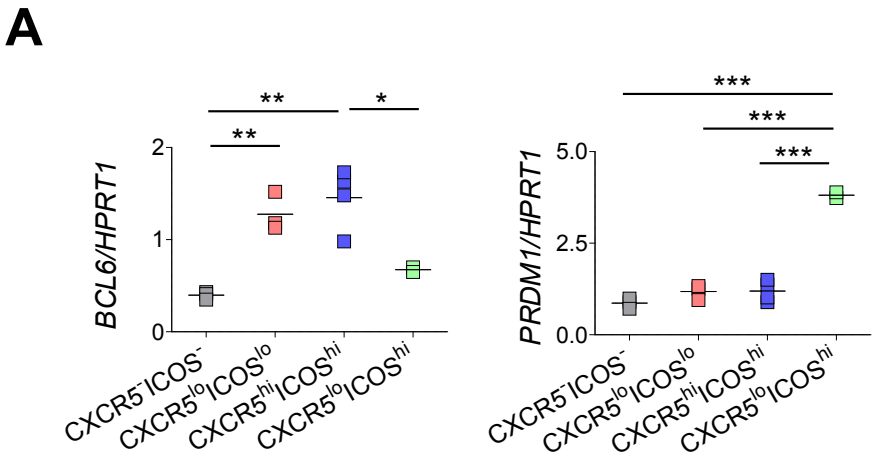


Figure 14: CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells express large amounts of *BCL6* transcript (A) Expression of *BCL6* and *PRDM1* transcripts by tonsillar Th populations was analyzed by real-time RT-PCR. Expression of each transcript was normalized to that of HPRT1 transcript. N=4.

As Bcl-6 expression is controlled by many post-transcriptional regulatory mechanisms (Crotty et al., 2010), the expression of Bcl-6 and Blimp-1 proteins was analyzed by Western blot. As shown in Figure 14B, CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells expressed more abundant Bcl-6 than CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells. Consistent results were obtained by flow cytometry (Figure 14C). Blimp-1 was not detected in any tonsillar Th populations (Figure 14B).

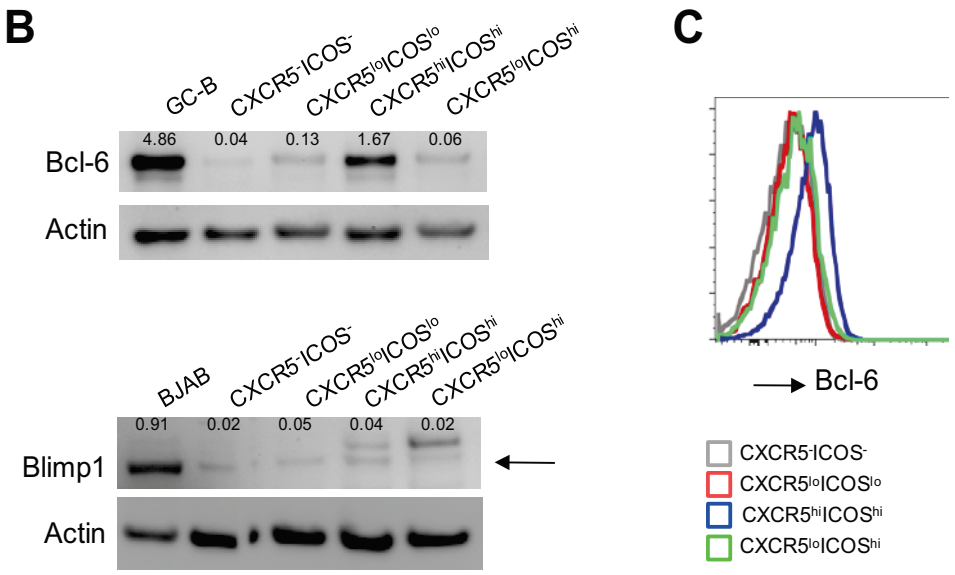


Figure 14. CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells express large amounts of *BCL6* (B) Expression of Bcl-6 and Blimp-1 proteins in tonsillar Th populations. Sorted Th populations were lysed, and equal amounts of protein were loaded per well to analyze the expression of Bcl-6 and Blimp-1 by Western blotting. For the positive controls, GC-B cell lysate was used for Bcl-6 detection and BJAB (a B cell line) cell lysate was used for Blimp-1 detection. Expected Blimp-1 band is indicated with an arrow. A representative of four experiments. (C) Analysis of the expression of Bcl6 expression in tonsillar CD4$^{+}$ Th population by flow cytometry (a representative of four experiments).

The difference in the expression of Bcl-6 between CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells and CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells might reflect the difference in the maturation stages. Indeed, whereas the expression of ICOS and PD-1 was low when isolated (Figure 6), CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells robustly upregulated the expression of ICOS and PD-1 upon interaction with naïve B cells at even higher densities than CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells (Figure 14D). Thus, analysis of *BCL6* and *PRDM1* transcript expression shows that CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells are committed to the Tfh lineage, but they express less Bcl-6 protein than CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells.

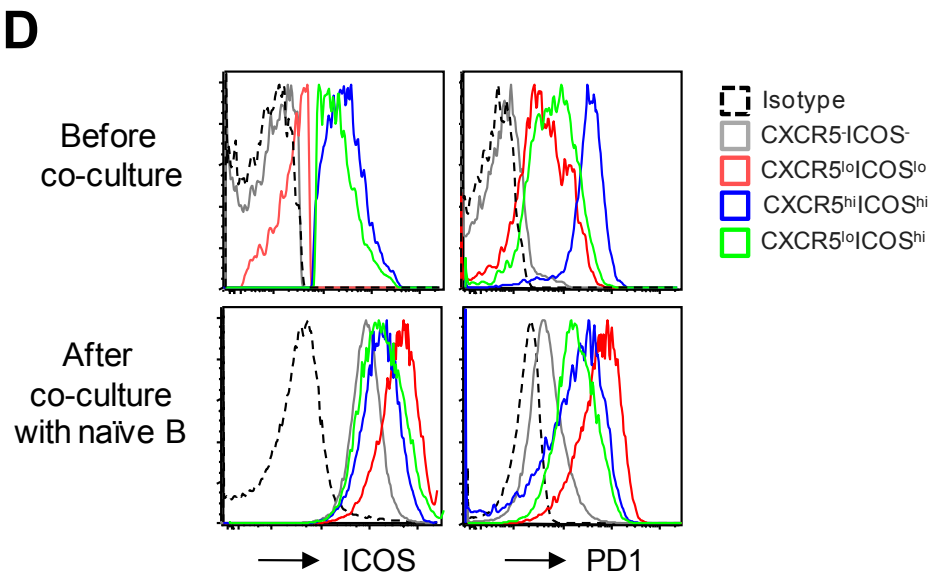


Figure 14. CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells express large amounts of *BCL6* (D) Upregulation of ICOS and PD-1. The four Th populations were cultured with naïve B cells, and the expression of ICOS and PD-1 on T cells was analyzed at day 5. A representative from two experiments.

*The Frequency of CXCR5$^{lo}$ICOS$^{lo}$ Tfh Cells is Different in Adult Tonsils*

We analyzed whether CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells were also present in adult tonsils and human spleen. Whereas pediatric tonsil samples contained significantly more CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells than CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells (Figure 6B), the frequency of CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells was slightly higher than that of CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells in adult tonsil samples (Figure 15A, B). CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells 32 ± 5%; CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells 22 ± 3%). In spleen, the majority of CD4$^{+}$ T cells were CXCR5$^{-}$ICOS$^{-}$CD127$^{+}$ CD4$^{+}$ T cells, and CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells were barely found. The separation between CXCR5$^{-}$ICOS$^{-}$ and CXCR5$^{lo}$ICOS$^{lo}$ Tfh cell populations was not clear, and CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells, if there were any, expressed only low levels of CXCR5 (Figure 15A). Thus, the frequency of CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells is different among human secondary lymphoid organs.

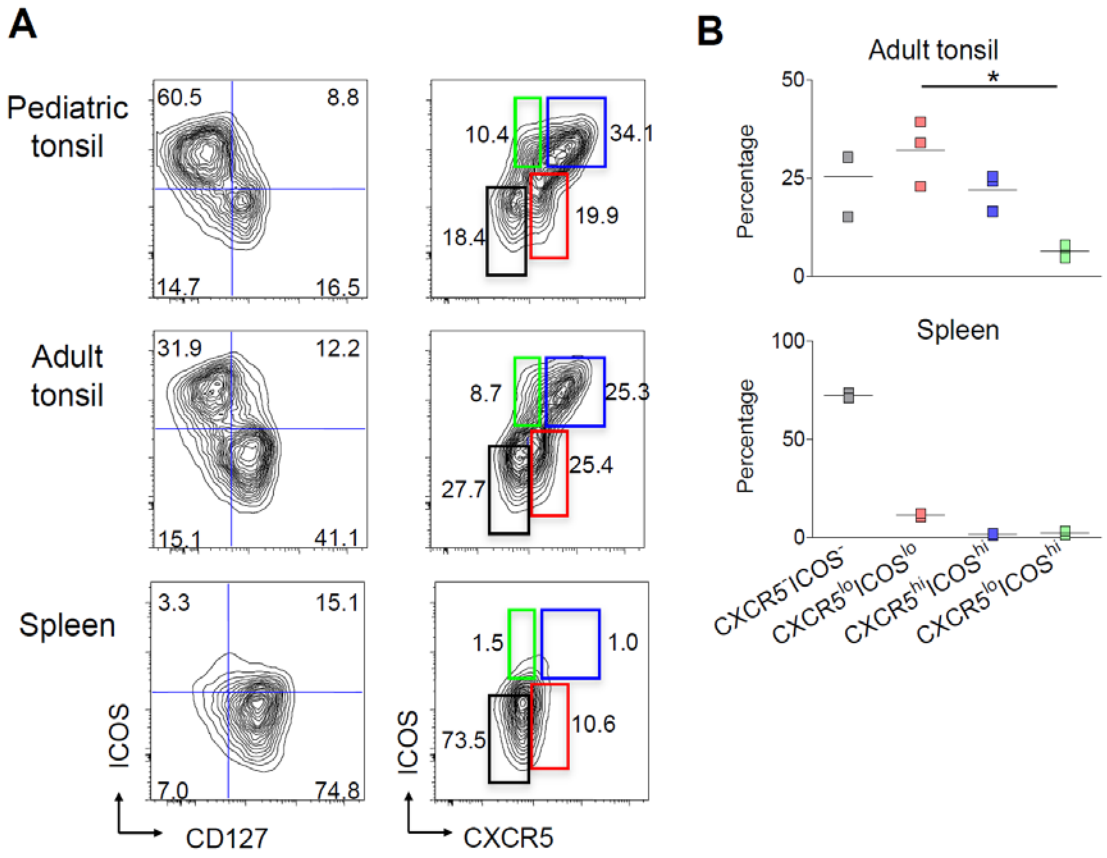


Figure 15: CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells in adult tonsils and human spleen samples. The four Th populations in pediatric tonsil samples, adult tonsil samples, and human spleen samples. The same number of T-enriched cells (after removing B cells with magnetic beads) was stained and analyzed in parallel in identical conditions. The four Th populations were determined based on the analysis of pediatric tonsil samples, and the same gates were applied for the adult and spleen samples. (A) A representative of at least two experiments gated to CD3$^{+}$CD4$^{+}$ T cells. (B) The frequency of each Th population in adult tonsils (n = 3) and spleen (n = 2). One-way ANOVA Bonferonni multiple comparisons test for adult tonsil samples. *P < 0.05.

## *Discussion*

Tfh cells have been established as a novel effector CD4$^{+}$ T cell subset specialized for B cell help. As the nomenclature indicates, Tfh cells are currently defined as CD4$^{+}$ T cells localized within B cell follicles. First, we demonstrate that Tfh cells are also found at extrafollicular sites in human tonsils, and can be identified as CXCR5$^{lo}$ICOS$^{lo}$IL-7R$^{+}$CD45RO$^{+}$ cells. The abundance of *BCL6* and *PRDM1* transcripts in CXCR5$^{lo}$ICOS$^{lo}$ CD4$^{+}$ T cells were similar to that in CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells, indicating that

$CXCR5^{lo}ICOS^{lo}$ $CD4^{+}$ T cells are committed to the Tfh lineage. Our study also demonstrates that tonsillar ICOS-expressing Th cells are not necessarily committed to the Tfh pathway. $CXCR5^{lo}ICOS^{hi}$ Th cells virtually lacked the capacity to help B cell responses. This subpopulation expressed high levels of *PRDM1*, but lacked the expression of *BCL6*, indicating that their developmental commitment to conventional Th cells, but not Tfh lineage. The two tonsillar Th subsets expressing abundant *BCL6* transcript, $CXCR5^{lo}ICOS^{lo}$ Tfh cells and $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells were found to differentially help B cells. Consistent with previous studies that show efficient help of B cells by GC-Tfh cells (Bryant et al., 2007; Kim et al., 2005; Rasheed et al., 2006), $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells were efficient at helping GC-B cells. Reciprocally, GC-B cells were able to maintain the survival of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells, as the recovery of viable $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells was significantly better when cultured with GC-B cells than with naïve B cells In contrast, $CXCR5^{lo}ICOS^{lo}$ Tfh cells were far more efficient than GC-Tfh cells at inducing naïve B cells to proliferate and differentiate into Ig-producing cells. The difference between the two Th populations in the ability to help naïve B cells was, at least partly, explained by the differences in the secretion of cytokines, in particular IL-21. Whereas less IL-21 secretion by GC-Tfh cells might be due to their poor survival upon co-culture with naïve B cells (Figure 10G), our study shows that the two Th populations display intrinsically distinct cytokine production properties. First, as reported previously (Chtanova et al., 2004; Kim et al., 2004; Rasheed et al., 2006), GC-Tfh cells secreted substantially higher levels of CXCL13 than $CXCR5^{lo}ICOS^{lo}$ Tfh cells upon interaction with B cells, showing that GC-Tfh cells maintain the capacity to secrete soluble factors. This was further supported by the

equivalent IL-4 secretion between GC-Tfh cells and CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells upon CD3/CD28 stimulation (Figure 16). Second, upon stimulation with CD3/CD28 stimulation, CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells secreted larger amounts of IL-10 and IL-21 than GC-Tfh cells (Figure 16).

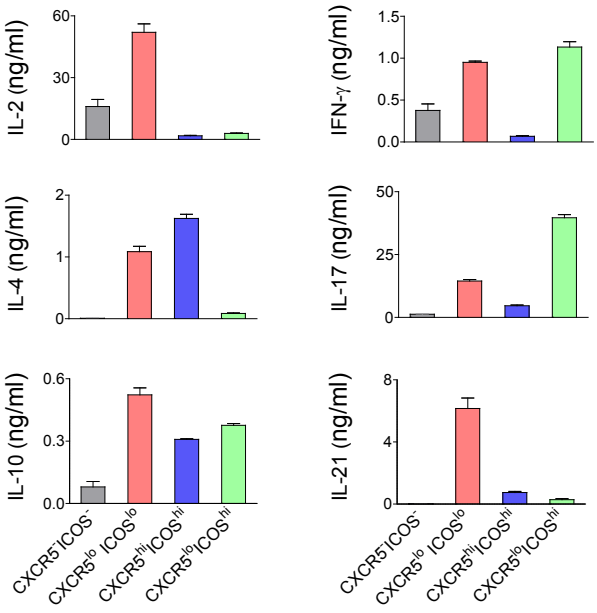


Figure 16: Cytokine secretion of CD3/CD28 stimulated tonsillar CD4$^+$ T cells. Tonsillar Th subpopulations (5 x 10$^4$ cells/well in triplicates) were stimulated with CD3/CD28 mAbs for 48 h to measure cytokine secretion. A representative of two experiments.

This was unexpected, as many reports demonstrate that GC-Tfh cells are the CD4$^+$ T cell subset expressing the highest levels of IL-21. We hypothesized that the discrepancy between previous studies and our study might be explained by the difference in the kinetics of IL-21 expression after activation in the two tonsillar CD4$^+$ T cell subsets. Thus, we analyzed *IL21* transcript expression in CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells and GC-Tfh cells before and after CD3/CD28 stimulation. Consistent with previous studies, isolated GC-Tfh cells expressed higher levels of *IL21* transcript than CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells (Figure 17). However upon stimulation, GC-Tfh cells rapidly lost IL21 expression

within 24 h, whereas CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells robustly upregulated IL21 expression (Figure 17). Rapid loss of IL21 expression by GC-Tfh cells was not due to cell death, as recovery of viable cells was comparable between GC-Tfh cells and CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells (data not shown). Furthermore, GC-Tfh cells, which also expressed largest amounts of *CXCL13* transcript when isolated, maintained CXCL13 expression for at least 2 days after CD3/CD28 stimulation (Figure 17).

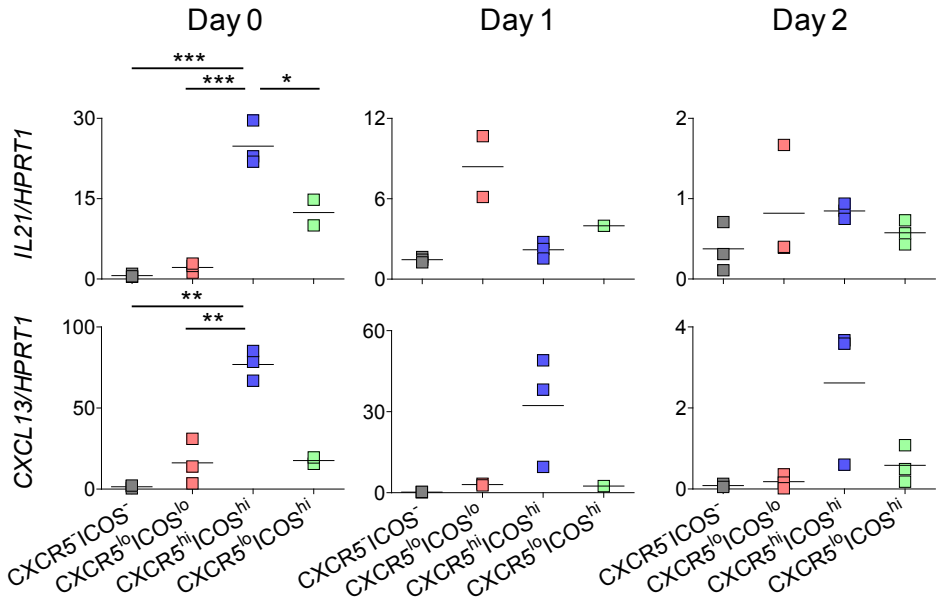


Figure 17: Kinetics of IL21 and CXCL13 expression. The four Th populations were analyzed for the expression of IL21 and CXCL13 by real-time RT-PCR before and after stimulation with CD3/CD28 mAb-coated beads (n = 2-3). One-way ANOVA Bonferonni multiple comparison test for n = 3 and paired t test for n = 2.

Thus, our study shows that CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells secrete largest amounts of IL-10 and IL-21 among tonsillar CD4$^{+}$ T cell populations, whereas CXCR5$^{hi}$ICOS$^{hi}$ GC-Tfh cells secrete largest amounts of CXCL13. Similar to GC-Tfh cells (Bryant et al., 2007), the help provided by CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells for naïve B cells was totally dependent on IL-21, ICOS, IL-10, and CD40L. CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells were also efficient at inducing memory B cells to produce Igs. CXCR5$^{lo}$ICOS$^{lo}$ Tfh cells were

exclusively localized outside GCs, and thus they might represent extrafollicular helper cells engaged in inducing differentiation of naïve and memory B cells into extrafollicular plasma cells. Consistently, recent studies using lupus-prone mice demonstrated the presence of Bcl-6-expressing extrafollicular helper cells (Odegard et al., 2008; Poholek et al., 2010). We cannot exclude, however, the possibility that $CXCR5^{lo}ICOS^{lo}$ Tfh cells represent precursors of GC-Tfh cells. Whereas the transcript was expressed at similar amounts, $CXCR5^{lo}ICOS^{lo}$ Tfh cells expressed lower amounts of Bcl-6 protein than $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells. This might reflect the fact that $CXCR5^{lo}ICOS^{lo}$ Tfh cells are in a transition stage of differentiating into mature GC-Tfh cells. Robust upregulation of ICOS and PD-1 by $CXCR5^{lo}ICOS^{lo}$ Tfh cells upon interaction with naïve B cells further supports this hypothesis. Alternatively, $CXCR5^{lo}ICOS^{lo}$ Tfh cells might constitute both extrafollicular helper cells and GC-Tfh precursors, and modify their balance according the microenvironment of secondary lymphoid organs. In contrast to the similar expression of *BCL6* and *PRDM1* transcripts, EBI2 and CCR7 were expressed at higher levels by $CXCR5^{lo}ICOS^{lo}$ Tfh cells than $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells. Although the mechanism whereby EBI2-expressing cells remain outside of GCs remains to be established, the differential expression of EBI2 appears to explain the differences in the localization of $CXCR5^{lo}ICOS^{lo}$ Tfh cells and $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells. Notably, $CXCR5^{lo}ICOS^{lo}$ Tfh cells were unable to help GC-B cells due to the induction of apoptosis of GC-B cells through the interaction of FAS/FAS-L. This might represent a fail-safe system to avoid the involvement of $CXCR5^{lo}ICOS^{lo}$ Tfh cells that erroneously entered into the follicles in the selection of GC-B cells undergoing somatic hypermutation. Alternatively, $CXCR5^{lo}ICOS^{lo}$ Tfh cells that have recently migrated into

GCs might be actively involved in the selection process of B cells in GCs. A mouse study indicated that FAS expression by GC-B cells is essential for the selection of high-affinity B cells in GCs (Hao et al., 2008). Given the minimal expression of FAS-L by GC-Tfh cells, it is possible that while recently GC-recruited $CXCR5^{lo}ICOS^{lo}$ Tfh cells are involved in the negative selection in GCs to eliminate B cells with suboptimal affinity or autoreactivity. Our study indicates that the differentiation of human Tfh cells occurs at outside of B cell follicles. This suggests that, similar to other Th subsets, the commitment of Th differentiation program to the Tfh pathway is initiated when naïve $CD4^+$ T cells interact with antigen-presenting dendritic cells. This observation supports our previous proposal that particular type of DC subset is specialized for the development of Tfh cells (Klechevsky et al., 2008). In humans, IL-12 secreted by DCs appears to be the major cytokine in the development of IL-21-producing Tfh-like cells (Schmitt et al., 2009). Furthermore, IL-12 was shown to induce Bcl-6 expression in human $CD4^+$ T cells (Lund et al., 2005; Ma et al., 2009). Therefore, IL-12-producing DC subsets, such as $CD14^+$ dermal DCs, likely represent the major human DC subset that promotes the development of precursors of Tfh cells. Taken together, we have identified two distinct Tfh-committed cells in human tonsils differentially help B cell subsets. While, $CXCR5^{lo}ICOS^{lo}$ Tfh cells localize exclusively outside GCs and induce naïve and memory B cells to become antibody-secreting cells. $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells are specialized to help B cells in GCs. Whereas $CXCR5^{lo}ICOS^{lo}$ Tfh cells produce higher levels of IL-21 upon interaction with B cells, GC-Tfh cells produce higher levels of CXCL13. $CXCR5^{lo}ICOS^{lo}$ Tfh cells and GC-Tfh cells express equivalent amounts of *BCL6* and *PRDM1* transcripts.

CHAPTER FOUR

# Results: Induction of ICOS$^+$CXCR3$^+$ Tfh Cells Correlates With Antibody Responses in Influenza Vaccination

## *Introduction and Rational*

While Tfh cells localize in secondary lymphoid organs, obtaining lymph node samples from patients is challenging. There is a strong need to establish surrogate strategies to assess the quality of Tfh responses in humans. We have previously demonstrated that human blood Tfh cells potently induce naïve B cells differentiation, immunoglobulin (Ig) secretion, and isotype switching and based on the expression of two chemokine receptor CXCR3 and CCR6, are composed of subsets including Tfh1 (CXCR3$^+$CCR6$^-$), Tfh2 (CXCR3$^-$CCR6$^-$), and Tfh17 (CXCR3$^-$CCR6$^+$) cells (Morita el al., 2011) (Figure 18).

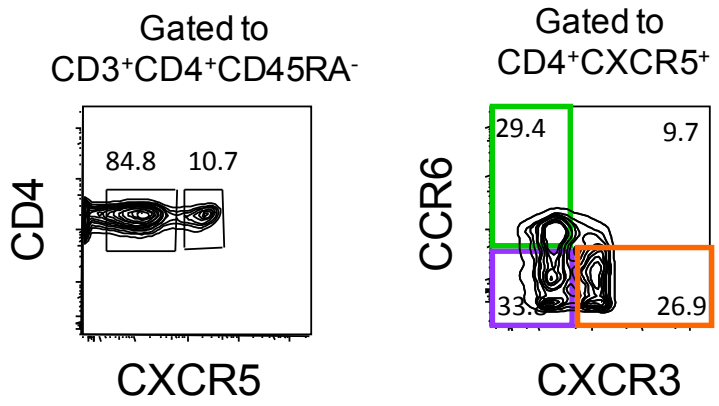


Figure 18: Human blood Tfh cells are composed of Tfh1, Tfh2, and Tfh17 cells, from Morita el al., 2011 CXCR3 and CCR6 expression on blood CXCR5$^+$CD4$^+$ T cell population. Gated to CD3$^+$CD4$^+$CD45RA$^-$ cells.

While, Tfh2 and Tfh17 cells efficiently induced naïve B cells to produce immunoglobulins via interleukin-21 (IL-21). Tfh1 cells were found to lack the capacity to secrete IL-21 and the B cell help (Morita et al., 2011)(Figure 19).

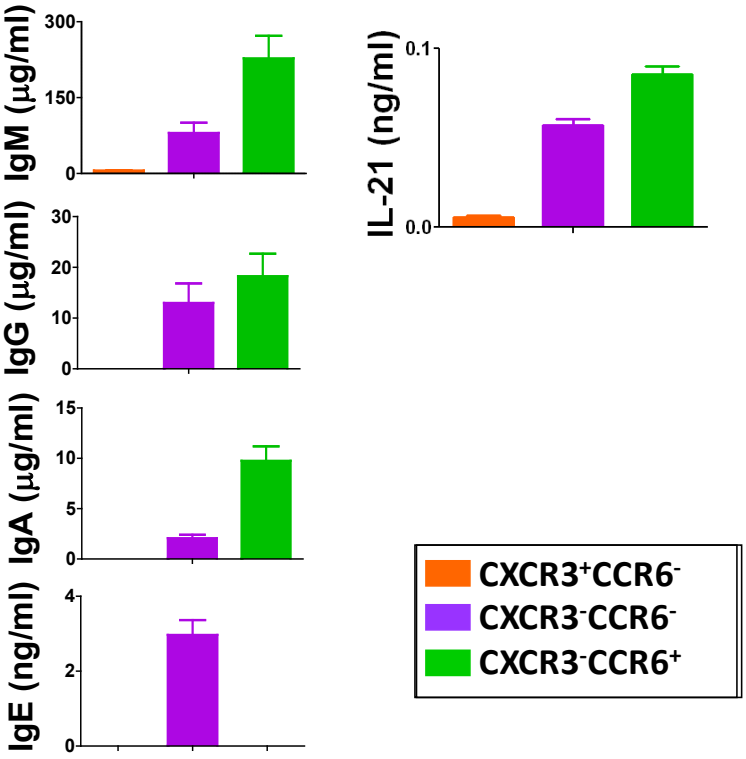


Figure 19: Tfh2 and Tfh17 Cells efficiently help naïve B cells, from Morita el al., 2011. Ig secretion by naive B cells cocultured with blood Th cell populations for 12 days. N = 3–4, mean ± SD. Representative data from three independent experiments.

More interestingly, patients with juvenile dermatomyositis, a systemic autoimmune disease, displayed a profound skewing of blood Tfh cell subsets toward Tfh2 and Tfh17 cells. Importantly, the skewing of subsets toward Tfh2 and Tfh17 cells correlated with disease activity (Figure 20).

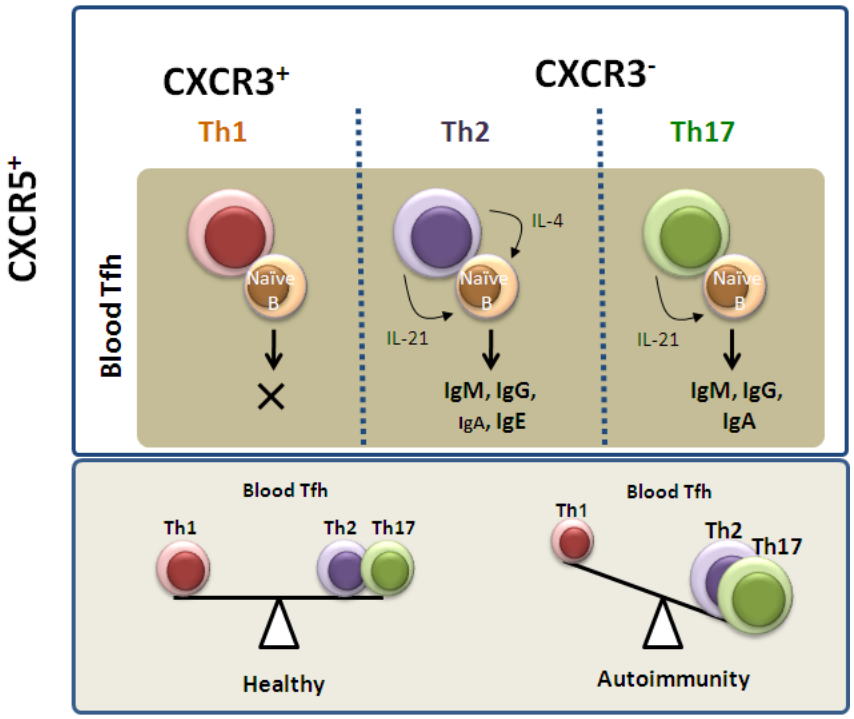


Figure 20: Human blood Tfh cell subsets and their alteration in autoimmunity, from Morita el al., 2011 Human blood Tfh cells comprise three subsets: Tfh1, Tfh2, and Tfh17 cells. Unlike Tfh2 and Tfh17 cells, Tfh1 cells do not help B cells. Importantly, Tfh cell subsets are skewed toward Tfh2 and Tfh17 cells in juvenile dermatomyositis.

However, the role of Tfh cells in vaccination remains poorly understood. Influenza vaccines provide protection by generating high-affinity antibodies against hemagglutinin thereby preventing virus entry (Nichol et al., 1994; Osterholm et al., 2012). However, early immunological events that lead to the development of protective immunity following vaccinations remain largely unknown (Crotty, 2011; King et al., 2008). In the present study, we aimed to determine whether influenza vaccination can result in the activation of blood Tfh cells, and whether the induction of such cells would represent a biomarker of successful immunization. Here we show that the emergence of $ICOS^{+}CXCR3^{+}$ Tfh cells in blood after influenza vaccinations is an early biomarker of antibody responses.

*Induction of ICOS on CXCR3$^+$ Tfh Cells After Influenza Vaccinations*

Two cohorts of healthy individuals were accrued in this study. A non-adjuvanted trivalent split seasonal influenza vaccine (Fluzone®) was administered to a cohort of healthy adult (n=12, called adult cohort) during winter 2009/2010, and to a cohort of healthy children (n=19, called children cohort) during winter 2010/2011. The two vaccines shared the influenza B strain (B/Brisbane/60/2008-like). The Influenza H3N1 strains were different (2009/2010: A/Brisbane/10/2007 (H3N2)-like; 2010/2011: A/Perth/16/2009 (H3N2)-like), but largely similar (for example, the identity of hemagglutinin sequences 98%). However, only the 2010/2011 vaccine did contain a component derived from swine-origin H1N1 influenza strain (A/California/7/2009 (H1N1)-like), a pandemic strain in the year 2009-10 (Jain et al., 2009), and the 2009/2010 vaccine contained A/Brisbane/59/2007 (H1N1)-like strain. The frequency of total CD4$^+$ T cells as well as CXCR5$^+$ CD4$^+$ T cells in blood did not change at any time points after vaccination (days 1, 3, 7, 10, 14, 21, and 28) (Figure 21).

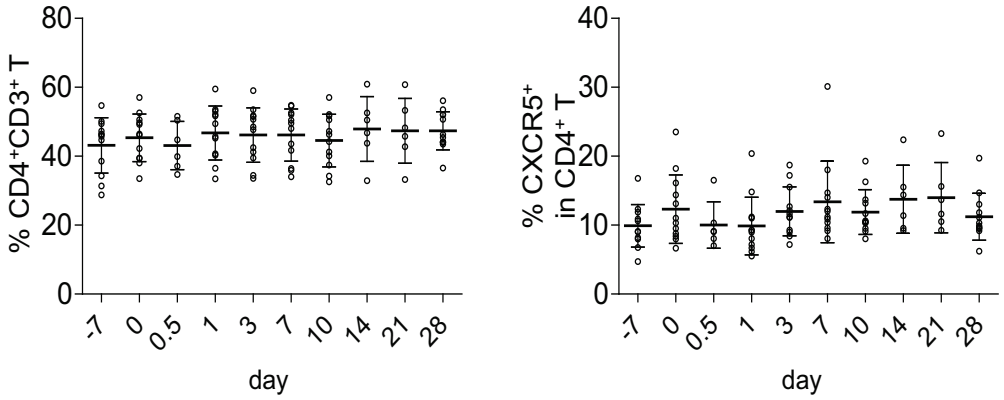


Figure 21: Total $CD4^+$ and Tfh cells did not change after influenza vaccination**.** Percentage of total $CD4^+$ T cells within lymphocytes and $CXCR5^+$ cells within $CD4^+$ T cells. Fresh blood samples were obtained from a healthy adult cohort (n=12) before and after vaccination with trivalent seasonal influenza vaccine (influenzazone® 2009/2010). No significance in One-way ANOVA test.

However, the frequency of $CD4^+$ T cells expressing ICOS increased post-vaccination and peaked on Day 7 (Figure 22A).

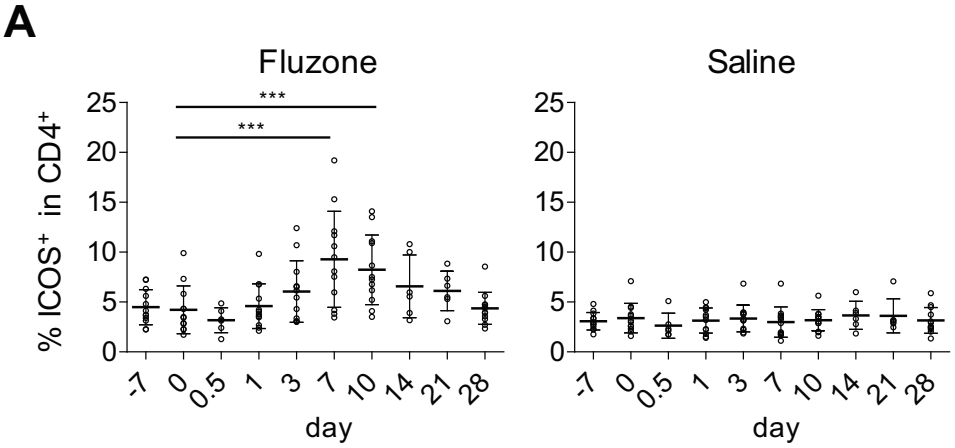


Figure 22: Seasonal influenza vaccines induce $ICOS^+$ Tfh cells in blood. (A) Percentage $ICOS^+$ cells within $CD4^+$ T cells. Fresh blood samples were obtained from a healthy adult cohort (n=12) before and after vaccination with trivalent seasonal influenza vaccine (Fluzone® 2009/2010). The right panel shows data from 6 adults who received injection of normal saline. One-way ANOVA. p-value *** <0.001.

The upregulation of ICOS was largely limited to $CXCR5^+$ Tfh cells, as the frequency of $ICOS^+$ cells within the memory $CXCR5^{neg}CD4^+$ T cells did not change (Figure 22B).

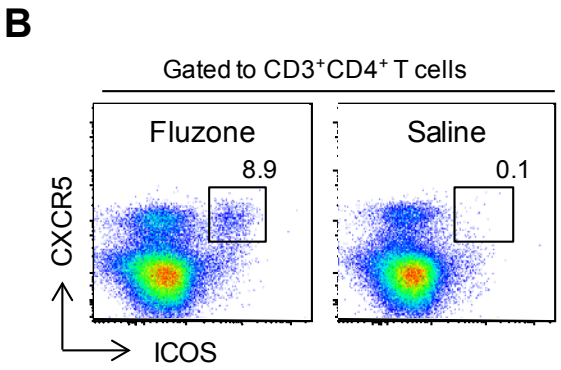


Figure 22: Seasonal influenza vaccines induce ICOS$^+$ Tfh cells in blood (B) A representative results of ICOS expression on Tfh cells on 7 d after influenza vaccination or injection of normal saline.

Furthermore, upregulation of ICOS expression was found to be limited to Tfh cells that co-expressed CXCR3 but not CCR6 (Figure 23).

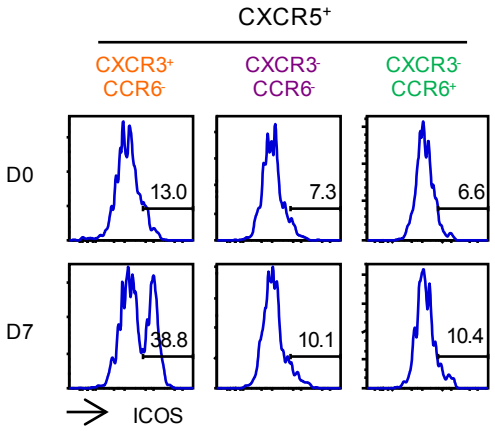


Figure 23: ICOS is expressed by CXCR3$^+$ Tfh cells. ICOS expression on Tfh subsets before and 7 d after influenza vaccination. A representative flow data.

Therefore, in this study, we refer CXCR3$^+$CCR6$^-$ Tfh cells as CXCR3$^+$ Tfh cells for simplicity. The extent of ICOS expression within CXCR3$^+$ Tfh cells varied among subjects (in adult, mean range 2.9 to 34.4 %; in children, range 4.9 to 45.5 %, data not shown). The induced ICOS$^+$CXCR3$^+$ Tfh cells co-expressed high levels of PD-1, but did not express detectable levels of Bcl-6 (Figure 24), and thus were distinct from bona fide

Tfh cells present in secondary lymphoid organs (Bentebibel et al., 2011; Crotty, 2011; King et al., 2008).

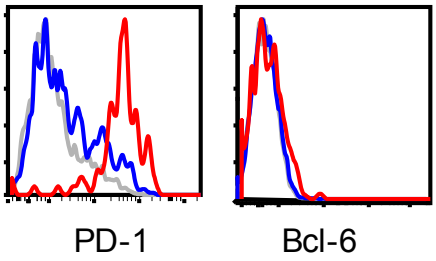


Figure 24: CXCR3$^{+}$ Tfh cells express PD1 but not Bcl6. Expression of PD-1 and Bcl-6 by ICOS$^{+}$ CXCR3$^{+}$ Tfh cells (red), ICOS$^{neg}$ CXCR3$^{+}$ Tfh cells (blue), and CXCR3$^{neg}$ CD4$^{+}$ T cells (gray). A representative out of three experiments.

*Emergence of ICOS$^{+}$CXCR3$^{+}$ Tfh Cells Serves as a Predictive Biomarker*

We wondered whether the emergence of ICOS$^{+}$CXCR3$^{+}$ Tfh cells might predict the development of antibody responses. Hemagglutinin inhibition (HI) and virus neutralization (VN) antibody titers against each influenza virus strains incorporated in the vaccines were measured by our collaborators in Mount Sinai school of medicine at baseline and at day 28 post-vaccination in both children (Figure 25), and adult cohort (Figure 26).


Figure 25: HI and VN titers in children cohort. Hemagglutinin inhibition (HI, shown in panel A) and virus neutralization (VN, shown in panel B) antibody titers against each influenza virus strains incorporated in the vaccines were determined at baseline and at day 28 post-vaccination in the adult cohort.


Figure 26: HI and VN titers in adult cohort. Hemagglutinin inhibition (HI, shown in panel A) and virus neutralization (VN, shown in panel B) antibody titers against each influenza virus strains incorporated in the vaccines were determined at baseline and at day 28 post-vaccination in the adult cohort.

The increase of blood ICOS$^+$CXCR3$^+$ Tfh cells positively correlated with the fold increase of global antibody titers, for both adults and children (Figure 27A). The global antibody response was determined as the maximum fold increase of the titer from the baseline among the three influenza strains in the vaccine.

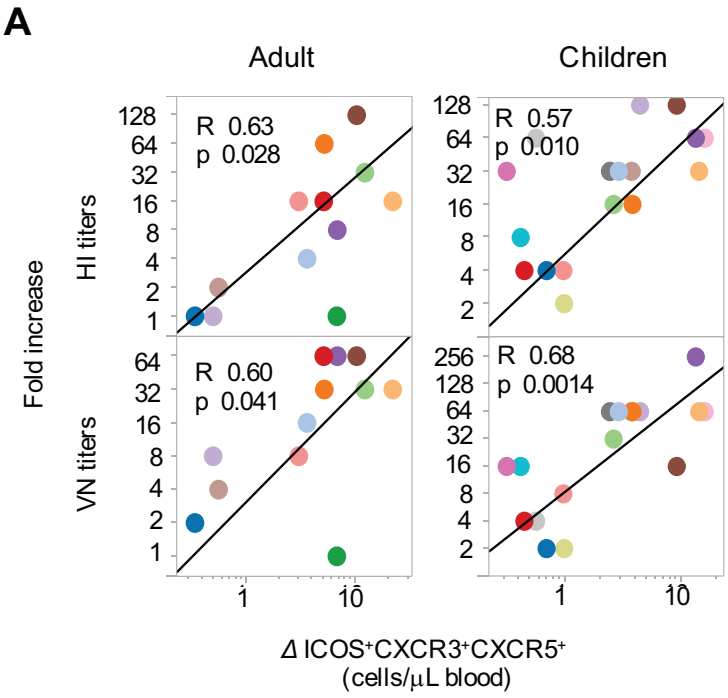


Figure 27: ICOS$^+$CXCR3$^+$ Tfh cells correlate with antibody responses (A) Correlation between the increase of global antibody (HI and VN) titers and the increase of ICOS$^+$CXCR3$^+$ Tfh cells. R: Pearson R; p: p-value.

Furthermore, the absolute number of ICOS$^+$CXCR3$^+$ Tfh cells in blood measured at day 7 alone was sufficient to predict the global antibody responses in both adult and children cohorts (Figure 27B).

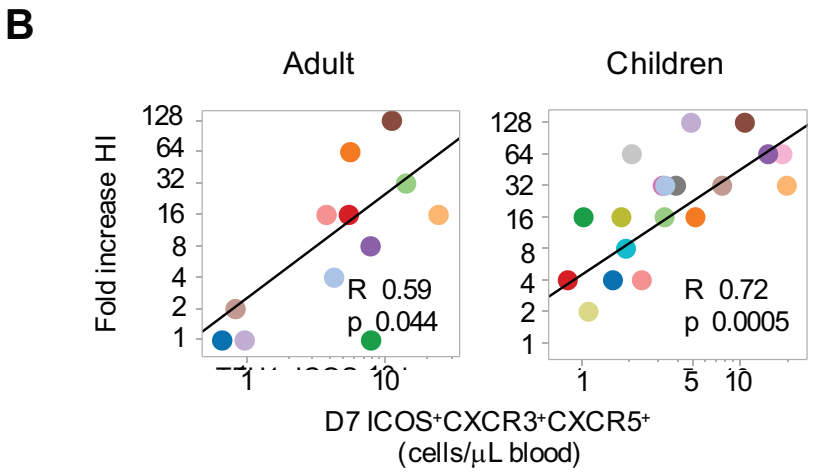


Figure 27: ICOS$^{+}$CXCR3$^{+}$ Tfh cells correlate with antibody responses (B) Correlation between the increase of global antibody (HI and VN) titers and absolute number of ICOS$^{+}$CXCR3$^{+}$ Tfh cells at day 7.

No other immune parameters on blood Th subsets, including the frequency of Tfh cells or that of ICOS$^{+}$ cells among CXCR5$^{neg}$ CD4$^{+}$ T cell subsets, showed a significant correlation in both cohorts (Table 1).

Table 1: Absolute cell number of ICOS$^+$CXCR3$^+$ Tfh cells in blood at day 7 represents a biomarker. Correlation with the increase of HI titers is shown. Spearman R and p-value are shown in the table.

| | Adult | | Children | |
|---|---|---|---|---|
| | Spearman R | p-value | Spearman R | p-value |
| CD4 | -0.18 | 0.57 | 0.35 | 0.15 |
| ICOS$^+$ CD4 | 0.13 | 0.7 | 0.43 | 0.07 |
| CXCR5$^+$ | 0.26 | 0.42 | 0.35 | 0.14 |
| ICOS$^+$ CXCR5$^+$ | 0.58 | 0.05 | 0.35 | 0.15 |
| CXCR5$^+$ CXCR3$^+$ | 0.45 | 0.14 | 0.54 | 0.02 |
| ICOS$^+$ CXCR5$^+$ CXCR3$^+$ | 0.59 | 0.04 | 0.67 | 0.001 |
| CXCR5$^+$ CXCR3$^-$CCR6- | 0.27 | 0.4 | -0.06 | 0.81 |
| ICOS$^+$ CXCR5$^+$ CXCR3$^-$CCR6$^-$ | 0.57 | 0.05 | 0.08 | 0.75 |
| CXCR5$^+$ CXCR3$^-$CCR6$^+$ | 0.26 | 0.42 | 0.04 | 0.86 |
| ICOS$^+$ CXCR5$^+$ CXCR3$^-$CCR6$^+$ | 0.24 | 0.45 | -0.12 | 0.61 |
| CXCR5$^-$ | 0.33 | 0.3 | 0.24 | 0.33 |
| ICOS$^+$CXCR5$^-$ | -0.28 | 0.38 | 0.38 | 0.11 |
| CXCR5$^-$CXCR3$^+$CCR6$^-$ | -0.11 | 0.73 | 0.42 | 0.07 |
| ICOS$^+$CXCR5$^-$CXCR3$^+$CCR6$^-$ | -0.4 | 0.2 | 0.52 | 0.02 |
| CXCR5$^-$CXCR3$^-$CCR6$^-$ | -0.13 | 0.68 | -0.04 | 0.87 |
| ICOS$^+$CXCR5$^-$CXCR3$^-$CCR6$^-$ | -0.3 | 0.35 | 0.24 | 0.32 |
| CXCR5$^-$CXCR3$^-$CCR6$^+$ | 0.54 | 0.07 | 0.02 | 0.94 |
| ICOS$^+$CXCR5$^-$CXCR3$^-$CCR6$^+$ | 0.2 | 0.54 | 0.02 | 0.93 |
| CXCR5$^-$CXCR3$^+$CCR6$^+$ | 0.31 | 0.32 | 0.3 | 0.22 |
| ICOS$^+$CXCR5$^-$CXCR3$^+$CCR6$^+$ | -0.078 | 0.81 | 0.26 | 0.29 |
| CD8 | 0.53 | 0.08 | 0.05 | 0.84 |
| PB+PC | 0.65 | 0.02 | 0.02 | 0.93 |

Of note, while the increase of plasmablasts (CD38$^+$CD27$^+$CD19$^+$) was observed at day 7 in the two cohorts, an observation consistent with previous studies (Nakaya et al., 2011), this parameter failed to show a good correlation with the increase of global antibody titers in the children cohort (Table 1). To validate our observation, we obtained blood samples from another adult cohort during the 2011/12 winter. Fluzone 2011/12 was administered to 37 healthy adults, and the correlation between the increase of blood ICOS$^+$CXCR3$^+$ Tfh cells at day 7 and the fold increase of global antibody titers at day 28

was analyzed. As shown in Figure 27C, the two parameters showed a strong positive correlation (HI: R=0.56, p=0.0003; VN: R=0.61, p<0.0001).

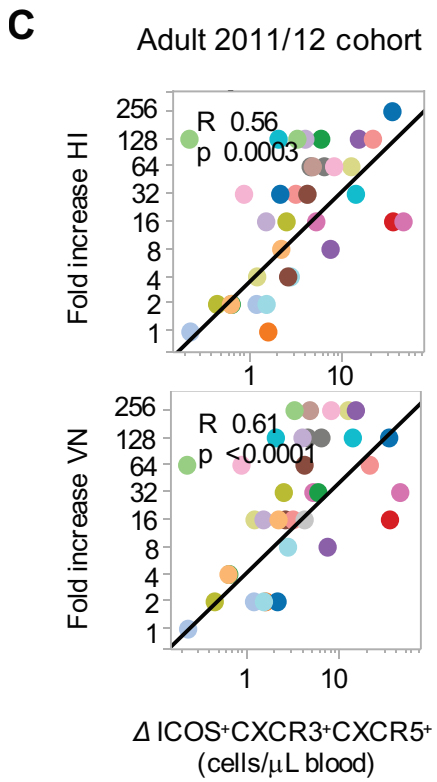


Figure 27: ICOS$^+$CXCR3$^+$ Tfh cells correlate with antibody responses (C) Correlation between the increase of global antibody (HI and VN) titers and the increase of ICOS$^+$CXCR3$^+$ Tfh cells in the 2011/12 adult cohort. N=37

Collectively, the data collected from three independent cohorts of vaccinees show that emergence of ICOS$^+$CXCR3$^+$ Tfh cells at day 7 serves as an early biomarker of the magnitude of global protective antibody responses in seasonal influenza vaccination.

*ICOS$^+$CXCR3$^+$ Tfh Cell Numbers Correlate With Recall Antibody Responses*

The increase of ICOS$^+$CXCR3$^+$ Tfh cells positively correlated with the increased antibody titers against each viral strain in the vaccines with the exception, in the children cohort of the titers against swine-origin H1N1 2009 (Figure 28A, Table 2).

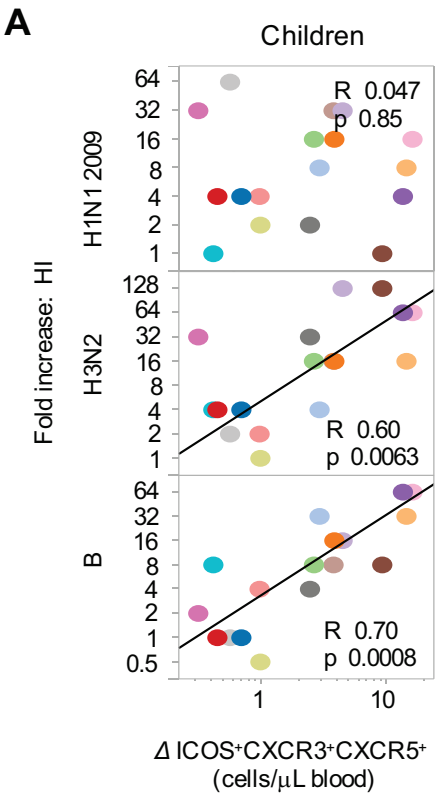


Figure 28: ICOS$^+$CXCR3$^+$ Tfh cell numbers correlate with recall antibody responses (A) Correlation between the increase of HI titers against three different hemagglutinin included in the vaccines and the increase of ICOS$^+$CXCR3$^+$ Tfh cells in the children cohort.

Table 2: Absolute cell number of ICOS$^+$CXCR3$^+$ Tfh cells in blood at day 7 represents a biomarker. Correlation with the increase of HI titers is shown. Spearman R and p-value are shown in the table.

| | | | | Spearman R | p-value |
|---|---|---|---|---|---|
| Adult (2009/2010) | ΔICOS$^+$CXR3$^+$CXCR5$^+$ (cells/µL) | HI | H1N1 | 0.61 | 0.035 |
| | | | H3N2 | 0.47 | 0.13 |
| | | | B | | |
| | | VIN | H1N1 | 0.32 | 0.31 |
| | | | H3N2 | 0.65 | 0.021 |
| | | | B | 0.57 | 0.056 |
| Adult (2011/2012) | ΔICOS$^+$CXR3$^+$CXCR5$^+$ (cells/µL) | HI | H1N1 | 0.56 | 0.0003 |
| | | | H3N2 | 0.58 | 0.002 |
| | | | B | 0.6 | <0.0001 |
| | | VIN | H1N1 | 0.54 | 0.0005 |
| | | | H3N2 | 0.54 | 0.0006 |
| | | | B | 0.61 | <0.0001 |
| Children (2010/2011) | ΔICOS$^+$CXR3$^+$CXCR5$^+$ (cells/µL) | HI | H1N1 | 0.12 | 0.63 |
| | | | H3N2 | 0.59 | 0.0008 |
| | | | B | 0.64 | 0.0032 |
| | | VIN | H1N1 | 0.12 | 0.63 |
| | | | H3N2 | 0.54 | 0.017 |
| | | | B | 0.64 | 0.0028 |

Given that seasonal influenza vaccines induce antibody responses mainly through a boost of recall response (Doherty et al., 2006), this lack of correlation might indeed reflect the lack of H1N1 2009 specific memory in some children. This is consistent with the fact that these children had not been vaccinated or naturally exposed to the H1N1 virus prior to being vaccinated in that 2010/11 winter. Indeed, there was no correlation between H1N1 2009 HI titer increase and the induction of ICOS$^+$CXCR3$^+$ Tfh cells in those children who did not carry pre-existing H1N1 2009 HI antibodies (R=0.07, p=0.84, n=11). In contrast, a positive correlation was observed in children who had pre-existing specific antibodies (R=0.70, p=0.046, n=8. HI titer ≥ x40) (Figure 28B). These data show that the increase of ICOS$^+$CXCR3$^+$ Tfh cells predicts the boost of pre-existing antibody titers, but not the induction of primary antibody responses.

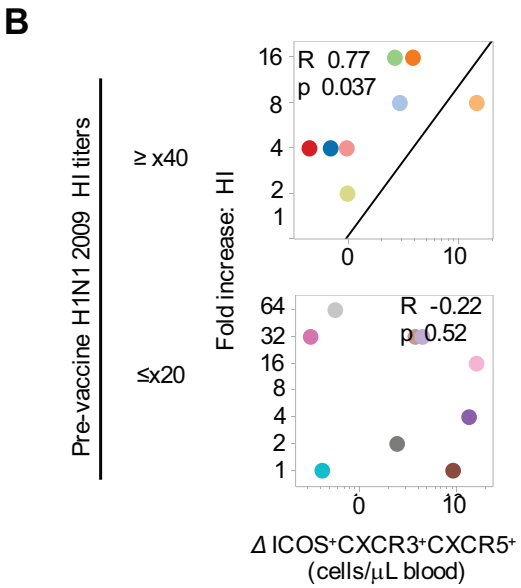


Figure 28: ICOS$^+$CXCR3$^+$ Tfh cell numbers correlate with recall antibody responses (B) Correlation between the increase of HI titers against H1N1 2009 and the increase of ICOS$^+$CXCR3$^+$ Tfh cells, in children who did not carry pre-existing antibodies (bottom), and in children who showed pre-existing specific antibodies (HI titer ≥ x40, top).

### *Induced ICOS$^+$CXCR3$^+$ Tfh Cells Recognize Influenza Antigens*

The increase of ICOS$^+$CXCR3$^+$ Tfh cells after vaccination suggests that they might have undergone proliferation. Indeed, a large fraction of these cells expressed Ki67 (Figure 29A), indicating that they were in cell cycle.

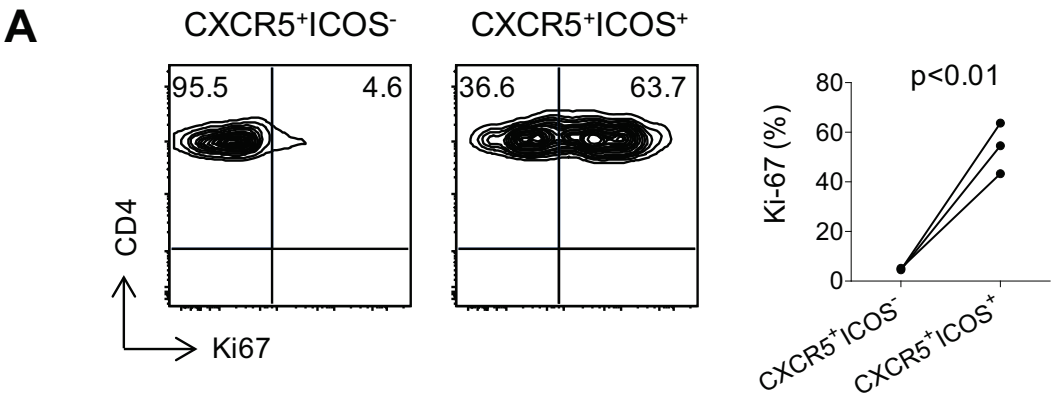


Figure 29: ICOS$^+$CXCR3$^+$ Tfh cells are specific for influenza antigens (A) Ki67 expression by ICOS$^+$CXCR3$^+$ Tfh cells at day 7 post-vaccination (n=3). A representative flow data result is shown on the left panels.

To test their specificity for vaccine antigens, PBMCs at day 7 post-vaccination were stimulated for 6 h with Fluzone® or killed Flu-virus (PR8) in the presence of brefeldin A and monensin. The expression of intracytoplasmic cytokines was analyzed by flow cytometry together with that of CD154, as this permits distinction of antigen-specific $CD4^+$ T cells from antigen non-specific cells (Chattopadhyay et al., 2005). Both stimulations induced a fraction of Tfh cells to express CD154, demonstrating the presence of influenza antigen-specific T cells (Figure 29B).

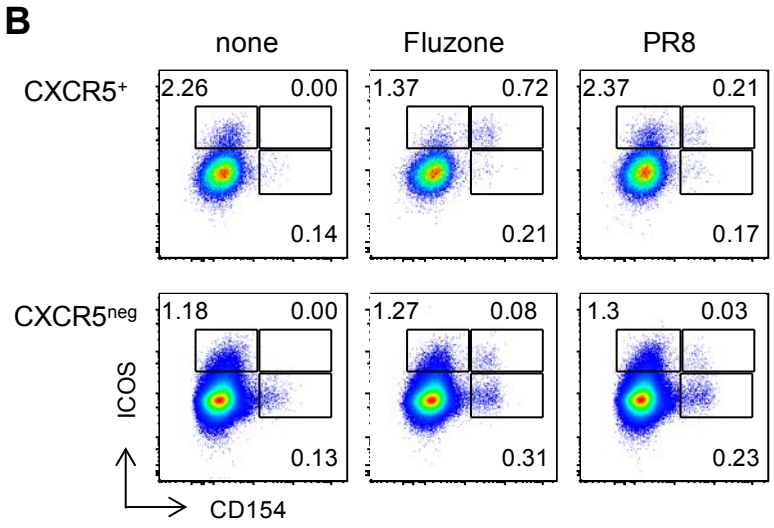


Figure 29: $ICOS^+CXCR3^+$ Tfh cells are specific for influenza antigens (B) CD154 assay. PBMCs obtained at day 7 post-vaccination were stimulated with either Fluzone or heat killed PR8 influenza virus for 6 h. $CD154^+$ cells represent antigen-specific cells. Gated to $CXCR5^+CD4^+$ and $CXCR5^{neg}CD4^+$ T cells. A representative from experiments with 4 donors.

### *Induced $ICOS^+CXCR3^+$ Tfh Cells Express Multiple Cytokines*

$CXCR5^+$ Tfh cells that expressed CD154 upon influenza antigen stimulation co-expressed several cytokines, including IL-2, IFN-γ, IL-10, and IL-21 (Figure 30A). Among the $CD154^+$ $CXCR5^+$ Tfh cells, these cytokines were expressed dominantly by $ICOS^+$ cells (Figure 30B). Approximately 70% of $CD154^+$ $ICOS^+$ Tfh cells expressed

IFN-γ, 50% of cells expressed IL-2, and 40% of cells expressed IL-21 (Figure 30B). In contrast, very few CD154$^{+}$ ICOS$^{neg}$CXCR5$^{+}$ CD4$^{+}$ T cells expressed IL-21, and a majority of them lacked expression of any of the tested cytokines (Figure 30B). Thus, the cytokine expression profiles were largely distinct between influenza-specific ICOS$^{+}$ and ICOS$^{neg}$ CXCR5$^{+}$CD4$^{+}$ T cells, and ICOS$^{+}$ cells expressed a broader range of cytokines, including IL-21.

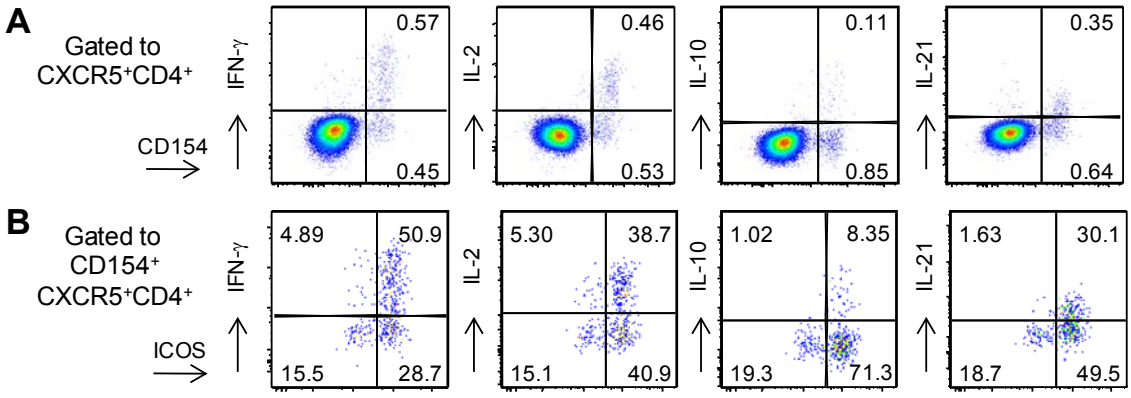


Figure 30: ICOS$^{+}$CXCR3$^{+}$ Tfh cells express multiple cytokines including IL-21 (A) Intracytoplasmic cytokine expression by Tfh cells stimulated with Fluzone. (B) Cytokine expression by CD154$^{+}$ Tfh cells induced by Fluzone stimulation. Expression of the indicated cytokines and ICOS. A representative from experiments with 4 donors.

*Induced ICOS$^{+}$CXCR3$^{+}$ Tfh Cells Efficiently Help Memory B cells*

To analyze the capacity of ICOS$^{+}$CXCR3$^{+}$ Tfh cells to help B cells, these cells were isolated from blood samples at day 7 post-vaccination, and cultured with both naïve and memory B cells from the same blood samples. Staphylococcal enterotoxin B, a superantigen, was added to the cultures to enhance T and B cell interactions (Morita et al., 2011). Naïve B cells cultured with ICOS$^{+}$CXCR3$^{+}$ Tfh cells did not proliferate or differentiate into antibody-secreting cells (CD38$^{hi}$CD138$^{-}$ plasmablasts and CD38$^{hi}$CD138$^{+}$ plasma cells) (Figure 31A, B). In contrast, consistent with our previous

study (Morita et al., 2011), naïve B cells cultured with CXCR3$^{-}$CCR6$^{-}$ Tfh2 and CXCR3$^{-}$CCR6$^{+}$ Tfh17 cells proliferated and differentiated into antibody-secreting cells (Figure 31A, B).

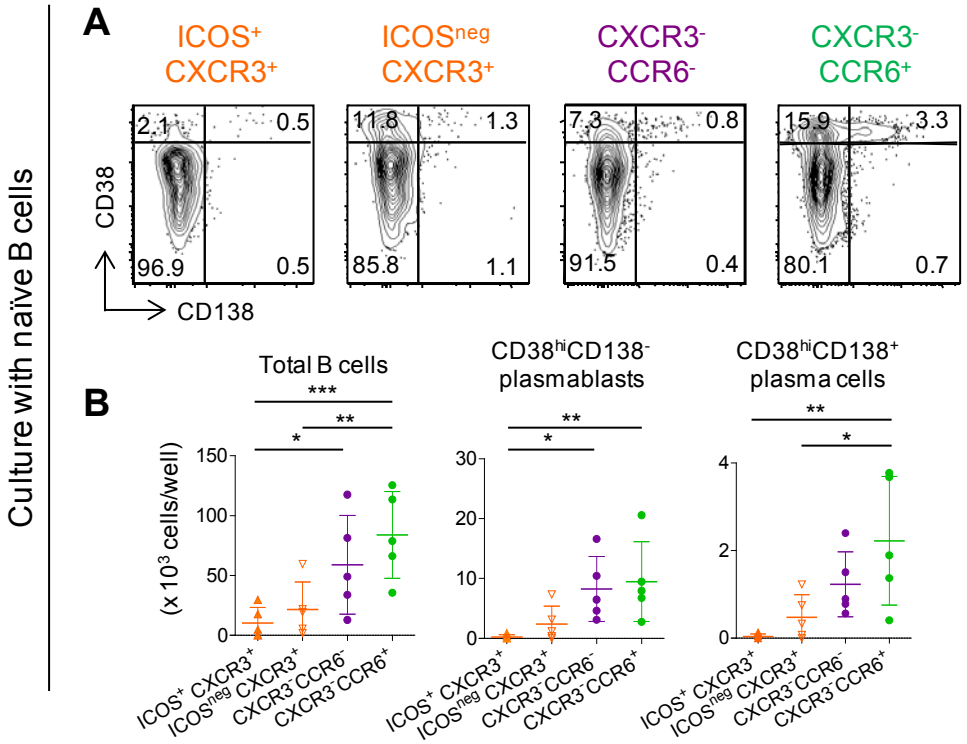


Figure 31: ICOS$^{+}$CXCR3$^{+}$ Tfh cells help memory B cells (A) Differentiation of naïve B cells co-cultured with Tfh subsets. Naïve B cells cultured for 12 d with the indicated Tfh subsets (from day 7 post-vaccination) were analyzed for the expression of CD38 and CD138 A representative data result of 4 experiments is shown (B) The number of total B, plasmablasts, and plasma cells in the cultures were determined on day 12. N=4. One way ANOVA. p-values ***<0.001, ** <0.01, * <0.05.

However, ICOS$^{+}$CXCR3$^{+}$ Tfh cells were the most efficient Tfh subset at inducing memory B cells to differentiate into plasma cells (Figure 31C, D).

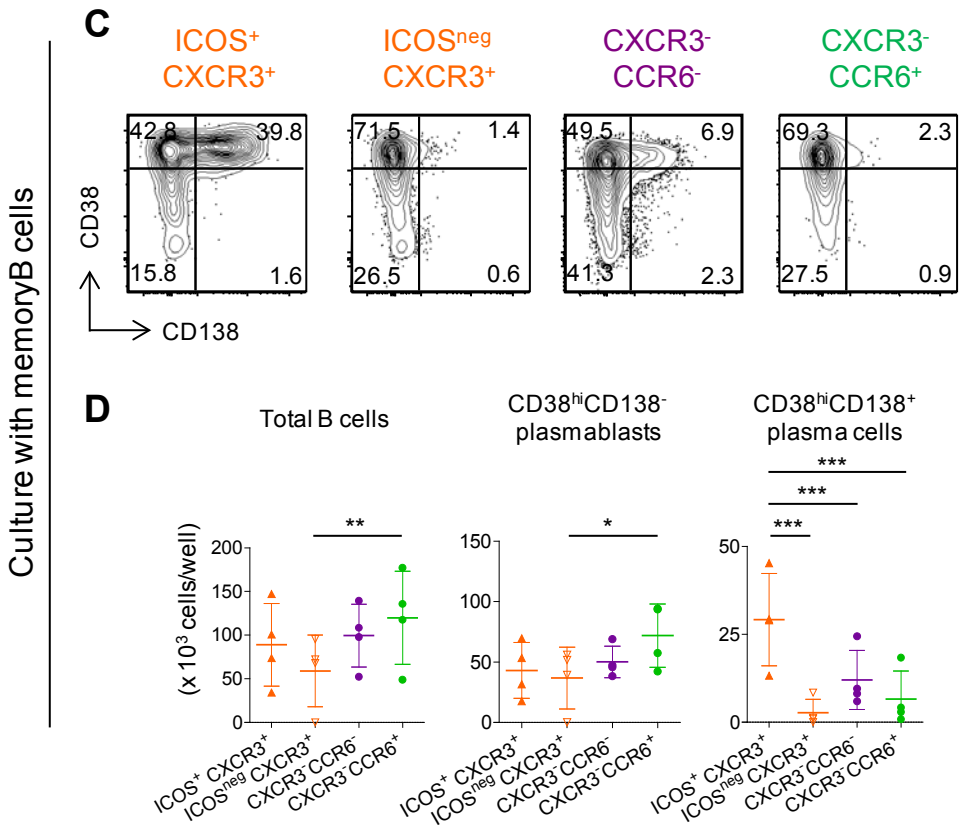


Figure 31: ICOS$^+$CXCR3$^+$ Tfh cells help memory B cells. Memory B cell differentiation (C) Memory B cells cultured for 6 d with the indicated Tfh subsets were analyzed for the expression of CD38 and CD138. A representative data result of 4 experiments is shown (D) The number of total B, plasmablasts, and plasma cells in the cultures were determined on day 6. N=4. One way ANOVA.

The memory B cell help was dependent on IL-10 and IL-21, as the blocking of both cytokines completely inhibited the proliferation of memory B cells and their differentiation into plasma cells (Figure 31E). Furthermore, blocking these two cytokines resulted in a complete inhibition of Ig production (Figure 31F).

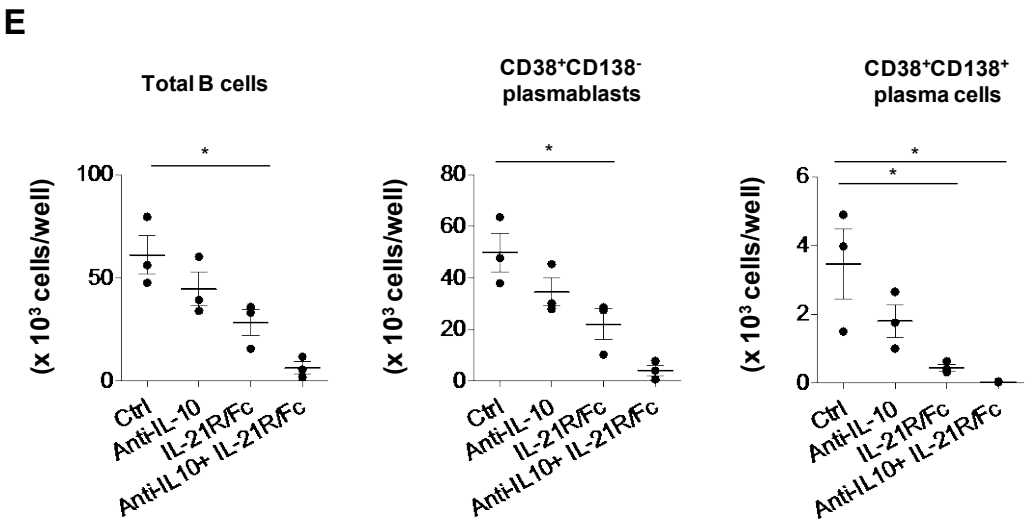


Figure 31: ICOS$^+$CXCR3$^+$ Tfh cells help memory B cells (E) Anti-IL-10 mAb and/or IL-21R-Fc chimera protein were added to the cultures of memory B cells and ICOS$^+$CXCR3$^+$ Tfh cells. The number of B cells, plasmablasts, and plasma cells recovered from the cultured wells was determined. N=3. One way ANOVA.

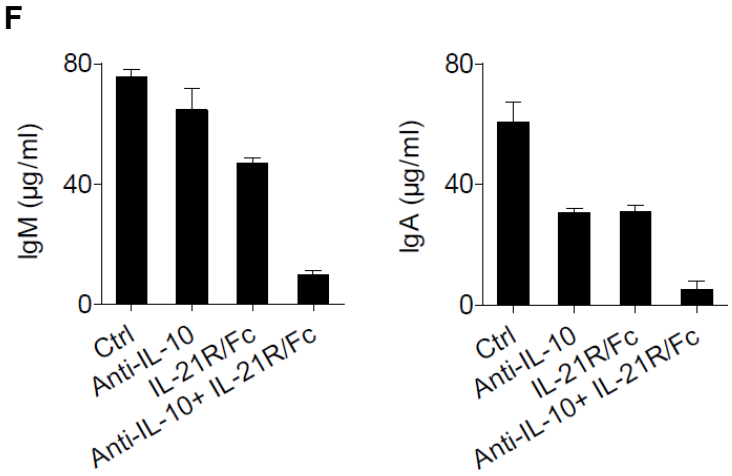


Figure 31: ICOS$^+$CXCR3$^+$ Tfh cells help memory B cells (F) Anti-IL-10 mAb and/or IL-21R-Fc chimera protein were added to the cultures of memory B cells and ICOS$^+$CXCR3$^+$ Tfh cells. Produced Igs were measured by ELISA at day 6. IgG was not measured due to the cross-reactivity with the Fc portion of the IL-21R-Fc chimera protein.

Notably, the recovery of viable ICOS$^+$ CXCR3$^+$ Tfh cells after the co-culture with memory B cells was less than that of viable ICOS$^{neg}$CXCR3$^+$CXCR5$^+$ CD4$^+$ T cells (Figure 31G, left panel). Furthermore, IL-2 concentration in the supernatant was lower in the culture with ICOS$^+$CXCR3$^+$ Tfh cells than with ICOS$^{neg}$CXCR3$^+$CXCR5$^+$CD4$^+$ T cells (Figure 26G, right panel). Therefore, the superior function of ICOS$^+$CXCR3$^+$ Tfh

cells in the induction of memory B cell differentiation towards plasma cells was not due to their superior survival and/or proliferation in culture.

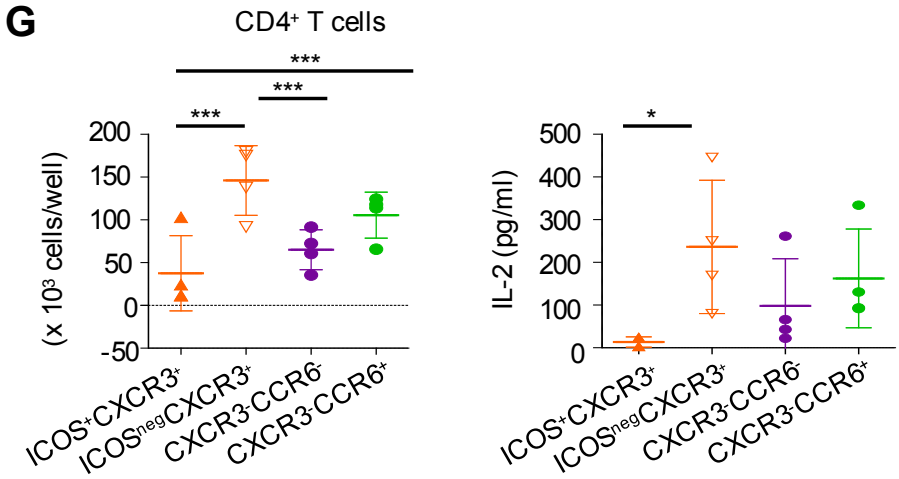


Figure 31: ICOS$^+$CXCR3$^+$ Tfh cells help memory B cells (G) T cell recovery and IL-2 production. The indicated Tfh subsets were cultured with memory B cells. The number of recovered viable CD4$^+$ T cells at day 6 (left panel), and IL-2 levels in the supernatants at day 2 (right panel) are shown. N=4. One way ANOVA.

Lastly, we determined whether ICOS$^+$CXCR3$^+$ Tfh cells were capable of inducing specific antibody responses. Sorted ICOS$^+$CXCR3$^+$ Tfh cells were co-cultured with memory B cells loaded with Fluzone. Consistent with Ki67 expression (Figure 29A), ICOS$^+$CXCR3$^+$ Tfh cells underwent considerable proliferation and cytokine secretion upon co-culture with Fluzone-loaded memory B cells (Figure 32A, B).

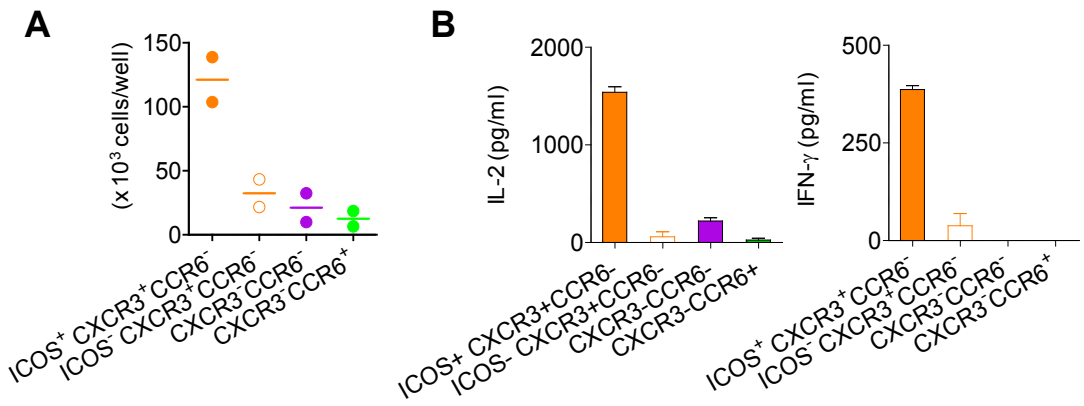


Figure 32: ICOS$^+$CXCR3$^+$ Tfh cells induce antigen-specific antibody response (A) Proliferation of CD4$^+$ T cells. CXCR5$^+$ Th subsets were cultured with memory B cells in the presence of the vaccine. The number of CD4$^+$ T cells in the cultures was determined on day 6. Two independent experiments (B) Cytokine levels on day 2 of culture supernatants. A representative of two experiments.

Furthermore, ICOS$^+$CXCR3$^+$ Tfh cells efficiently induced memory B cells to proliferate and differentiate into plasma cells (Figure 32 C, D), producing influenza-specific IgG (Figure 32E). Taken together, these observations show that ICOS$^+$CXCR3$^+$ Tfh cells induced by influenza vaccination recognize influenza antigens, and are capable of inducing memory B cells to produce influenza-specific Igs.

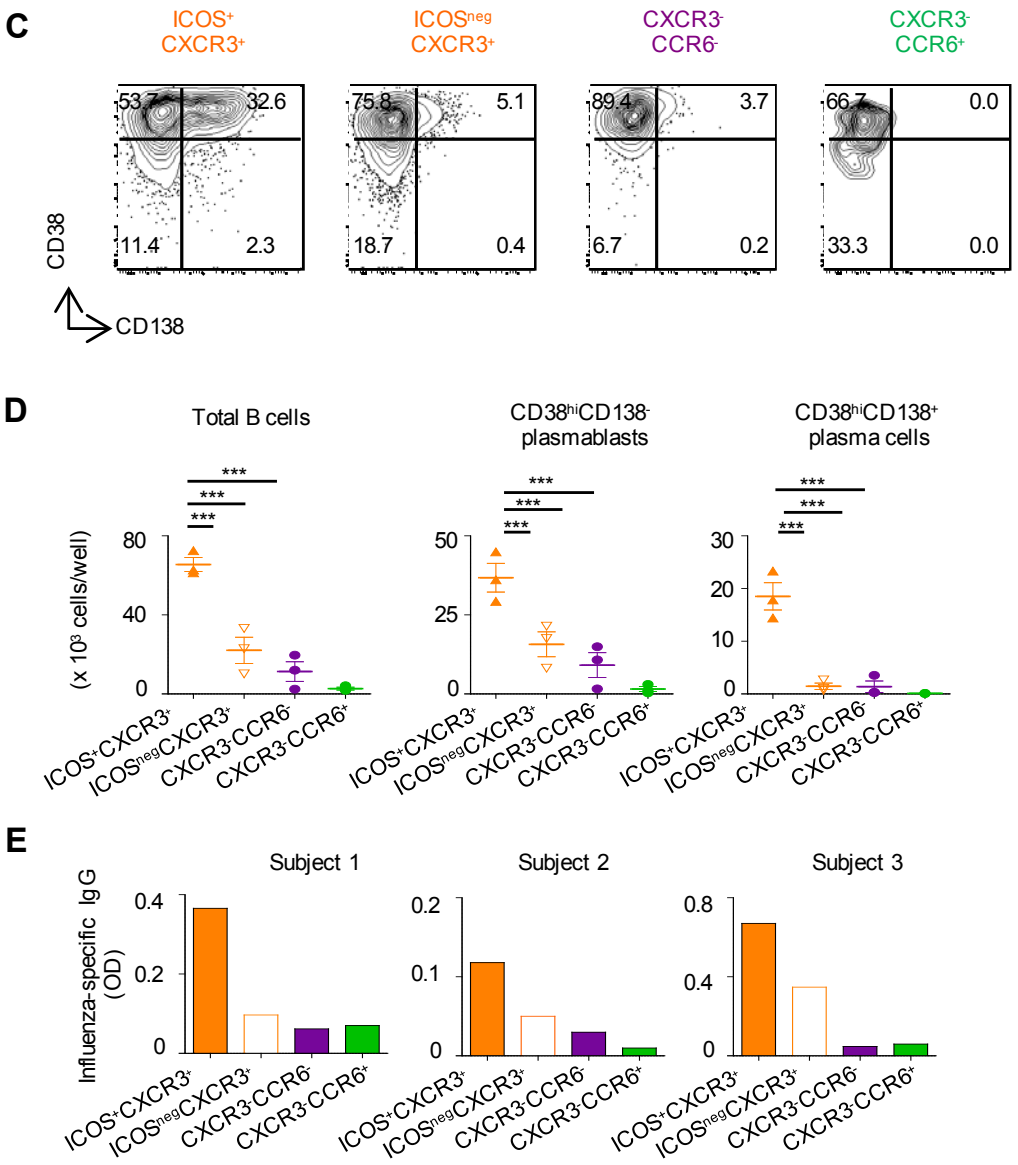


Figure 32: ICOS$^+$CXCR3$^+$ Tfh cells induce antigen-specific antibody response (C) The expression of CD38 and CD138 on memory B cells were determined on day 6. A representative data result of 3 experiments is shown (D) The number of total B, plasmablasts, and plasma cells in the cultures were determined on day 6. N=3 (E) Influenza-specific IgG levels on day 6, n=3. One way ANOVA

## *Discussion*

Administration of seasonal trivalent split influenza vaccines provides protection from influenza in approximately 60-90% of vaccinees (Osterholm et al., 2012). Impaired generation of neutralizing antibodies causes inadequate protection. Our study shows that

the induction of $ICOS^+CXCR3^+$ Tfh cells represents one of the key immunological events that are associated with protective antibody responses following seasonal influenza vaccination. Importantly, the emergence of $ICOS^+CXCR3^+$ Tfh cells in blood correlated with the increase of pre-existing antibodies, but not with the induction of primary antibody responses. Furthermore, our in vitro studies show that the induced $ICOS^+CXCR3^+$ Tfh cells efficiently induce memory B cells to differentiate into plasma cells. These $ICOS^+CXCR3^+$ Tfh cells are however unable to help naïve B cells. These observations suggest that the $ICOS^+CXCR3^+$ Tfh cells induced by split influenza vaccines contribute to the antibody response primarily through boosting memory responses. This is consistent with recent data showing that split influenza vaccines, including ones for H1N1 2009, induce antibody responses mainly via activation of memory B cells (Doherty et al., 2006; Li et al., 2012a). $ICOS^+CXCR3^+$ Tfh cells induced by influenza vaccination express Ki67, and thus are in cell cycle. Furthermore, this cell population was enriched with cells specific for influenza antigens. These observations suggest that $ICOS^+CXCR3^+$ Tfh cells were induced through the interaction with antigen-presenting cells carrying influenza antigens. Most likely this encounter occurs in secondary lymphoid organs, and the induced T cells interact with memory B cells carrying influenza antigens. However, the sites where the $ICOS^+CXCR3^+$ Tfh cells contribute to antibody responses might not be limited to the draining lymph nodes. Mouse studies with pulmonary influenza models showed that memory B cells and effector T cells migrating to the lung express CXCR3, and $CXCR3^+$ memory B cell persist at sites for a long time (Onodera et al., 2012; Pallikkuth et al., 2012). Furthermore, numerous bona fide Tfh cells were found in the lung infected by influenza virus (Strutt et

al., 2012). Therefore, it is possible that the induced $ICOS^+CXCR3^+$ Tfh cells might migrate to the lung through CXCR3, particularly when active inflammatory responses are ongoing, and that they help the generation of influenza-specific antibody responses through interaction with resident memory B cells. It is intriguing that the emergence of plasmablasts and $ICOS^+CXCR3^+$ Tfh cells in blood peaks at the same time after influenza vaccination (at day 7). This suggests that these different types of cells develop in a similar kinetics. However, it is notable that the emergence of $ICOS^+CXCR3^+$ Tfh cells can be detected even at 3 days after vaccination in some individuals. Therefore, induction of such T cells might be a limiting factor for the generation of plasmablasts and plasma cells. Accordingly, it will be important to define their developmental mechanisms, and eventually to identify pathways and/or adjuvants that promote their generation. While the current split influenza vaccines provide protection in many cases, our study suggests that this vaccine design might be suboptimal in some conditions, in particular where primary antibody responses are required, such as immunization to young children or against H5N1 avian influenza viruses. This hypothesis is also supported by the observations that a split influenza vaccine does not immunize naïve mice, unless adjuvants stimulating toll-like receptors are co-administered (Strutt et al., 2012). In the same line, recent studies in humans show that conjugation of adjuvants (for instance with MF59) with influenza vaccines significantly enhance the diversity and the affinity of antibody responses (Khurana et al., 2011). Therefore, such adjuvant might be required to induce different types of helper T cells which efficiently induce primary antibody responses. This study provides a proof of principle for the assessment of blood Tfh cell responses to predict the magnitude of antibody responses induced by vaccinations. It will be important and also

valuable to determine whether this approach reveals predictive biomarkers in different vaccines.

# CHAPTER FIVE

## Results: CXCR3$^+$ GC-Tfh Cells Suppress Germinal Center Response

### *Introduction and Rational*

The studies including ours on human blood Tfh cells have shown that CXCR3$^+$ CXCR5$^+$CD4$^+$ T cells (Tfh1 cells) represent a subset with distinct phenotype and functions (Boswell et al., 2014; Locci et al., 2013; Morita et al., 2011). Patients with autoimmune disease display an alteration in the balance of blood memory Tfh1, Tfh2, and Tfh17 cells. In patients with juvenile dermatomyositis (Morita et al., 2011), adult SLE (Le Coz et al., 2013), and Sjogren's syndrome (Li et al., 2012b), Tfh1 cells are underrepresented among blood memory Tfh cells, whereas Tfh2 and/or Tfh17 cells are overrepresented. Such alterations were found to correlate with disease activity, serum autoantibody titers, and/or the frequency of blood plasmablasts (Le Coz et al., 2013; Li et al., 2012b; Morita et al., 2011). This observation suggests that Tfh1 cells might act as a regulatory subset among Tfh cells.

A previous study demonstrated that CD4$^+$CXCR3$^+$ T cells can be found in GCs light zone of human tonsils (Rabin et al., 2003). However, very little is known regarding the biology of Tfh1 cells in human tonsils. Here, we isolated Tfh1 cells from GC-Tfh cells from tonsils, and analyzed their phenotype, gene expression profiles, and functions in vitro. We confirmed that GC-Tfh cells in tonsils contain a small fraction of cell

expressing CXCR3 (GC-Tfh1 cells). While GC-Tfh1 cells largely shared the phenotype and gene expression profiles with GC-Tfh cells, we found that GC-Tfh1 cells co-expressed Bcl-6 and T-bet, transcription factors associated with Tfh and Th1 cells respectively. Functionally, GC-Tfh1 cells lacked the capacity to help GC-B cells. Importantly, we found that GC-Tfh1 cells displayed a regulatory function and suppressed the helper function of GC-Tfh cells to induce GC-B cells to differentiate into Immunoglobulin-producing plasmablasts. Furthermore, GC-Tfh1 cells suppressed the proliferation and the cytokine production by GC-Tfh cells in a cell-contact dependent manner. GC-Tfh1 cells were different from naturally occurring Tregs as the expression of CD25 and Foxp3 was much lower than Tregs. Mechanistically, three factors expressed by GC-Tfh1 cells are associated with their regulatory functions: surface FAS-L, IFN-γ secretion, and the lack of CD40L expression. Thus, our study shows that GC-Tfh cells represent FAS-L-expressing Tfh cells and act as a regulatory subset of GC responses in humans.

*GC-Tfh cells Contained a Fraction of Cells Expressing CXCR3*

First, we wanted to confirm that Tfh cells in GCs contain cells expressing the chemokine receptor CXCR3. To this end, we collected tonsils from pediatric subjects undergoing tonsillectomy (age between 3 and 12 years), then we stained the CD19 negative fraction with anti-CD4, anti-ICOS, anti-CXCR5, a cocktail of antibodies composed of anti-CD8, anti-CD20, anti-CD56, and anti-CXCR3 mAbs and we analyzed the expression of CXCR3 by single GC-Tfh cells by flow cytometry and also by confocal microscopy.

Consistent with a previous study (Rabin et al., 2003), flow analysis showed that Tfh cells in GCs contain a small fraction of cells expressing CXCR3 (Figure 33A). The frequency of this fraction is approximately 10 % of total GC-Tfh cells (7.96 ± 0.6 %, Mean ± SEM n=28) (Figure 33B). Furthermore, confocal microscopy analysis confirmed the presence of $CD4^+$ T cells expressing CXCR3 inside GCs (Figure 33C).

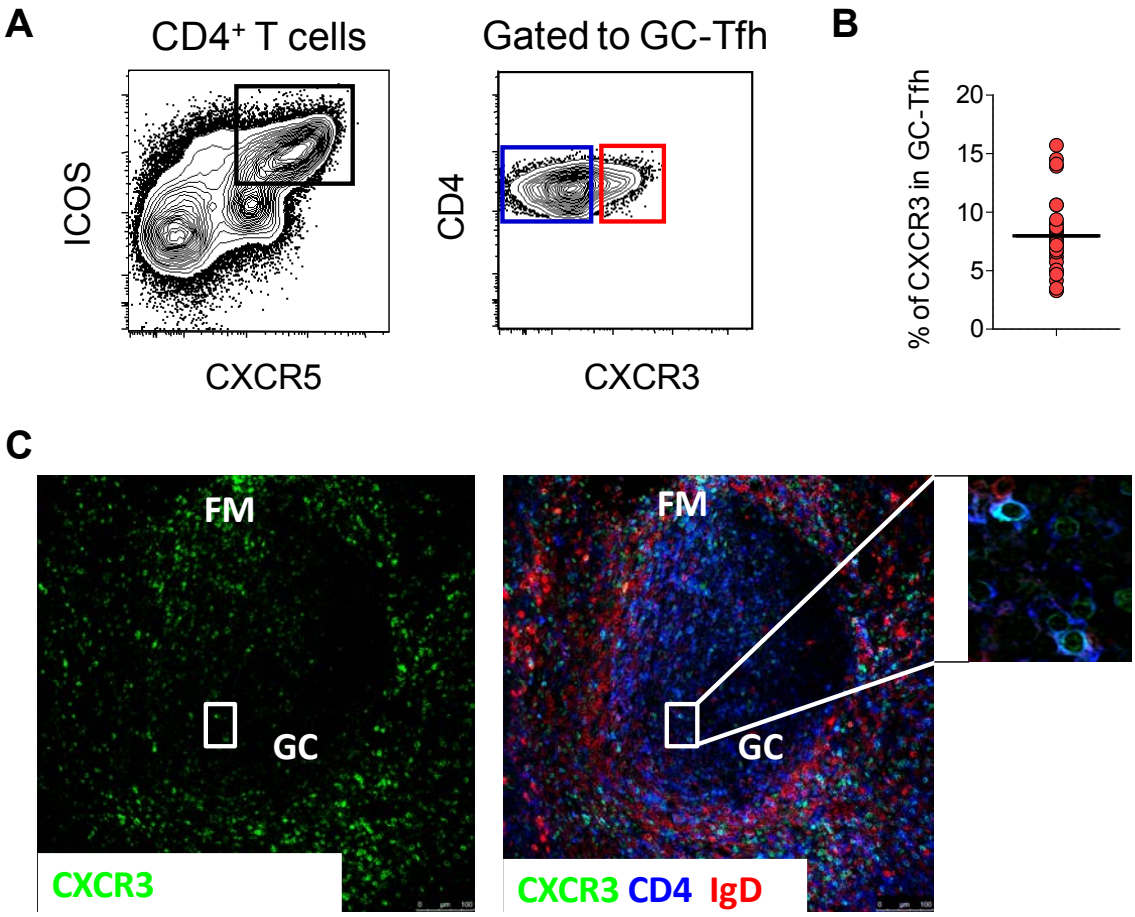


Figure 33: Tfh cells in GCs contain a fraction of cells expressing the chemokine receptor CXCR3 (A) Two tonsillar subsets in GCs were defined according to the expression of ICOS, CXCR5 and CXCR3. $CXCR3^-$ Tfh cells in blue color and $CXCR3^+$ Tfh in red color, (B) the frequency of CXCR3 in GC-Tfh cells from different pediatric tonsils (n=27), (C) Confocal Microscopy of CXCR3 in GCs.

*$CXCR3^+$ GC-Tfh Cells Co-express Bcl-6 and T-bet*

The chemokine receptor CXCR3 is typically expressed by Th1 cells that express the transcription factor T-bet. Here we wanted to determine whether $CXCR3^+$ GC-Tfh cells express Bcl-6 and/or T-bet. As shown in Figure 34A, Bcl-6 protein was abundantly expressed by both $CXCR3^-$ and $CXCR3^+$ GC-Tfh cells (Figure 34A). The expression of T-bet was higher in $CXCR3^+$ GC-Tfh cells (Figure 34A), and 35 ± 3 % of $CXCR3^+$ GC-Tfh cells co-expressed Bcl-6 and T-bet (Figure 34B. Mean ± SEM, n=6). $CXCR5^-ICOS^-$ naïve $CD4^+$ T cells did not express Bcl-6 or T-bet.

Hereafter *we call $CXCR3^+$ GC-Tfh cells as GC-Tfh1 cells and $CXCR3^-$ GC-Tfh cells as GC-Tfh cells for simplicity.*

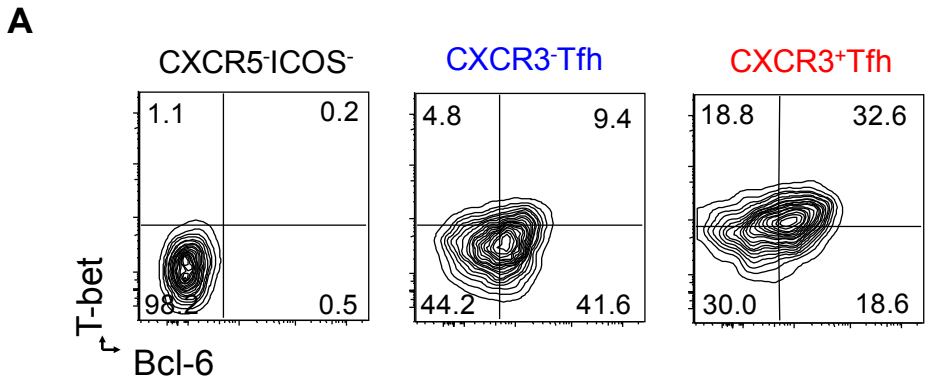


Figure 34: $CXCR3^+$ GC-Tfh cells co-express Bcl-6 and T-bet (A) Bcl-6 and T-bet expression in $CXCR3^-$ GC-Tfh and $CXCR3^+$ GC-Tfh cells. The expression of these two transcription factors was compared to $CXCR5^-ICOS^-$. A representative from six donor tonsils

**B**

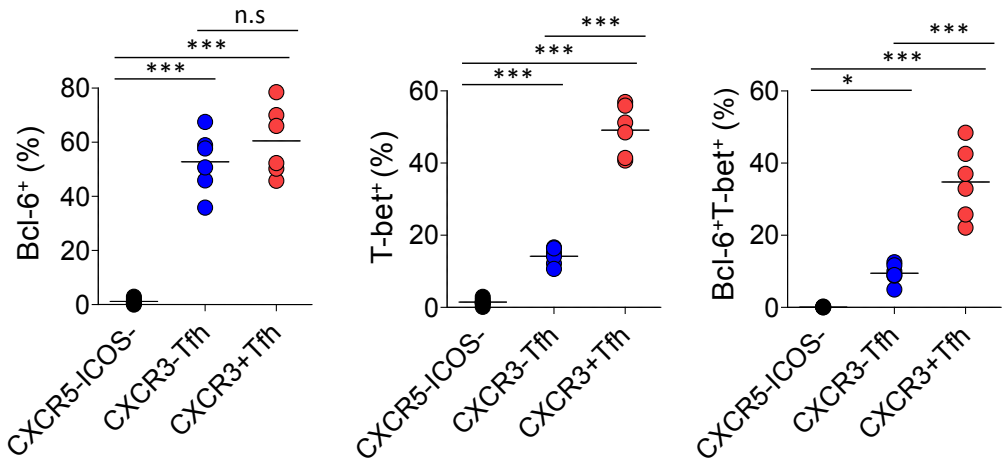


Figure 34. CXCR3$^{+}$ GC-Tfh cells co-express Bcl-6 and T-bet (B) Bcl6 and T-bet expression in CXCR3$^{-}$ GC-Tfh and CXCR3$^{+}$ GC-Tfh cells. The expression of these two transcription factors was compared to CXCR5$^{-}$ICOS$^{-}$. N=6. One-way ANOVA Bonferonni multiple comparisons test.

*GC-Tfh1 cells Largely Share Phenotype and Gene Expression Profiles of GC-Tfh Cells*

Assessment of the expression of Tfh associated molecules demonstrated that GC-Tfh1 cells largely shared the phenotype of GC-Tfh cells (Figure 35A). GC-Tfh1 cells expressed high levels of PD1, CD57, CD84, CD200, BTLA, FAS and the activation marker CD69. Furtemore, they expressed low levels of CD127 and CCR7 (Figure 35A).

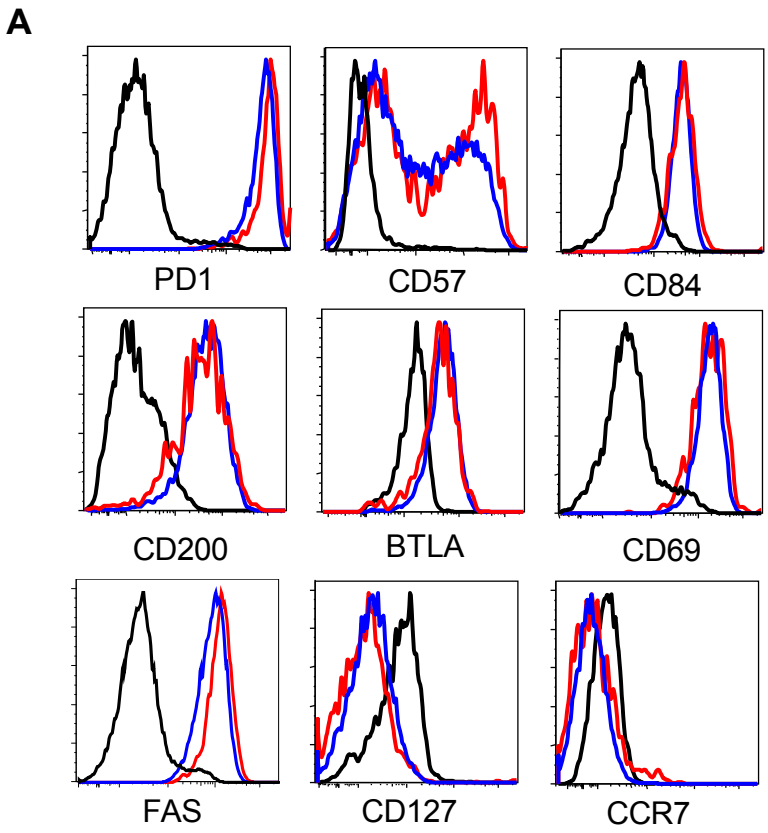


Figure 35: CXCR3$^+$ GC-Tfh cells largely share phenotype and gene expression profile of CXCR3$^-$ GC-Tfh cells (A) Expression of cell surface molecules (PD1, CD84, CD200, BTLA, CD57, CD127, CCR7, CD69) on ICOS$^-$CXCR5$^-$ (black), CXCR3$^-$ GC-Tfh (blue), and CXCR3$^+$ GC-Tfh (red). A representative from at least five donor tonsils.

Gene analysis with NanoString revealed that GC-Tfh1 cells largely shared the gene expression profile with GC-Tfh cells (Figure 35B). For instance, *BCL6, PDCD1, IL-21, MAF, TOX2* transcripts were abundantly expressed by GC-Tfh1 cells when compared to naïve CD4$^+$ T cells, and the expression was equivalent to that of GC-Tfh cells. The expression of *EBI2, RORA, RORC, FOXP3* transcripts were low in both GC-Tfh1 and GC-Tfh cells. Collectively, these data demonstrate that GC-Tfh1 cells largely share phenotype and gene expression profile with GC-Tfh cells.

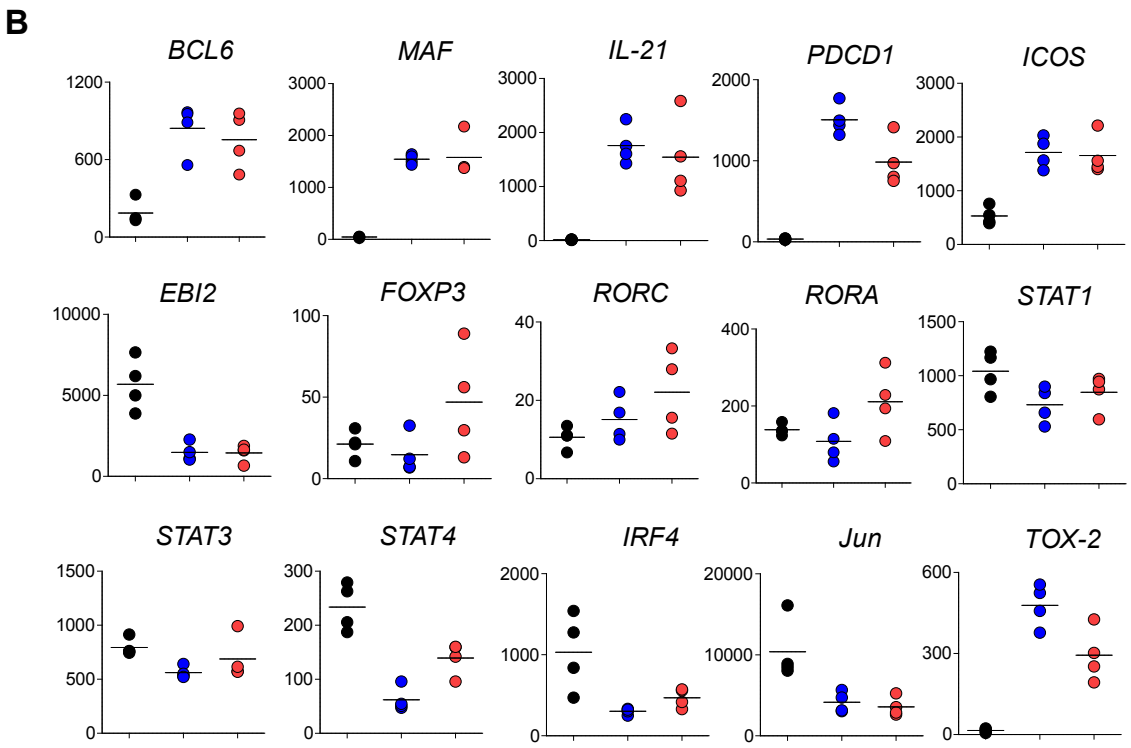


Figure 35: CXCR3$^+$ GC-Tfh cells largely share phenotype and gene expression profile of CXCR3$^-$ GC-Tfh (B) Multiplexed gene expression of freshly sorted naïve CD4$^+$ CD45RA$^+$ CXCR5$^-$ICOS$^-$ T cells (black), CXCR3$^-$ GC-Tfh (blue) and CXCR3$^+$GC-Tfh (red) analyzed by Nanostring. Data were normalized with at least 7 housekeeping genes included in the code set (n=4).

### *Cytokine Profile of GC-Tfh1 Cells*

As we described earlier in chapter three, cytokines secreted by Tfh cells play a major role in the regulation of B cell proliferation, differentiation, and class-switching. Thus, we analyzed the cytokine profiles of GC-Tfh1 cells. Upon stimulation with CD3/CD28, GC- Tfh1 cells secreted larger amounts of IL-2 and IFN-γ than GC-Tfh cells. The secretion of IL-4 and IL-21, the major cytokines associated with help of GC-B cells, was significantly lower by GC-Tfh1 cells. IL-10 production was similar between the two GC-Tfh subsets (Figure 36).

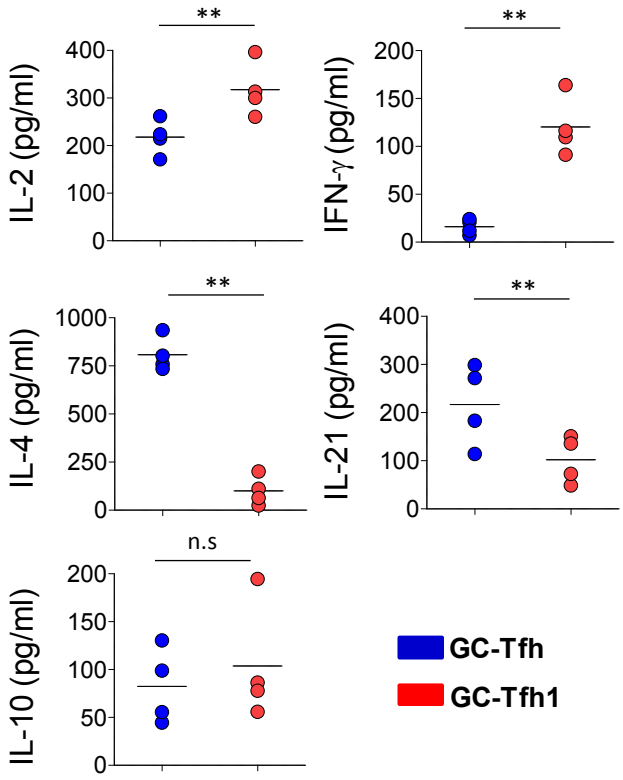


Figure 36: Cytokine secretion of CD3/CD28 stimulated tonsillar GC-Tfh and GC-Tfh1 cells. Tonsillar Th subpopulations (5 x $10^4$ cells/well) were stimulated with CD3/CD28 mAbs for 48 h to measure cytokine secretion. N=4. Paired T test.

### *GC-Tfh1 Cells do not Help GC-B Cells*

We next assessed whether GC-Tfh1 cells were able to help GC-B ($IgD^-$ $CD38^+CD19^+$) cells in vitro. To this end, GC-B cells (2 x $10^4$ /well) were cultured with autologous GC- Tfh1 cells (2 x $10^4$ /well) or GC-Tfh cells in the presence of staphylococcal enterotoxin B (SEB) for 7 days. The recovery of live cells (Live dead $aqua^-$ cells), T cells ($CD3^+CD4^+$), B cells ($CD3^-CD4^-$), and plasma cells ($CD38^{hi}CD20^{lo}$) in the cultures was analyzed by flow cytometry (Figure 37A).

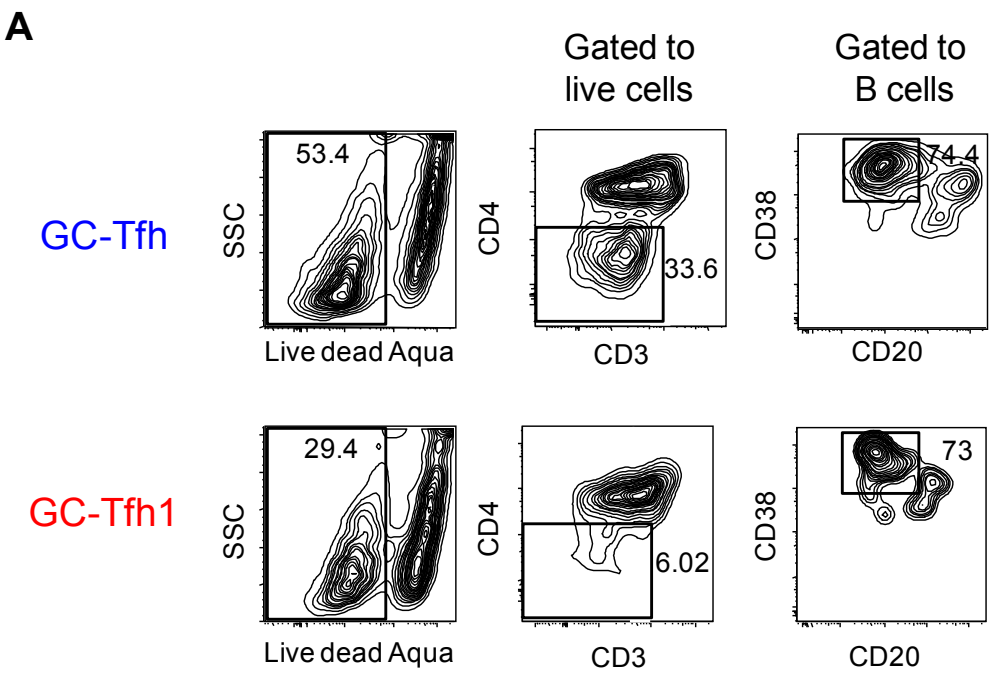


Figure 37: GC-Tfh1 cells are not efficient at helping GC-B cells (A) Recovery of GC-B cells co-cultured with GC-Tfh populations. GC-B cells cultured for 7 d with either GC-Tfh or GC-Tfh1 population were analyzed for the expression of CD20 and CD38. A representative from 11 experiments.

Consistent with our previous study (Bentebibel et al., 2011), GC-Tfh cells efficiently maintained the survival of GC-B cells during the co-culture (Figure 37B. 21.5 ± 2.5 x $10^3$ cells/well, Mean ± SEM n=13) and induced their differentiation into plasma cells (Figure 37B. 17.4 ± 1.9 x $10^3$ cells/well, Mean ± SEM n=13). In contrast, GC-Tfh1 cells failed to maintain the survival of GC-B cells (Figure 37A, 37B. 2.08 ± 0.5 x $10^3$ cells/well, Mean ± SEM n=13). Furthermore, GC-Tfh1 cells did not induce the differentiation of plasma cells (Figure 37B. 1.36 ± 0.32 x $10^3$ cells/well, Mean ± SEM n=13).

**B**

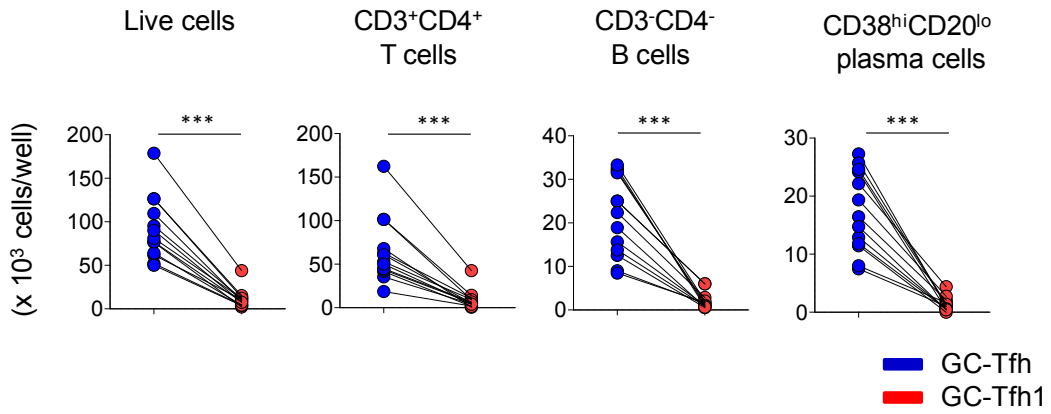


Figure 37. GC-Tfh1 cells are not efficient at helping GC-B cells. (B) The number of total live cells, T cells, B cells, plasma cells in the cultures was determined on day 7. N=11. Paired t test.

We also measured the secreted Igs during the 7 days of culture (Figure 37 C). We found that GC-Tfh1 cells were poor at inducing GC-B cells to produce Igs including IgG, IgA, and IgM (Figure. 37C. IgM 0.8 ± 0.4, IgG 1.6 ± 0.4, IgA 0.2 ± 0.1 μg/ml, Mean ± SEM, n=13), when compared to GC-Tfh cells (Figure. 37C. IgM 3.3 ± 0.5, IgG 6.3 ± 1.1, IgA 1.5 ± 0.3 μg/ml, Mean ± SEM, n=13). The recovery of viable GC-Tfh1 cells was much lower than GC-Tfh cells (Figure 37B. GC-Tfh1 9 ± 3 x $10^3$ cells/well, Mean ± SEM n=13, GC-Tfh 64.9 ± 10 x $10^3$ cells/well, Mean ± SEM n=3). Taken together, these data indicate that GC-Tfh1 cells were poor at maintaining the survival of GC-B cells and inducing their differentiation to plasma cells.

C

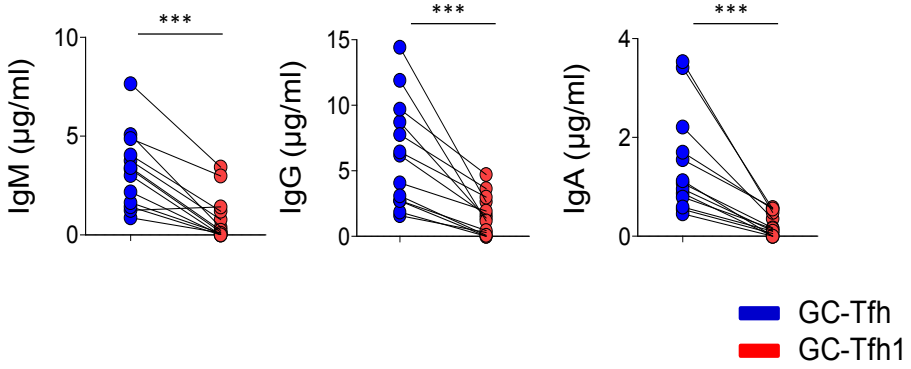


Figure 37. GC-Tfh1 cells are not efficient at helping GC-B cells. (C) Ig production by GC-B cells co-cultured with tonsillar Th populations in the presence of SEB for 7 d. N=11 Paired T test.

*GC-Tfh1 Cells are less Efficient than GC-Tfh Cells at Helping Memory B Cells*

As our previous study showed that activated blood Tfh1 cells were efficient at helping memory B cells (Bentebibel et al., 2013), we wanted to address whether GC-Tfh1 cells were able to help memory B cells ($IgD^-CD38^-CD19^+$). GC-Tfh or GC-Tfh1 populations ($2 \times 10^4$ /well) were cultured with each autologous memory B cell subset ($2 \times 10^4$ /well) in the presence of SEB, then the recovery of T cells ($CD3^+CD4^+$), B cells ($CD3^-CD4^-$), plasmablasts ($CD38^+CD138^-$), and plasma cells ($CD38^+CD138^+$) was analyzed by flow cytometry (Figure 38A). The recovery of T cells, B cells, plasmablasts, and plasma cells in the cultures was also calculated and secreted Igs were measured at day 6 of the culture (Figure 38 A-C). We found that both GC-Tfh1 and GC-Tfh cells were capable of maintaining the survival of memory B cells and inducing their differentiation to plasmablasts and plasma cells (Figure 38A).

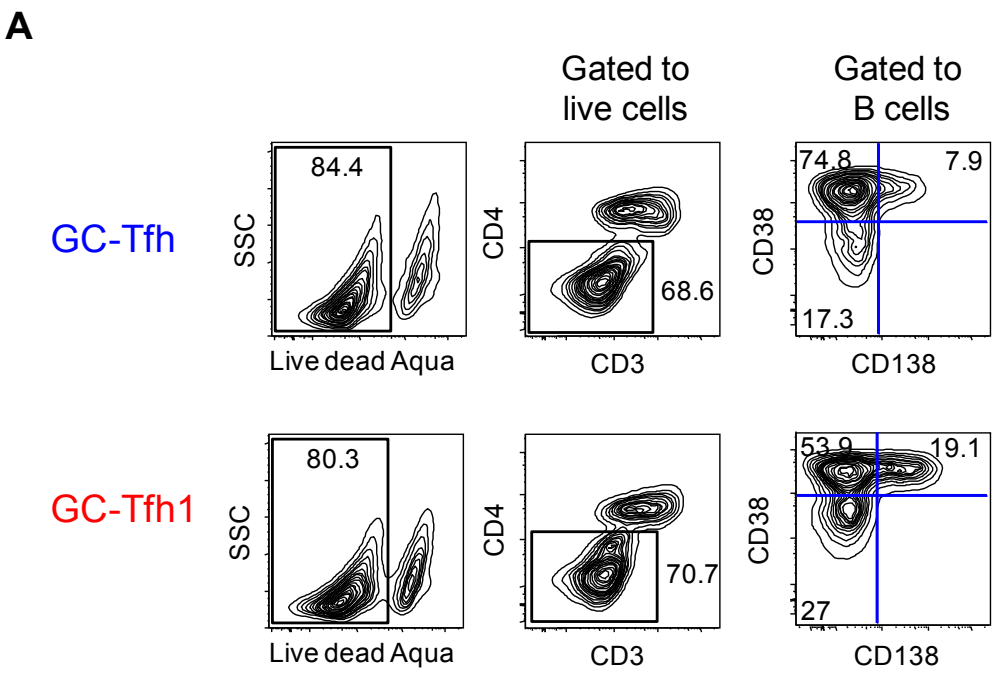


Figure 38: GC-Tfh1 cells are less efficient than GC-Tfh cells at helping memory B cells (A) Recovery of memory B cells co-cultured with GC-Tfh populations. Memory B cells cultured for 6 d with either Tfh or Tfh1 population were analyzed for the expression of CD38 and CD138. A representative from 7 experiments.

When we calculated the number of recovered cells in the culture at day 6, we found that fewer B cells and plasmablasts ($CD38^{+}CD138^{-}$) were recovered in the culture with GC- Tfh1 cells (Figure 38B. B cell recovery, GC-Tfh1 cells 42.3 ± 6.1 x $10^3$ cells/well, GC-Tfh cells 76.4 ± 8.7 x $10^3$ cells/well; plasmablast recovery, GC-Tfh1 cells 21.1 ± 3.2 x $10^3$ cells/well, GC-Tfh cells 53.1 ± 6.9 x $10^3$ cells/well, Mean ± SEM n=7). Furthermore, the recovery of GC-Tfh1 cells was less than GC-Tfh cells (Figure 38B, GC-Tfh1 cells 13.3 ± 1.5 x $10^3$ cells/well, GC-Tfh cells 35.4 ± 3.8 x $10^3$ cells/well, Mean ± SEM n=7). Interestingly, however, GC-Tfh1 cells yielded more plasma cells than GC-Tfh cells (Figure 38B, GC-Tfh1 cells 15.9 ± 1.9 x $10^3$ cells/well, GC-Tfh cells 10.3 ± 1.7 x $10^3$ cells/well, Mean ± SEM n=7).

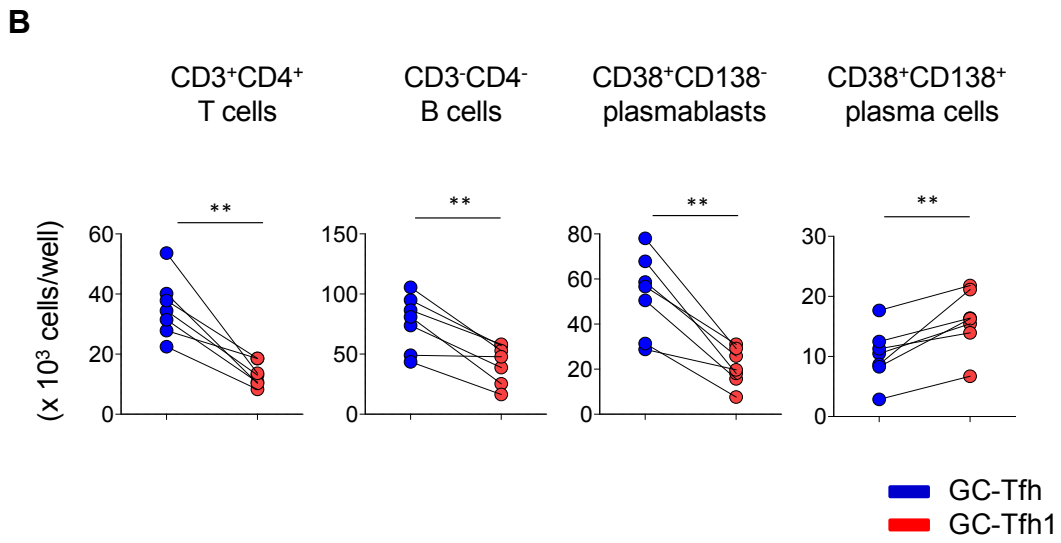


Figure 38. GC-Tfh1 cells are less efficient than GC-Tfh cells at helping memory B cells (B) The number of T cells, B cells, plasmablasts, and plasma cells in the cultures was determined on day 6. N=7. Paired T test.

Nonetheless, the production of IgG, IgA, and IgM by memory B cells was lower when cultured with GC-Tfh1 cells (Figure. 38C, GC-Tfh1 cells IgM 0.8 ± 0.4, IgG 1.6 ± 0.4, IgA 0.2 ± 0.1 μg/ml, GC-Tfh IgM 0.83 ± 0.2, IgG 7.9 ± 2.6, IgA 6.7 ± 1.7 μg/ml, Mean ± SEM, n=9). Thus, while less efficient than GC-Tfh cells at promoting the growth of memory B cells, GC-Tfh1 cells are able to induce memory B cells to differentiate into plasma cells.

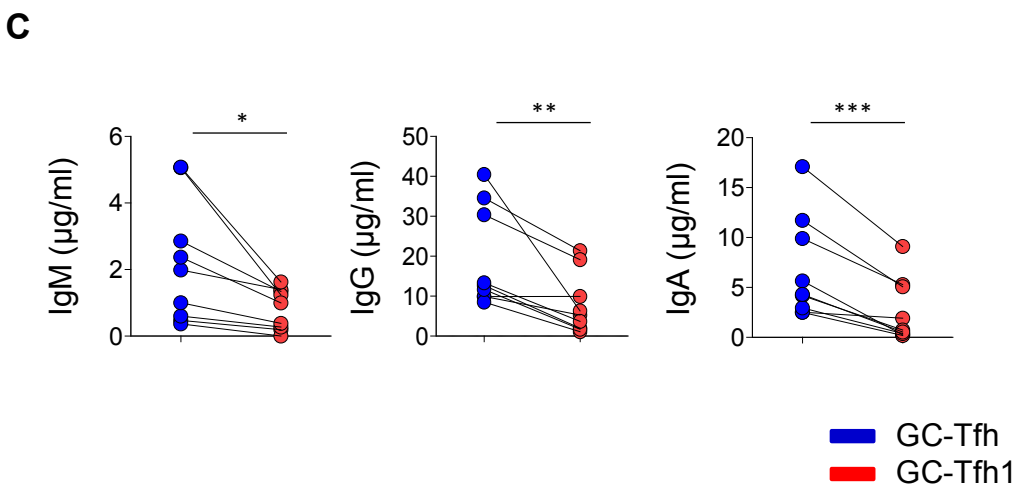


Figure 38. GC-Tfh1 cells are less efficient than GC-Tfh cells at helping memory B cells (C) Ig production by memory B cells co-cultured with tonsillar Th populations in the presence of SEB for 6 d. N=11 Paired T test.

### *IL-21 Supplementation is not Sufficient to Transform GC-Tfh1 Cells into Helpers of GC-B Cells*

We wondered whether the inability of GC-Tfh1 cells to help GC-B cells was due to their insufficient secretion of IL-21 (Figure 39). Thus, we examined whether supplementation of IL-21 into the co-cultures results in the secretion of higher amounts of Igs. As shown in Figure 39, IL-21 supplementation did not increase IgM, IgG, and IgA secretion. Thus, the inability of GC-Tfh1 cells to help GC-B cells is independent from their poor capacity to produce IL-21.

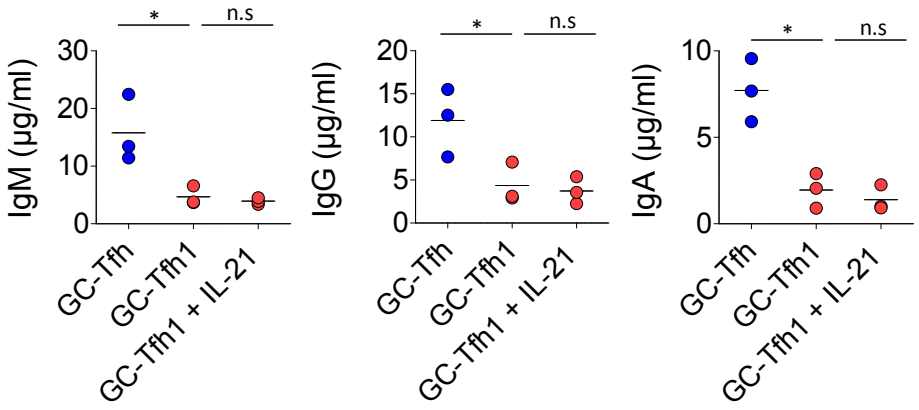


Figure 39: IL-21 supplementation is not sufficient to induce GC-Tfh1 cells to help GC-B cells. Ig secretion by GC-B cells co-cultured with GC-Tfh, GC-Tfh1, or GC-Tfh1 cells supplemented with recombinant IL-21 (20 ng/ml). Ig secretion measured at day 7. N=3

*IFN-γ Inhibits the Survival and/or Growth of GC-B Cells*

GC-Tfh1 cells secrete larger amount of IFN-γ than GC-Tfh cells (Figure 36). We wondered whether the inability of GC-Tfh1 cells to help GC-B cells was related to IFN-γ secretion. To address this question, we first cultured GC-Tfh cells (2 x $10^4$ /well) with GC-B cells (2 x $10^4$ /well) in the presence of SEB for 7 days, and recombinant human IFN-γ (R&D systems) was added to the co-culture. Then the frequency of T cells ($CD3^+CD4^+$), B cells ($CD3^-CD4^-$), and plasmablasts ($CD38^+CD20^{lo}$) in the cultures was analyzed by flow cytometry and the recovery of T cells, B cells, and plasmablasts in the co-cultures was calculated (Figure 40A). Igs secretions were also measured at day 7 of the culture (Figure 40B). The addition of IFN-γ resulted in a decrease of recovery of total viable cells (none 116.2 ± 11.4 x $10^3$ cells/well, + IFN-γ 66.1 ± 12.1 x $10^3$ cells/well, Mean ± SEM n=4), and T cells (none 78.37 ± 13.5 x $10^3$ cells/well, + IFN-γ 55.32 ± 12.1 x $10^3$ cells/well, Mean ± SEM n=4). The addition of IFN-γ resulted also in >70% decrease of GC-B cell recovery (none 35.63 ± 3.2 x $10^3$ cells/well, + IFN-γ 9.57 ± 0.3 x $10^3$ cells/well, Mean ± SEM n=4) as well as their differentiation to plasma cells (none

25.29 ± 2.3 x $10^3$ cells/well, + IFN-γ 6.21 ± 0.2 x $10^3$ cells/well, Mean ± SEM n=4) (Figure 40A).

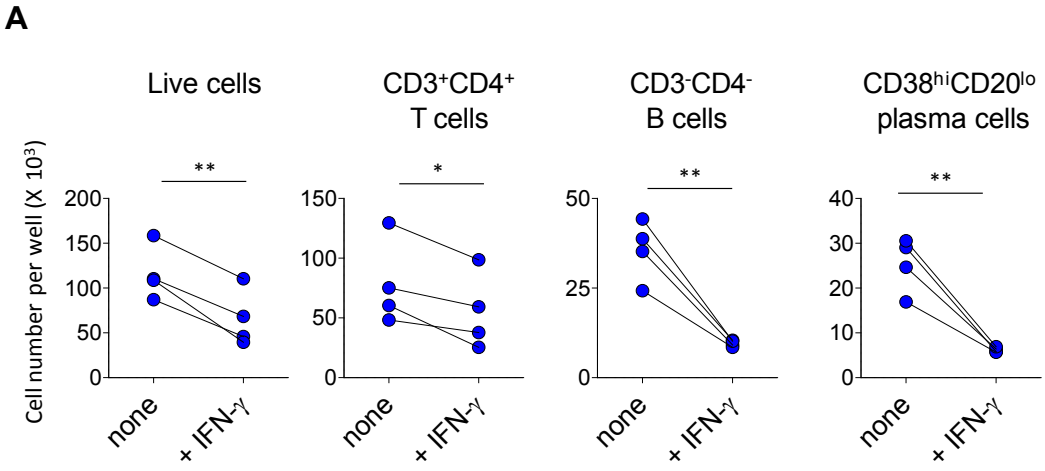


Figure 40: IFN-γ inhibits the proliferation of GC-B cells and memory B cells (A) inhibition of GC-B cells proliferation, the number of T cells, B cells and plasma cells in the cultures of GC-Tfh and GC-B cells was determined on day 7. N=4. Paired T test.

Furthermore, the addition of IFN-γ decreased the secretion of IgM, IgG, and IgA (Figure. 40B. IgM 0.47 ± 0.1, IgG 0.96 ± 0.4, IgA 0.46 ± 0.1 γ µg/ml vs. IgM 2.7 ± 0.2, IgG 3.3 ± 0.4, IgA 1.57 ± 0.2 µg/ml, Mean ± SEM, n=4). Taken together, these data show that IFN-γ inhibits the survival of GC-B cells and their differentiation into plasma cells.

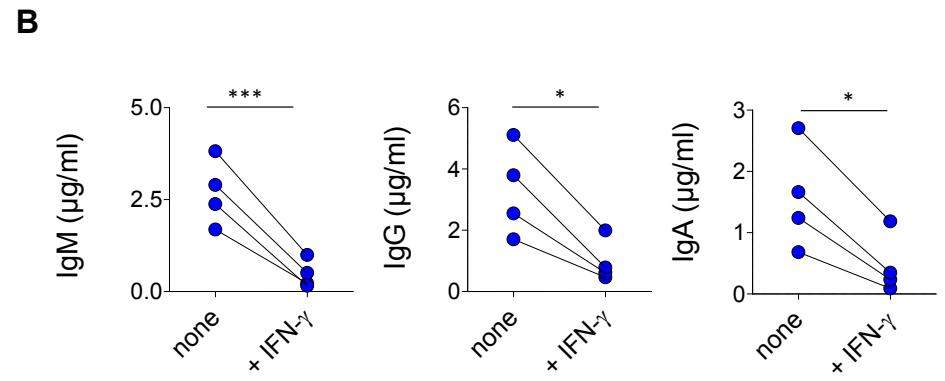


Figure 40. IFN-γ inhibits the proliferation of GC-B cells and memory B cells. (B) Inhibition of Ig production by GC-B cells co-cultured with GC-Tfh cells in the presence of SEB for 7 d. N=4 Paired T test.

*IFN-γ Inhibits the Survival and/or Growth of Memory B Cells*

We also examined the effect of IFN-γ on the proliferation and differentiation of memory B cells. An addition of IFN-γ decreased T cell recovery cultured with memory B cells (none 72.45 ± 25.2 x $10^3$ cells/well, + IFN-γ 30.02 ± 13.5 x $10^3$ cells/well, Mean ± SEM n=4), as well as B cell recovery (none 57.37 ± 11.5 x $10^3$ cells/well, + IFN-γ 18.03 ± 4.8 x $10^3$ cells/well, Mean ± SEM n=4) and development of plasmablasts (none 33.82 ± 2.3 x $10^3$ cells/well, + IFN-γ 11.69 ± 3.6 x $10^3$ cells/well, Mean ± SEM n=4) (Figure 40C).

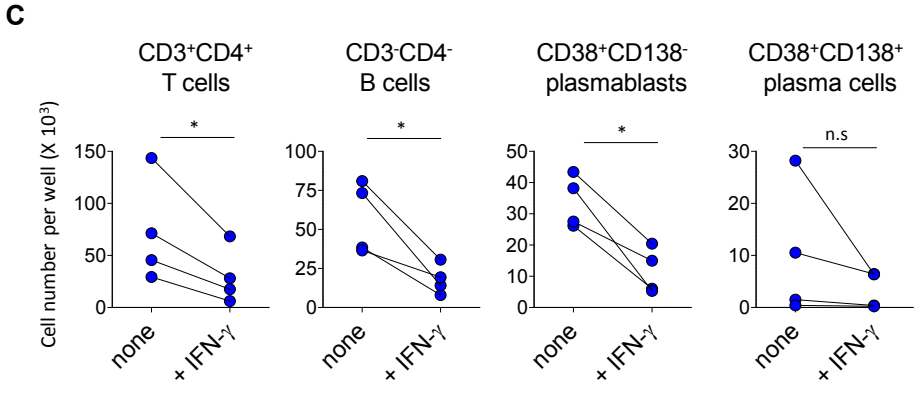


Figure 40. IFN-γ inhibits the proliferation of GC-B cells and memory B cells (C) Inhibition of memory B cells proliferation, the number of T cells, B cells, plasmablasts, and plasma cells in the cultures of GC-Tfh cells and memory B cells was determined on day 6. N=4. Paired T test.

An addition of IFN-γ also decreased the production of IgM, IgG, and IgA by memory B cells (Figure 40D. IgM 0.11 ± 0.1, IgG 13.88 ± 2.9, IgA 3.05 ± 0.6 μg/ml vs. IgM 1.22 ± 0.3, IgG 37.32 ± 5.3, IgA 8.12 ± 0.8 μg/ml, Mean ± SEM, n=4).

**D**

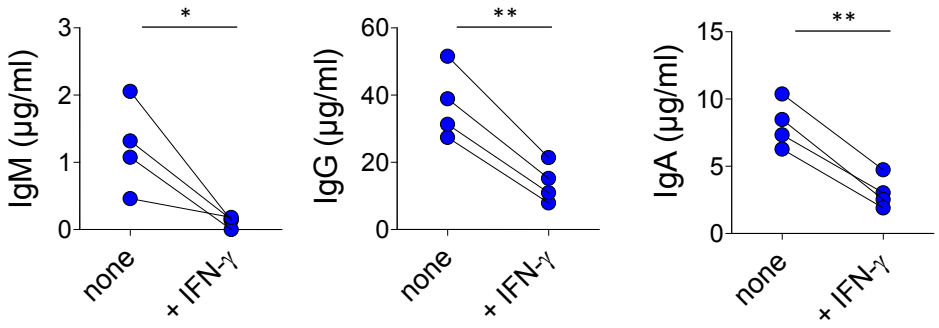


Figure 40. IFN-γ inhibits the proliferation of GC-B cells and memory B cells (D) Inhibition of Ig production by memory B cells co-cultured with GC-Tfh cells in the presence of SEB for 7 d. N=4 Paired T test.

In opposite, blocking IFN-γ during the co-cultures of GC-Tfh1 and memory B cells increased the recovery of T cells (none 22.56 ± 3.6 x $10^3$ cells/well, + anti-IFN-γ 38.49 ± 7.7 x $10^3$ cells/well, Mean ± SEM n=4), the recovery of memory B cells (none 18.03 ± 4.8 x $10^3$ cells/well, + anti-IFN-γ 48.05 ± 8.3 x $10^3$ cells/well, Mean ± SEM n=4), and the generation of plasmablasts (none 14.5 ± 3.7 x $10^3$ cells/well, + anti-IFN-γ 35.25 ± 6.9 x $10^3$ cells/well, Mean ± SEM n=4) and plasma cells (none 4.33 ± 1.2 x $10^3$ cells/well, + anti-IFN-γ 9.5 ± 0.5 x $10^3$ cells/well, Mean ± SEM n=4) (Figure 40E).

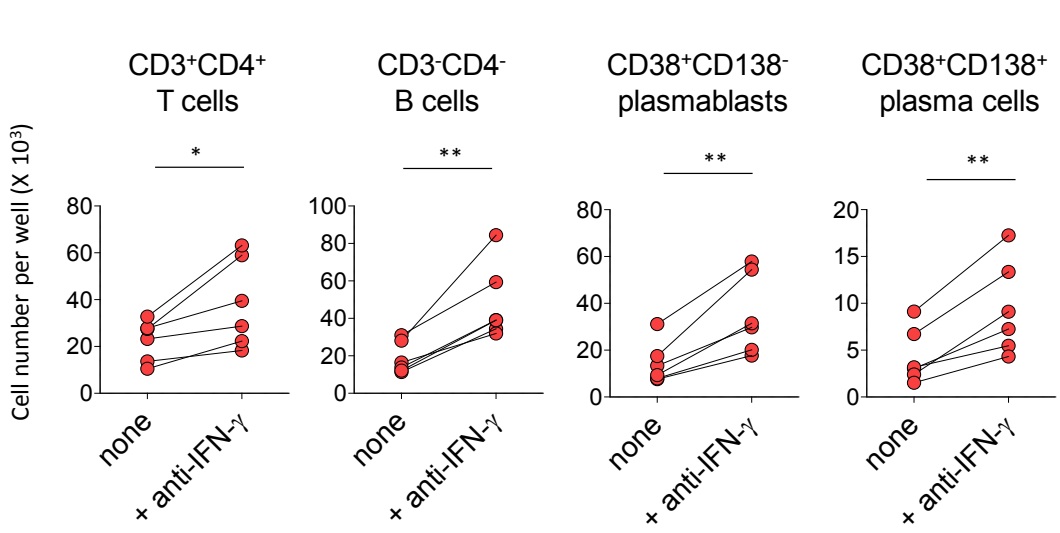


Figure 40. IFN-γ inhibits the proliferation of GC-B cells and memory B cells (E) IFN-γ blocking increases the proliferation of memory B cells, the number of T cells, B cells, plasmablasts, and plasma cells in the cultures was determined on day 6. N=6. Paired T test.

The addition of anti-IFN-γ into the co-cultures also increased the secretion of IgG (Figure 40F. 11.01 ± 2.1 μg/ml vs. 5.25 ± 1.9 μg/ml, Mean ± SEM, n=4). The secretion of IgM and IgA was not affected by anti-IFN-γ.

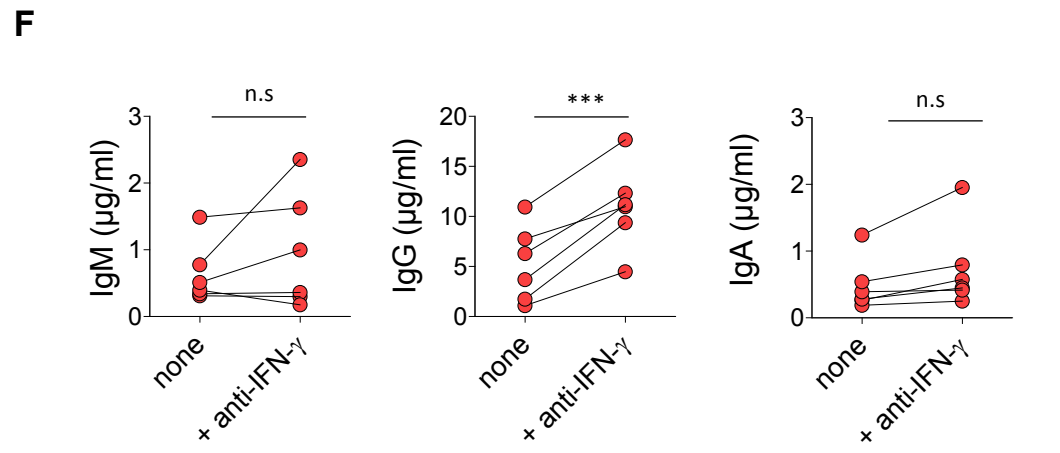


Figure 40. IFN-γ inhibits the proliferation of GC-B cells and memory B cells (F) IFN-γ blocking increases IgG production from memory B cells, Ig production by GC-B cells co-cultured with GC-Tfh1 cells in the presence of SEB for 6 d. N=6 Paired T test.

Collectively, these results show that while GC-Tfh1 cells have a capacity to help memory B cells to differentiate into antibody-producing cells, IFN-γ secreted by GC-Tfh1 cells is detrimental for their helper function.

*IFN-γ Blocking is not Sufficient to Transform GC-Tfh1 Cells into Helpers of GC-B Cells*

We next analysed whether blocking IFN-γ can transform GC-Tfh1 cells into helpers of GC-B cells. A supplementation of anti-IFN-γ in the cultures of GC-Tfh1 cells with GC-B cells moderately increased the recovery of GC-B cells (none 0.89 ± 0.1 x $10^3$ cells/well, + anti-IFN-γ 2.78 ± 0.5 x $10^3$ cells/well, Mean ± SEM n=3) and the generation of plasma cells (none 0.64 ± 0.1 x $10^3$ cells/well, + IFN-γ 1.95 ± 0.3 x $10^3$ cells/well, Mean ± SEM n=3) (Figure 40G). But neither reached a statistical significance in a paired t-test. Furthermore, we did not observe any increase of Ig secretion by IFN-γ blocking (Figure 40H). Thus, IFN-γ secreted by GC-Tfh1 cells might have a detrimental effect on GC-B cells, these observations suggest an involvement of other factors derived from GC-Tfh1 cells for their inability to help GC-B cells.

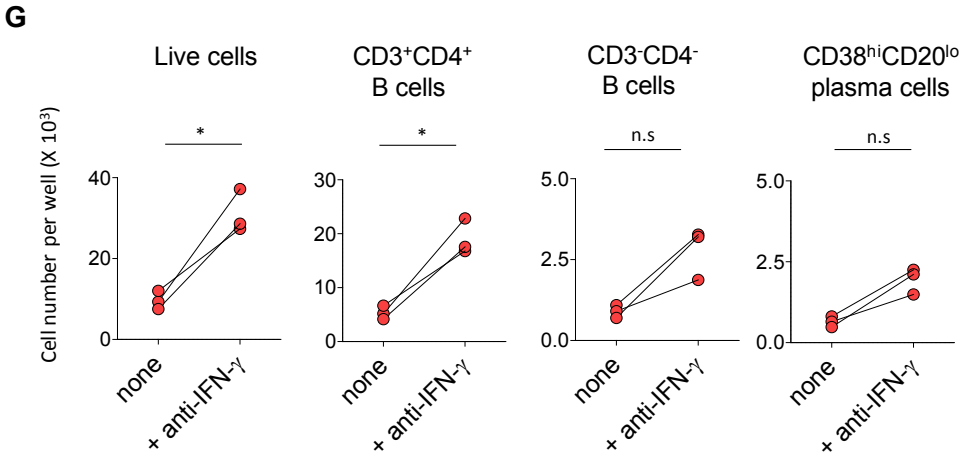


Figure 40. IFN-γ inhibits the proliferation of GC-B cells and memory B cells (G) IFN-γ blocking does not affect the proliferation of GC-B cells, the number of live cells, T cells, B cells, and plasma cells in the cultures was determined on day 7. N=3. Paired T test.

**H**

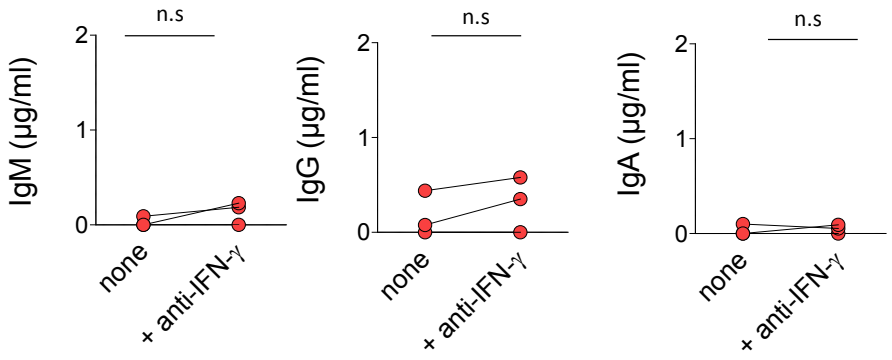


Figure 40. IFN-γ inhibits the proliferation of GC-B cells and memory B cells (H) IFN-γ blocking does not affect Igs production from GC-B cells, Ig production by GC-B cells co-cultured with Tfh cells in the presence of SEB for 6 d (n=3). Paired T test.

### *FAS-L$^+$CD4$^+$ T Cells in GCs*

FAS is highly expressed in GC-B cells and has an essential role in GC-B cell apoptosis both *in vitro* (Hennino et al., 2001) and *in vivo* (Takahashi et al., 2001). FAS expressing autoreactive B cells are eliminated upon interaction with FAS-L$^+$ CD4$^+$ T cells (Rathmell et al., 1995). Furthermore, ablation of FAS specifically in GC-B cells in mice leads to the development of fatal lymphoproliferation due to activation of B cells and T cells (Hao et al., 2008). Thus, FAS expressed by GC-B cells play a fundamental regulatory role in the elimination of autoreactive B cells as well as in the maintenance of immune homeostasis. However, the identity of FAS-L$^+$ T cells in GCs remains largely unclear. In our previous study on human tonsillar Tfh subsets, we failed to detect FAS-L$^+$ cells in GC-Tfh cell population (Bentebibel et al., 2011). Therefore, we first examined whether FAS-L$^+$ T cells can be detected by immuno-fluorescent microscopy. We confirmed the presence of FAS-L$^+$ CD4$^+$ T cells in GCs in human tonsils (Figure 41). The FAS-L expression was not equally distributed on the T cell surface, but profoundly polarized. This suggests that FAS-L is accumulated into the immunological synapse with

GC-B cells. Of note, FAS-L expression was limited to $CD4^+$ cells in GCs, suggesting the absence of FAS-$L^+$ $CD8^+$ T cells in GCs.

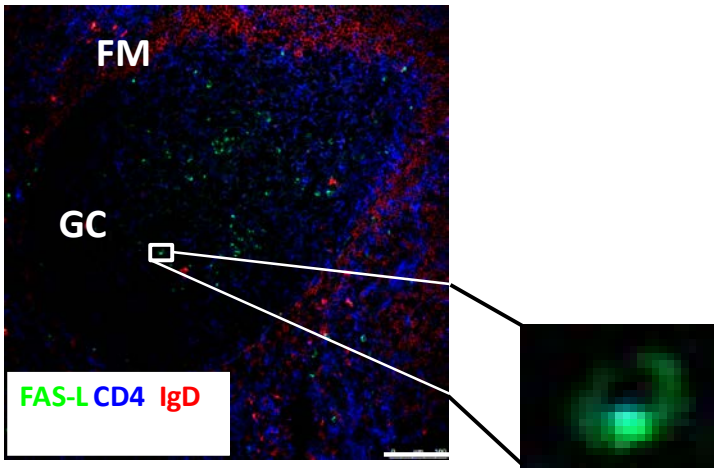


Figure 41: FAS-$L^+CD4^+$ T cells can be found in GCs. Localization of FAS-$L^+CD4^+$ T cells in tonsils was analyzed by immunohistochemistry using a frozen tonsil section. FM: follicular mantle. Localization was analyzed by the co-expression of CD4 (blue), FAS-L (green), and IgD (red) by immunofluorescence microscopy.

*A Fraction of GC-Tfh1 Cells Express Surface FAS-L*

We next examined which GC-Tfh cells express FAS-L by flow cytometry. As shown in Figure 42, we found that surface FAS-L expression was largely limited to GC-Tfh1 cells (21.2 ± 2.5 % of GC-Tfh1 cells vs. 3.1 ± 0.4 % of GC-Tfh cells. Mean ± SEM, n=9).

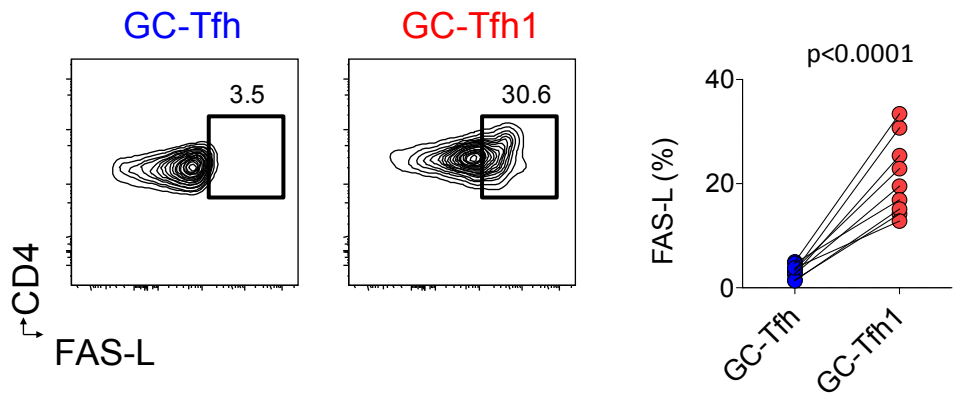


Figure 42: A fraction of GC-Tfh1 cells express surface FAS-L. FAS-L expression by GC-Tfh1 cells (n=9). A representative flow data result is shown on the left panel. Paired T test.

*FAS Blocking in GC-Tfh1/GC-B Cell Culture Promotes B Cells to Proliferate and Secrete IgG*

FAS-L is expressed by T cells after receiving activation signals through T cell receptor (TCR). To examine whether FAS-L expression by GC-Tfh1 cells is simply due to the active TCR signaling at sites, we cultured GC-Tfh cells and GC-Tfh1 cells with GC-B stimulated with SEB and analysed FAS-L expression at day 5. As shown in Figure 43A, while GC-Tfh1 cells maintained FAS-L expression after culture with GC-B cells, GC- Tfh cells did not upregulate FAS-L expression. Furthermore, analysis of Ki67 expression showed that approximately 20% of GC-Tfh and GC-Tfh1 cells expressed Ki67, indicating a majority of GC-Tfh and GC-Tfh1 cells were not in cell cycle (Figure 43B). These results suggest that FAS-L expression by GC-Tfh1 cells does not simply reflect the cell activation status, but rather is a characteristic of GC-Tfh1 cells.

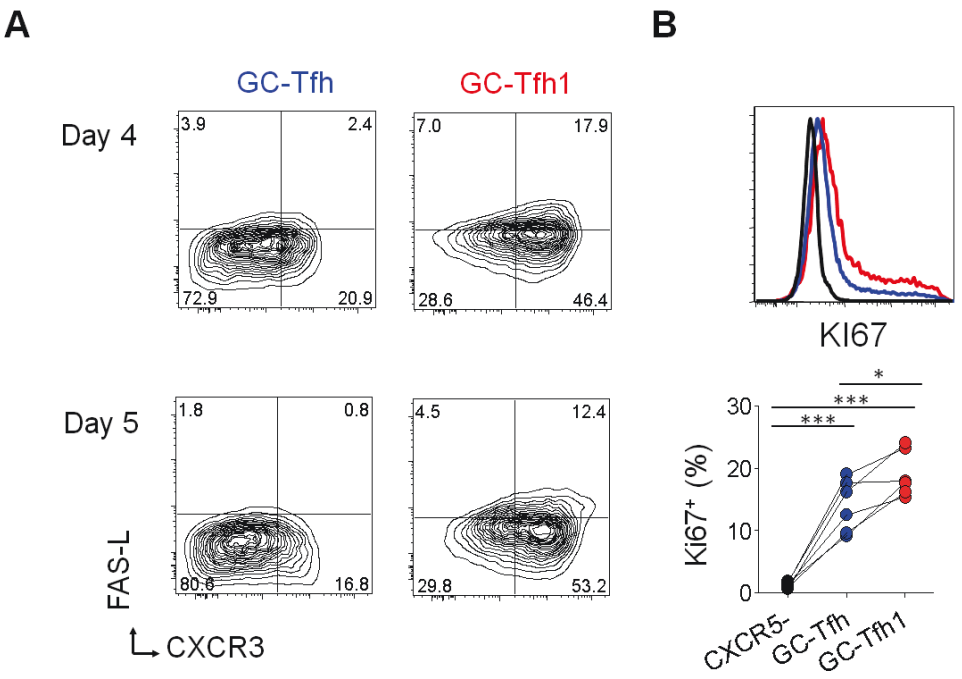


Figure 43: GC-Tfh cells do not express FAS-L even after activation (A) GC-Tfh and GC-Tfh1 cells were cultured with GC-B cells for 4 or 5 days in the presence of SEB and surface FAS-L expression on T cells was analyzed by flow cytometry. A representative from two experiments (B) Ki67 expression by $CD4^{+}CXCR5^{-}$ T cells, GC-Tfh and GC-Tfh1 cells analyzed by flow cytometry (n=6). One-way ANOVA Tukey multiple comparison tests. A representative flow data result is shown on the top panel.

Considering that co-cultures of GC-B cells and GC-Tfh1 cells resulted in few remaining viable GC-B cells (Figure 44A), we hypothesized that GC-Tfh1 cells might induce the apoptosis of GC-B cells via FAS/FAS-L interactions. Upon blocking the FAS/FAS-L interaction with a neutralizing anti-FAS antibody, GC-Tfh1 cells significantly improved the survival of GC-B cells (none 2.2 ± 0.5 % Mean ± SEM, n=6, + anti-FAS mAb 17.5 ± 3.5 % Mean ± SEM, n=6) and their differentiation into plasma cells (none 2.2 ± 0.5 % Mean ± SEM, n=6, + anti-FAS mAb 15.4 ± 3.3 % Mean ± SEM, n=6) (Figure 44A).

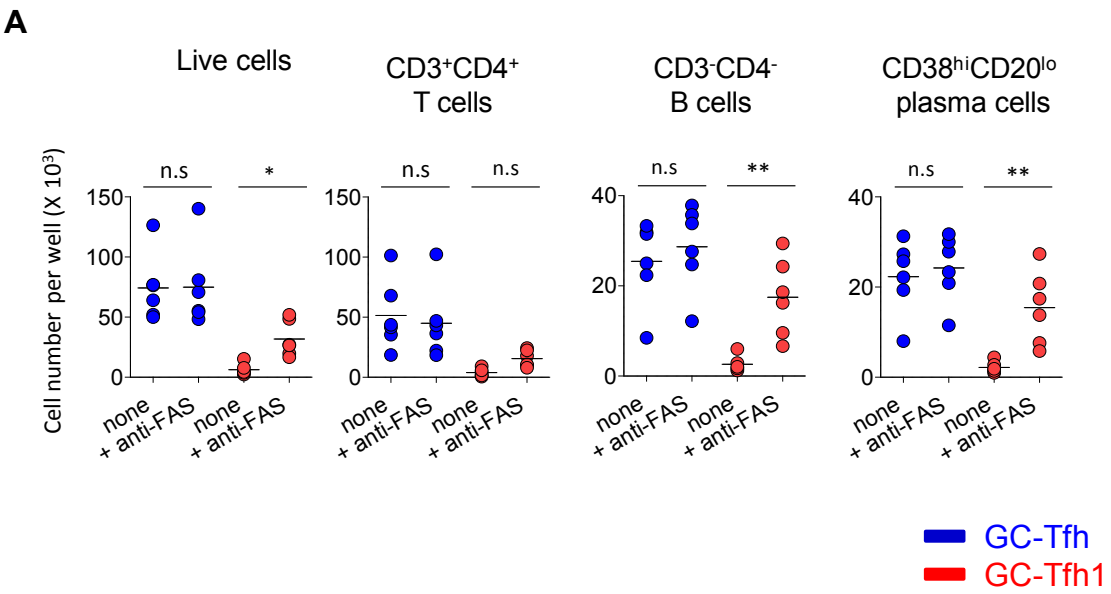


Figure 44: FAS blocking in GC-Tfh1/GC-B cell culture promotes B cells to proliferate and secrete IgG (A) Absolute number per well of live cells, T cells, B cells, and plasma cells in the co-culture of GC-B cells with either GC-Tfh or GC-Tfh1 cells in the absence or presence of FAS mAb. One-way ANOVA Tukey multiple comparison tests.

Furthermore, blocking the FAS/FAS-L interaction resulted also in an increase in the amount of IgG produced by GC-B cells cultured with GC-Tfh1 cells (none 2.9 ± 0.5 µg/ml, Mean ± SEM, n=6, + anti-FAS mAb 6.9 ± 1.7 µg/ml, Mean ± SEM, n=6) (Figure 44B). However, the production of IgM (none 1.6 ± 0.6 µg/ml, Mean ± SEM, n=6, + anti-FAS mAb 2.1 ± 0.7 µg/ml, Mean ± SEM, n=6) and IgA (none 0.4 ± 0.1 µg/ml, Mean ± SEM, n=6, + anti-FAS mAb 0.3 ± 0.1 µg/ml, Mean ± SEM, n=6) remained unchanged (Figure 44B). These results show that GC-Tfh1 cells actively limit the growth of GC-B cells by inducing apoptosis via FAS/FAS-L interactions.

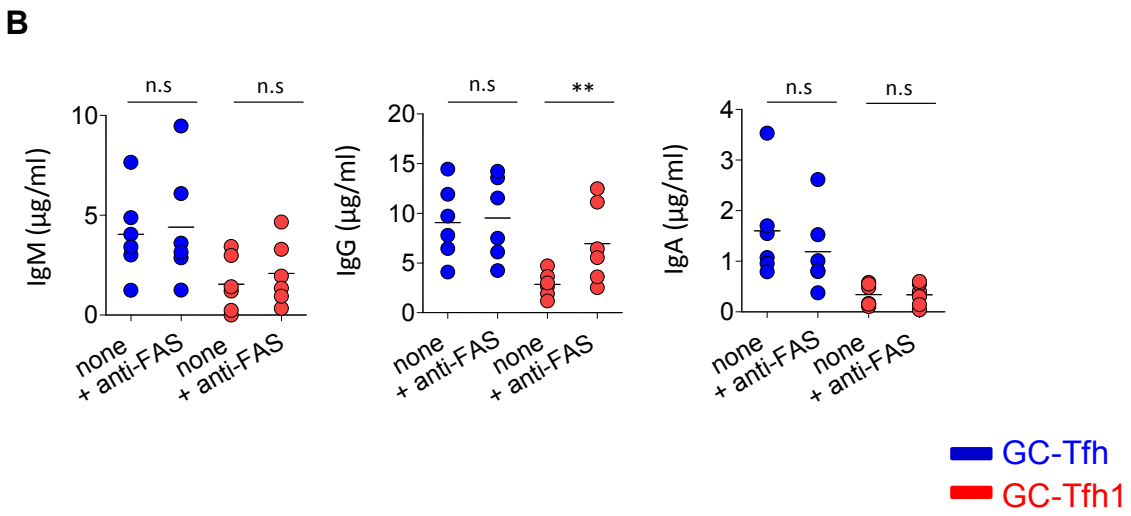


Figure 44. FAS blocking in GC-Tfh1/GC-B cells culture promotes B cells to proliferate and secrete IgG (B) Ig secretion by GC-B cells co-cultured with either GC-Tfh or GC-Tfh1 in the absence or presence of FAS mAb. Ig concentrations were measured at day 7. A representative from six experiments

### *GC-Tfh1 Cells Express Low Level of CD40L*

CD40L is a crucial factor that binds to CD40 on the surface of B cells and induces the activation, proliferation, survival, and differentiation of B cells. Here we examined the expression of CD40L on naïve $CD4^+$ T cells, GC-Tfh and GC-Tfh1 cells. First, the expression of CD40L was determined at a transcriptional level by NanoString. We found that GC-Tfh1 cells expressed less *CD40L* transcript than GC-Tfh cells and the level of expression was equivalent to naïve $CD4^+$ T cells (Figure 45A). While neither GC-Tfh1 or GC-Tfh cells expressed CD40L upon isolation from tonsils (not shown), CD40L was expressed by a higher frequency of GC-Tfh cells than GC-Tfh1 cells 24 h after culture with SEB-pulsed GC-B cells (22.4 ± 6.1 % vs. 12.2 ± 3.1 %, Mean ± SEM, n=3) (Figure 45B). Furthermore, the culture of GC-Tfh1 cells with GC-B cells resulted in lower number of recovered $CD4^+CD40L^+$ cells (2.01 ± 0.8 %, Mean ± SEM n=3) compared to the number of $CD4^+CD40L^+$ cells in the culture of GC-Tfh cells with GC-B cells (10.7 ± 1.9 % Mean ±

SEM n=3) (Figure 45C, right panel). These results show that GC-Tfh1 cells displayed a reduced capacity to express CD40L than GC-Tfh cells.

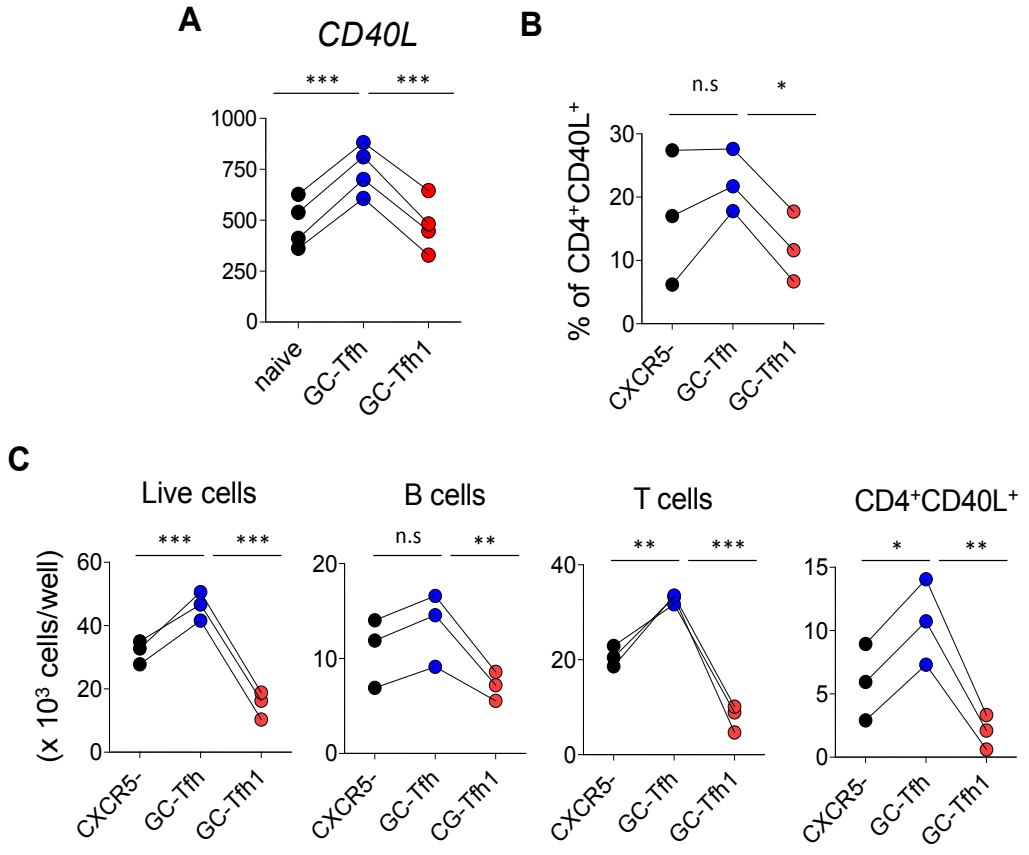


Figure 45: GC-Tfh1 cells express low level of CD40L (A) *CD40L* expression by tonsillar $CD4^+$ Th subsets. Naïve $CD4^+$ T cells ($CD45RA^+CXCR5^-ICOS^-$), GC-Tfh and GC-Tfh1cells were analyzed for the expression of *CD40L* by Nanostring. Data were normalized with at least 7 housekeeping genes included in the code set, n=4 (B) The expression of CD40L (after 24 hs of culture with GC-B cells) by naive $CD4^+$ T cells, GC-Tfh and GC-Tfh1 cells, n=3 (C) Absolute number of live cells, T cells, B cells, and $CD4^+CD40L^+$ cells in the co-culture of GC-B cells with either $CXCR5^-$, GC-Tfh or GC-Tfh1 cells.

### *CD40L Supplementation Combined With Blocking IFN-γ and FAS is Sufficient to Induce GC-Tfh1 Cells to Help GC-B Cells*

Next, we wondered whether the poor capacity of GC-Tfh1 cells to help GC-B cells was due to their lower expression of CD40L. Supplementation of CD40L in the culture of GC-Tfh1 cells with GC-B cells did not result in an increase of the receovry of live cells (none 4.1 ± 1.9 % Mean ± SEM, n=3, + CD40L 11.5 ± 2.7 % Mean ± SEM,

n=3), T cells (none 3.1 ± 1.3 % % Mean ± SEM, n=3, + CD40L 8.6 ± 2.6 % Mean ± SEM, n=3), GC-B cells (none 0.9 ± 0.6 % Mean ± SEM, n=3, + CD40L 2.5 ± 1.2 % Mean ± SEM, n=3) or their differentiation to plasma cells (none 0.7 ± 0.6 % Mean ± SEM, n=3, + CD40L 2.1 ± 1.1 % Mean ± SEM, n=3)(Figure 46A). In contrast, blocking IFN-γ and FAS in the culture of GC-Tfh1 cells and GC-B cells improved the recovery of live cells, proliferation of GC-Tfh1 cells, GC-B cells, and their differentiation to plasma cells (Figure 46B). Notably, supplementation of CD40L to the culture of GC-Tfh1 cells with GC-B cells combined with blocking IFN-γ and FAS resulted in the highest recovery of live cells (anti-FAS + anti-IFN-γ 30.4 ± 10.4 % Mean ± SEM, n=3, anti-FAS + anti-IFN-γ + CD40L 47.4 ± 11.2 % Mean ± SEM, n=3), T cells (anti-FAS + anti-IFN-γ 22.2 ± 9.1 % % Mean ± SEM, n=3, anti-FAS + anti-IFN-γ + CD40L 33.2 ± 8.9 % Mean ± SEM, n=3), GC-B cells (anti-FAS + anti-IFN-γ 8.2 ± 2.3 % Mean ± SEM, n=3, anti-FAS + anti-IFN-γ + CD40L 14.1 ± 4.4 % Mean ± SEM, n=3), and their differentiation to plasma cells (anti-FAS + anti-IFN-γ 7.3 ± 2.8 % Mean ± SEM, n=3, anti-FAS + anti-IFN-γ + CD40L 12.2 ± 4.9 % Mean ± SEM, n=3).

Collectively, our study indicates that the poor capacity of GC-Tfh1 cells to help GC-B cells is associated with multiple factors characteristics to GC-Tfh1 cells. A low expression of surface CD40L is associated with their poor capacity to maintain the survival and proliferation of GC-B cells. IFN-γ secreted by GC-Tfh1 cells reduces the viability of GC-B cells. FAS-L expressed by GC-Tfh1 cells induces active cell death of GC-B cells by delivering signals through FAS.

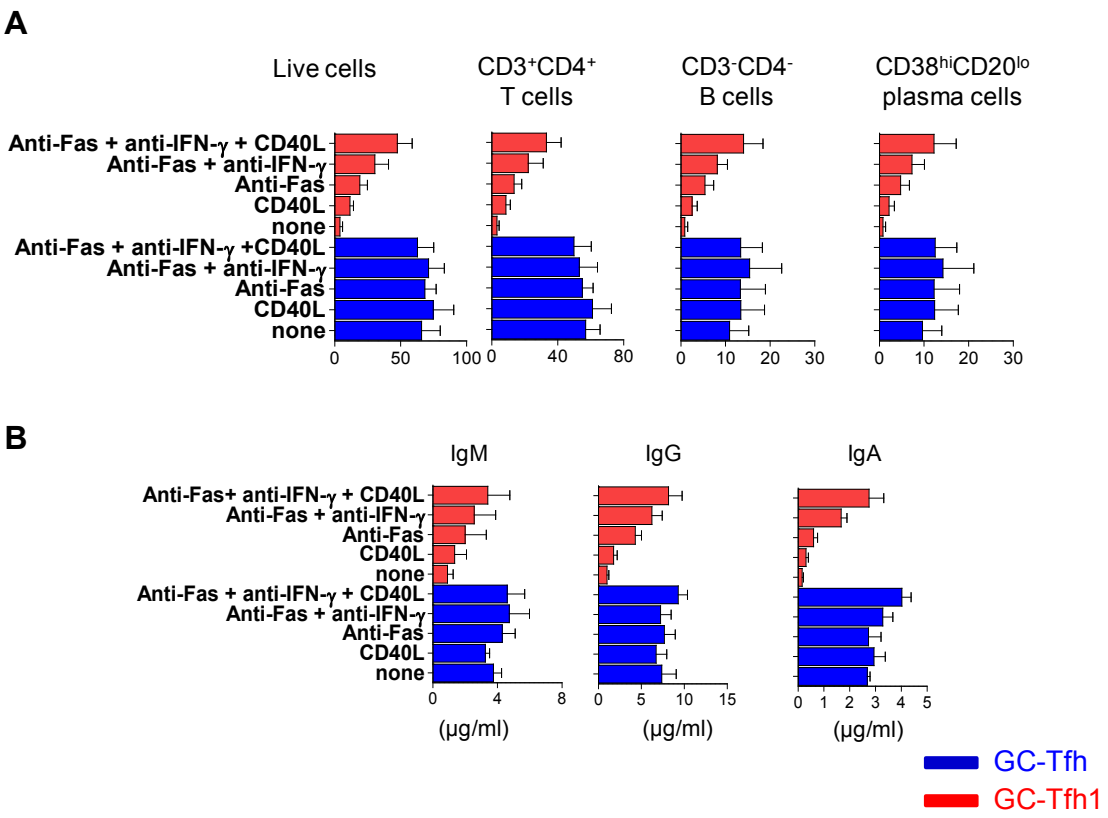


Figure 46: CD40L supplementation combined with blocking IFN-γ and FAS pathways is sufficient to induce GC-Tfh1 cells to help GC-B cells (A) Absolute number of live cells, T cells, B cells, and plasma cells in the co-culture of GC-B cells with either GC-Tfh or GC-Tfh1 cells in the absence or presence of recombinant CD40L combined with blocking IFN-γ and FAS. (B) Igs secretion in the co-culture of GC-B cells with either GC-Tfh or GC-Tfh1 cells in the absence or presence of recombinant CD40L combined with blocking IFN-γ and FAS (n=3)

### *GC-Tfh1 Cells Have Suppressive Function*

Our study indicates that GC-Tfh1 cells are equipped with factors that can actively inhibit GC-B cell growth and differentiation. Thus, it is possible that the function of GC-Tfh1 cells might dominate the function of GC-Tfh cells, and act as a regulatory component among GC-Tfh cells. To address this hypothesis, we first examined whether GC-Tfh1 cells can inhibit the function of GC-Tfh cells to help GC-B cells. We measured Igs secretion in the co-cultures of GC-Tfh cells (20 x $10^3$ cells/well), GC-B cells (20 x $10^3$ cells/well) and graded numbers of GC-Tfh1 at day 7. As shown in Figure 47, addition

of GC-Tfh1 cells into the co-culture of GC-Tfh cells and GC-B cells inhibited the secretion of Igs in a cell number-dependent manner. These results show that GC-Tfh1 cells suppress the helper function of GC-Tfh cells.

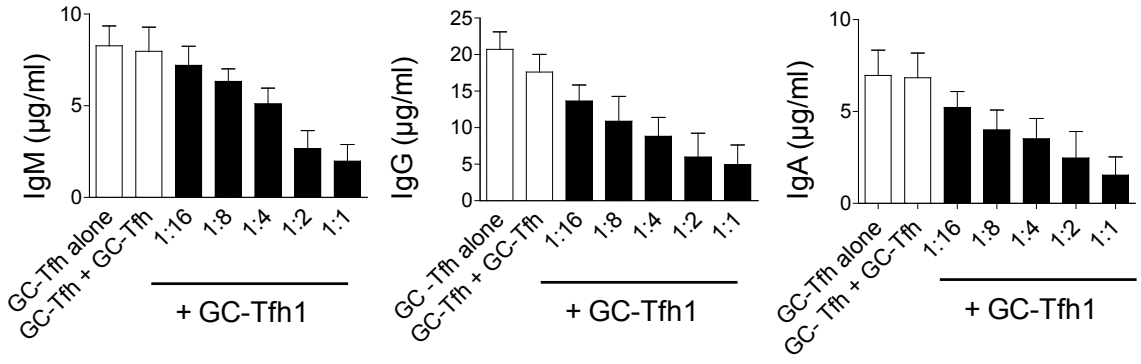


Figure 47: GC-Tfh1cells inhibit the secretion of Igs from GC-B cells cultured with GC-Tfh cells. Igs secretion were measured in the co-cultures of GC-Tfh cells (20 x $10^3$ cells/well) and GC-B cells (20 x $10^3$ cells/well) graded numbers of GC-Tfh1 as indicated (for 7 days), and produced Igs were analyzed. N=2

*GC-Tfh1 Cells Inhibit the Proliferation of GC-Tfh Cells*

The suppression of Ig secretion by GC-Tfh1 cells might be due to their direct effect on GC-B cells, and/or GC-Tfh cells. As GC-B cells, GC-Tfh cells express high levels of FAS on cell surface (Figure 35). Therefore, we next investigated whether GC-Tfh1 cells can directly suppress the function of GC-Tfh cells. GC-Tfh1 cells were co-cultured with autologous GC-Tfh cells labeled with CFSE and activated with anti-CD3/CD28 beads. We found that GC-Tfh1 cells suppressed the proliferation of GC-Tfh cells (Figure 48A). This was not due to larger cell numbers in culture, as GC-Tfh cells in the same cell number proliferated well in culture. GC-Tfh1 efficiently suppressed the proliferation of GC-Tfh cells in a dose dependent manner (Figure 48B). Thus, GC-Tfh1 cells directly suppress the proliferation of GC-Tfh cells.

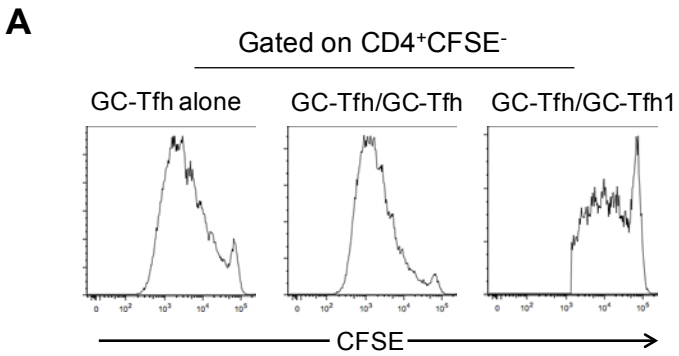


Figure 48: GC-Tfh1 cells inhibit the proliferation of GC-Tfh cells (A) GC-Tfh cell proliferation monitored using CFSE labeling. CFSE labeled GC-Tfh ($2\times10^4$/well) were cultured in the presence of autologous GC-Tfh1 or GC-Tfh cells ($2\times10^4$/well) and were stimulated with CD3/CD28 beads for 5 days, cells were harvested, stained for CD4 expression and analyzed by flow cytometry. CFSE histograms from one representative experiment is shown (n=4).

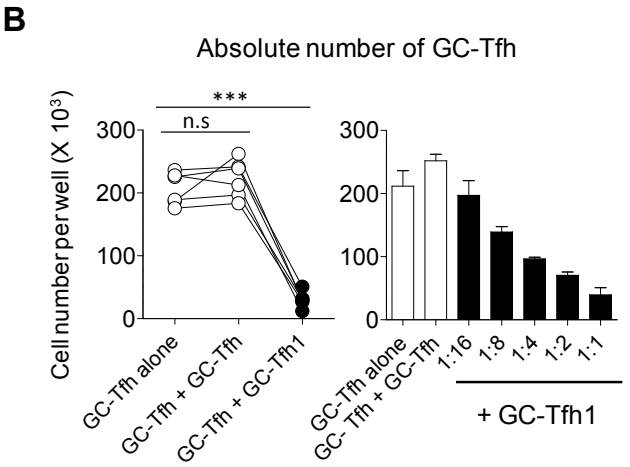


Figure 48. GC-Tfh1 cells inhibit the proliferation of GC-Tfh cells (B) absolute number of GC-Tfh cultured with either GC-Tfh or GC-Tfh1. CFSE labeled Tfh ($2\times10^4$/well) were stimulated alone or mixed with GC-Tfh1 cells ($2\times10^4$/well) with CD3/CD28 beads for 5 days, harvested cells were stained with anti-CD4 and the absolute number of GC-Tfh were calculated based on the CFSE signal and number of viable cells recovered in the culture (left panel). GC-Tfh1 cells were stimulated alone or mixed with GC-Tfh1 cells (1, 1:2, 1:4, 1:8, 1:16 ratio) (right panel).

### *GC-Tfh1 Cells Inhibit the Cytokines Secretion from GC-Tfh Cells*

We next examined whether GC-Tfh1 cells suppress cytokine production by GC-Tfh cells. Sorted GC-Tfh1 cells were co-cultured with GC-Tfh cells and activated with anti-CD3/CD28 beads for 2 days, and then the cytokine in the supernatant was measured by Luminex. As shown in Figure 49, GC-Tfh1 cells inhibited the secretion of IL-4 and

IL-21 secretion by GC-Tfh cells stimulated with CD3/CD28 microbeads. Furthermore, GC- Tfh1 cells suppressed IL-4 and IL-21 production from GC-Tfh cells in a dose dependent manner (Figure 49). However, we did not observe any suppression in the secretion of IL-2 or IFN-γ from GC-Tfh cells.

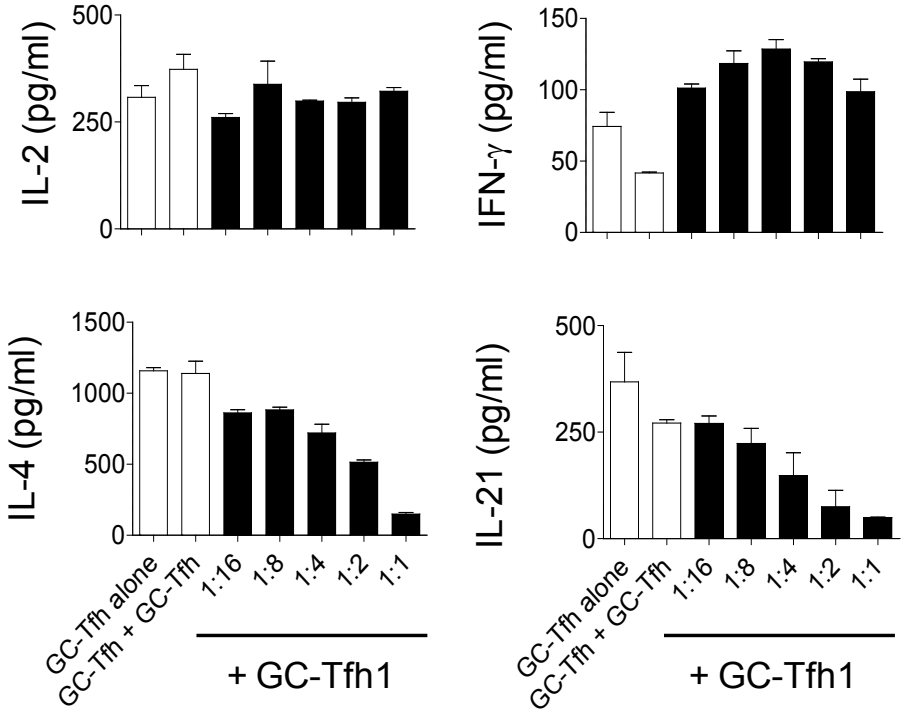


Figure 49: GC-Tfh1 cells inhibit the cytokines secretion from GC-Tfh cells. GC-Tfh1 cells abrogate cytokine production from GC-Tfh. Culture supernatants were harvested at day 2 of the suppression assay, and secreted cytokines were measured (a representative of 2 experiments).

*GC-Tfh1 Cells Require Cell-to-Cell Contact for Suppressive Function*

Naturally occurring regulatory T cells (nTregs) suppress the proliferation of responder T cells via cell contact dependent mechanisms. Some of the induced Tregs display suppressive functions via secreting inhibitory cytokines such IL-10 and TGFβ (Belkaid, 2007; McGeachy et al., 2005). We examined whether GC-Tfh1 cells mediate suppression via cell contact. In a transwell culture, separating GC-Tfh1 cells ($2\times10^4$/well)

in the top wells from GC-Tfh cells ($2\times10^4$/well) in the bottom wells completely prevented the suppression of GC-Tfh cells proliferation (Figure 50 A, B).

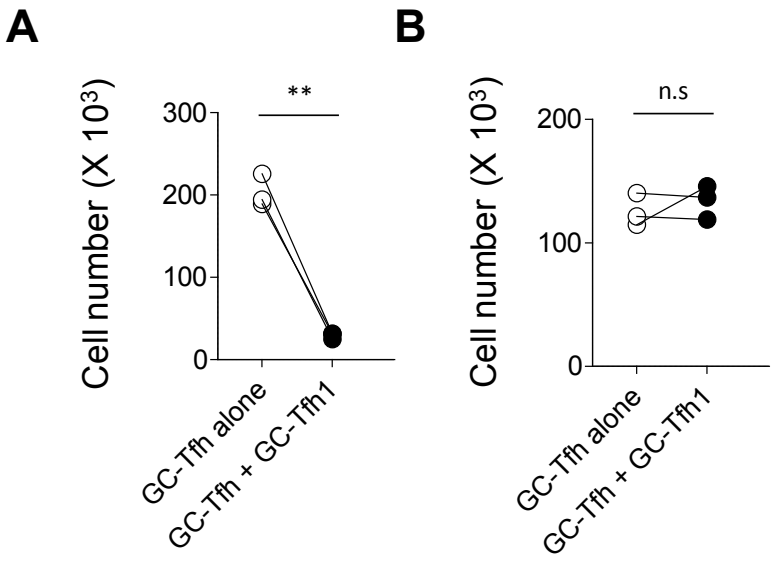


Figure 50: GC-Tfh1 cells require being proximity of GC-Tfh cells to suppress the proliferation of GC-Tfh cells (A) inhibition of the proliferation of GC-Tfh cells by GC-Tfh1 cells (absolute number of GC-Tfh cells is shown) (B) Revert of the proliferation suppression of GC-Tfh cells in suppression assay with transwell. GC-Tfh1 cells were plated in the top wells, while autologous CFSE labeled GC-Tfh cells were plated in the bottom wells (n=3). Paired T test.

Similar to the proliferation, inhibition of the direct contact of GC-Tfh1 cells with GC-Tfh cells abrogated the suppression of IL-4 and IL-21 secretion by GC-Tfh cells (Figure 51 A, B). Thus, cell contact is important for suppressive function of GC-Tfh1 cells.

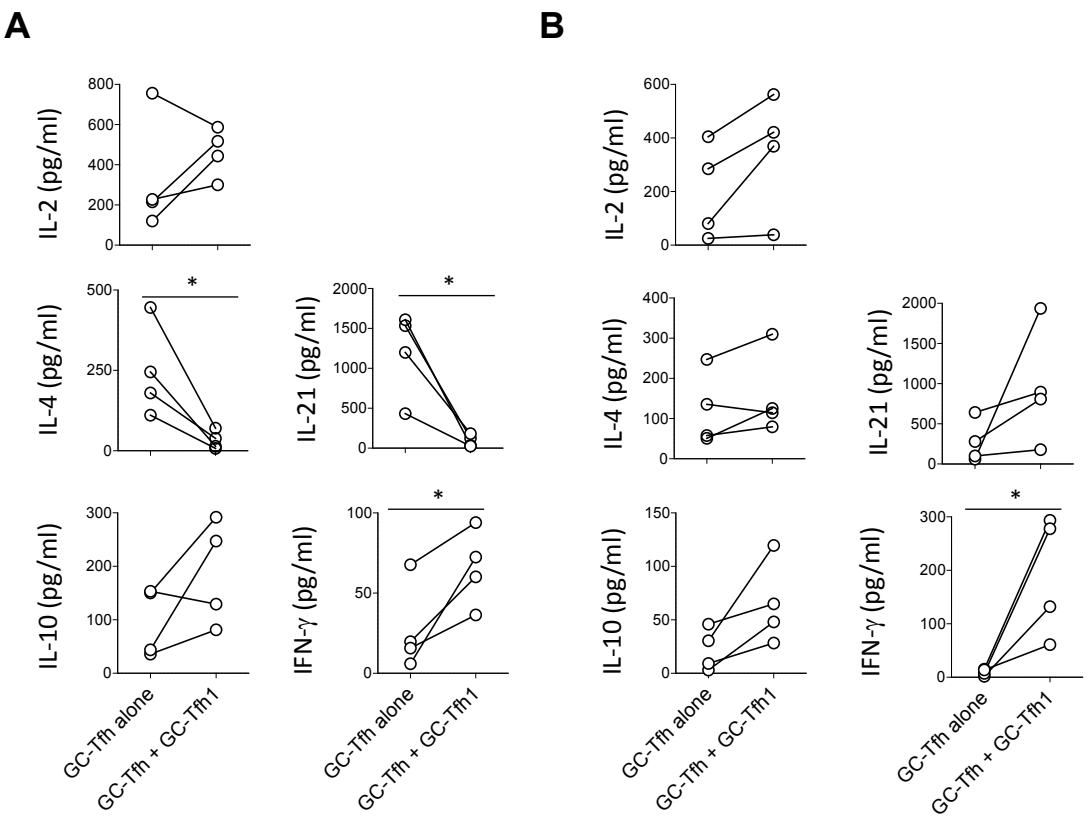


Figure 51: GC-Tfh1 cells require being proximity of GC-Tfh cells to suppress IL-4 and IL-21 secretion (A) inhibition of IL-4 and IL-21 secretion from GC-Tfh cells by GC-Tfh1 cells at day 2 of culture (B) Revert of IL-4 and IL-21 secretion in suppression assay with transwell. GC-Tfh1 cells were plated in the top wells, while autologous GC-Tfh cells were plated in the bottom wells. Both cells were stimulated with CD3/CD28 beads, and cytokine levels at day 2 in the bottom wells were measured (n=4). Paired T test.

We hypothesized that FAS-L/FAS signaling is involved in the suppression of GC-Tfh cells by GC-Tfh1 cells. Blocking FAS-L/FAS-L interaction in suppression assay using Anti-FAS mAb resulted in partial inhibition of the suppression of GC-Tfh cells by GC- Tfh1 cells (Figure 52A). However, we did not observe any revert of IL-4 and IL-21 suppression after blocking FAS-L/FAS interaction (Figure 52B). Taken together, these data indicate that GC-Tfh1 cells use at least in part surface FAS-L, to regulate GC-Tfh cell proliferation.

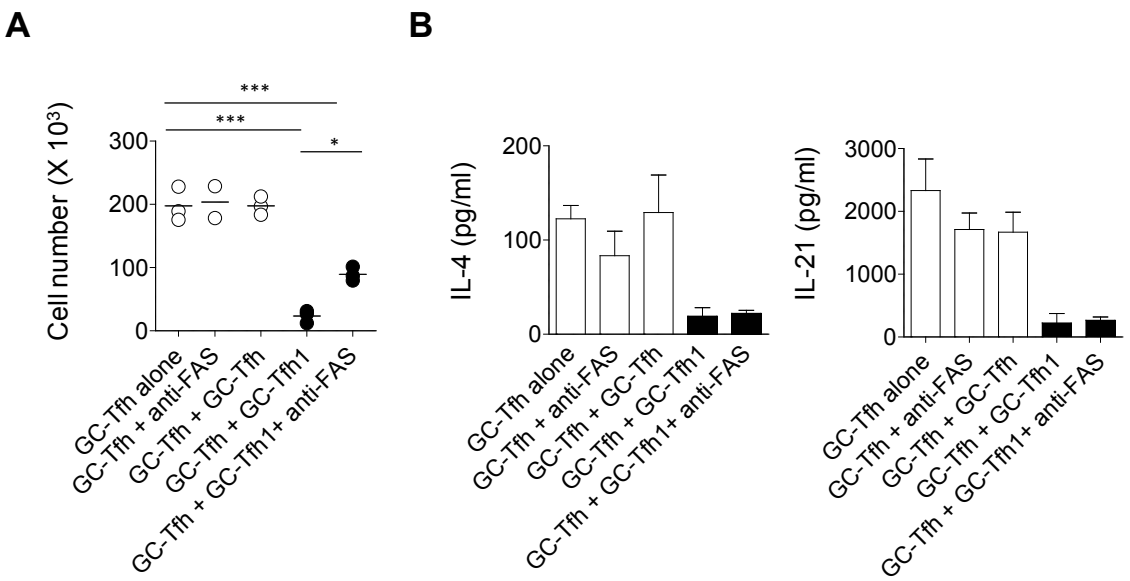


Figure 52: GC-Tfh1 cells use at least in part surface FAS-L, to regulate GC-Tfh cell proliferation in a contact-dependent manner (A) Blocking FAS/FAS-L interaction in the suppression assay resulted in a partial revert in the proliferation of GC-Tfh cells (B) Blocking FAS/FAS-L interaction in the suppression assay did not Revert the suppression of IL-4 and IL-21 secretion (n=2).

### *GC-Tfh1 Cells do not Express Cytotoxic Markers*

Several studies demonstrated that suppressive functions of regulatory T cells are in part mediated by cytotoxic granules (Gondek et al., 2005; Grossman et al., 2004). However, we did not detect the expression of granzymes including granzyme A, B, K, or perforin by GC-Tfh1 cells (Figure 53). This finding suggests that GC-Tfh1 cells are not cytotoxic cells and the mechanism of suppression by GC-Tfh1 cells likely does not involve cytolytic activity mediated by granzymes and perforin.

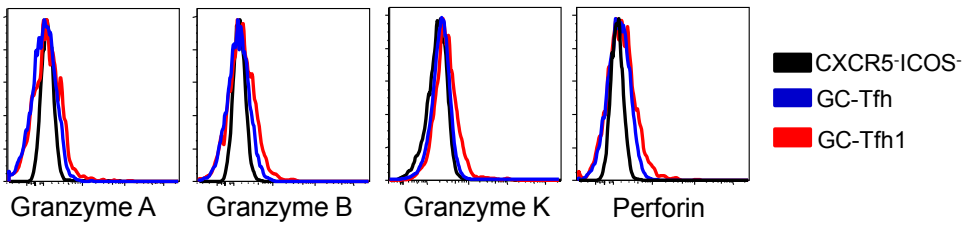


Figure 53: GC-Tfh1 cells are not cytotoxic cells. Expression of Granzyme A, B, K and perforin on GC-Tfh, GC-Th1 and $CXCR5^{-}ICOS^{-}$ cells. A representative from at least four donor tonsils.

*GC-Tfh1 Cells do not Express Foxp3*

Regulatory T cells (Tregs) negatively regulate immune responsiveness to inhibit self-reactivity and to guard against over-reactivity to pathogens (Fehervari and Sakaguchi, 2004). It has been shown that Tregs cells can up-regulate CXCR5 upon Ag priming and can suppress T cell-dependent Ig production (Lim et al., 2004). The same group demonstrated that $Foxp3^+$ Tregs can be found at the T-B border and within GCs of human tonsil and can directly suppress Igs secretion by B cells (Lim et al., 2005). To investigate whether GC-Tfh1 cells share characteristics with nTregs, we examined their expression of the transcription factor Foxp3. Using flow cytometry analysis, we found that Foxp3 expression was expressed only at very low intensity on GC-Tfh1 cells and the expression was comparable with GC-Tfh cells (Figure 54). In contrast, Foxp3 expression was very high in the cells expressing CXCR3 from the non GC-Tfh compartment $CXCR5^{lo}ICOS^{hi}$. Similar to Foxp3, CD25 marker was also expressed at very low intensity on GC-Tfh1 cells. Taken together, this finding suggests that GC-Tfh1 cells are distinct from $Foxp3^+$ Tregs. Thus, our study suggests that GC-Tfh1 cells represent a previously undefined subset of GC-Tfh cells that display suppressive roles in GC environment.

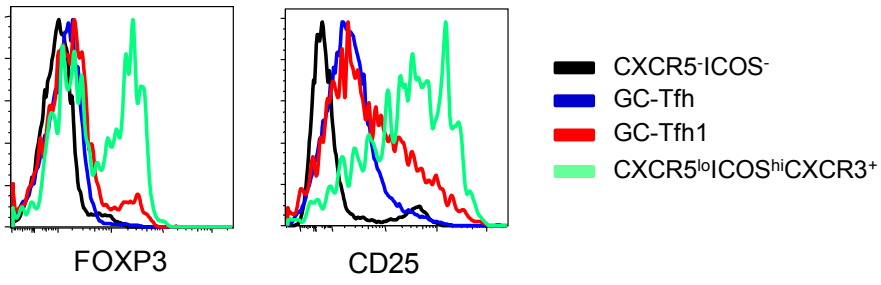


Figure 54: GC-Tfh1 cells are not naturally occurring $CD4^+$ regulatory T cells. Expression of CD25 and the transcription factor FOXP3 on GC-Tfh, GC-Th1 cells and $CXCR5^-ICOS^-$ cells. A representative from at least four donor tonsils.

*Discussion*

In this study, we show that GC-Tfh1 cells likely represent a regulatory component of GC- Tfh cells in humans. GC-Tfh1 cells actively suppress the survival and the growth of GC- B cells. GC-Tfh1 cells also suppress the proliferation and the cytokine production of GC- Tfh cells. Therefore, GC-Tfh1 cells have a capacity to suppress the activity of both B cells and Tfh cells in GCs, and thus likely regulate the magnitude of GC responses.

We have identified three features associated with the suppressive functions of GC-Tfh1 cells. First, we found that FAS-L was exclusively expressed by GC-Tfh1 cells. While previous mouse studies demonstrated the importance of FAS-L expression by GC-Tfh cells to maintain immunological homeostasis (Hao et al., 2008; Rathmell et al., 1995), the identity of FAS-L-expressing GC-Tfh cells has been unclear. Our previous study showed that FAS-L was expressed at higher levels by other tonsillar $CD4^+$ T cell subsets, such as $CXCR5^{lo}ICOS^{lo}$ Tfh cells and $CXCR5^{lo}ICOS^{hi}$ cells, than GC-Tfh cells (Bentebibel et al., 2011). Given that GC-B cells highly express FAS, it makes sense that terminally differentiated GC-Tfh cells diminish FAS-L expression to provide help to GC-B cells instead of inducing apoptosis. By using immune-fluorescent microscopy, we confirmed that a small fraction of GC-Tfh cells express FAS-L. While FAS-L is known to be expressed by also $CD8^+$ T cells and NK cells, FAS-L expression in GCs was limited to $CD4^+$ T cells. By using flow cytometry, we found that FAS-L expression was restricted to GC-Tfh1 cells. GC-Tfh cells did not upregulate FAS-L expression even after in vitro activation, while GC-Tfh1 cells maintained FAS-L expression. This suggests that FAS-L expression by GC-Tfh1 cells is cell intrinsic. We further found that blocking the

FAS/FAS-L interactions during the culture of GC-Tfh1 cells with GC-B cells and with GC-Tfh cells increased the recovery of GC-B cells and GC-Tfh cells. This shows that FAS-L on GC-Tfh1 cells delivers signals through FAS on GC-B cells and GC-Tfh cells and induces their apoptosis. In contrast, blocking the FAS/FAS-L interactions during the culture of GC-B cells and GC-Tfh cells did not affect recovery, differentiation, or Ig production of B cells, confirming that GC-Tfh cells lack the expression of FAS-L.

Second, we found that GC-Tfh1 cells produce IFN-γ, which inhibits the survival and growth of GC-B cells. In addition to the expression of CXCR3, a chemokine receptor typically expressed by Th1 cells, GC-Tfh1 cells express T-bet and IFN-γ. Thus, similar to Tfh1 cells in blood, GC-Tfh1 cells display features of Th1 cells. The major difference between GC-Tfh1 cells and blood Tfh1 cells is the expression of Bcl-6, and only GC-Tfh1 cells highly express Bcl-6.

Third, we found that GC-Tfh1 cells express less CD40L than GC-Tfh cells. CD40 signals are essential for B cells to differentiate into plasma cells and to undergo class-switching. The deficiency of CD40L causes a primary immunodeficiency called hyper-IgM syndrome. We found that GC-Tfh1 cells express less CD40L transcripts than GC-Tfh cells, and less cell surface CD40L protein upon activation.

The combination of the blocking of the FAS/FAS-L interactions and IFN-γ, and the supplementation of soluble CD40L transformed GC-Tfh1 cells to become efficient helpers of GC B cells, and yielded equivalent recovery, plasma cell differentiation, and Ig production (including IgG, IgA, and IgM) with GC-Tfh cells. These results show that the three features all contribute to the suppressive functions of GC-Tfh1 cells.

GC-Tfh1 cells require cell-to-cell contact to display suppressive functions. However, GC- Tfh1 cells are clearly distinct from Tregs and T follicular regulatory (Tfr) cells. First, GC-Tfh1 cells produce large amounts of IL-2 (more than GC-Tfh cells) upon activation. Second, GC-Tfh1 cells lack the expression of Foxp3 and CD25. Rather the analysis of phenotype and the gene expression profiles clearly show that GC-Tfh1 cells are largely similar to GC-Tfh cells. Thus, GC-Tfh1 cells likely represent a previously undefined subset of GC-Tfh cells, which display suppressive roles in GC environment.

Our conclusion is supported by the observations in the studies of blood Tfh subsets in autoimmune diseases. In patients with juvenile dermatomyositis (Morita et al., 2011), adult SLE (Le Coz et al., 2013), and Sjogren's syndrome (Li et al., 2012b), Tfh1 cells are underrepresented among blood memory Tfh cells, whereas Tfh1 and/or Tfh17 cells are overrepresented. Such alterations were found to correlate with disease activity, serum autoantibody titers, and/or the frequency of blood plasmablasts (Le Coz et al., 2013; Li et al., 2012b; Morita et al., 2011). This observation suggests that the balance between Tfh1 cells and non-Tfh1 cells is important for the regulation of Tfh response, and that an alteration towards dominance of non-Tfh1 cells increases of functional Tfh cells and causes autoimmune diseases. The altered blood Tfh subsets in autoimmune disease patients likely reflect the alteration in the balance between GC-Tfh1 and GC-Tfh cells in secondary lymphoid organs. Therefore, our study suggests that a strategy to increase the number of functional GC-Tfh1 cells might represent a novel therapeutic approach for human autoimmune diseases.

# CHAPTER SIX

## Conclusion and Summary

Our studies on tonsillar and blood Tfh cell subsets lead us to draw three main conclusions regarding their phenotype, function, and the critical role they play in generation of protective antibody responses against influenza virus.

First, we analyzed the phenotype and functions of distinct $CD4^+$ T cell subsets in human tonsils. We showed that tonsillar $CD4^+$ T cells included at least four populations defined according to the expression of CXCR5 and ICOS: $CXCR5^-ICOS^-$, $CXCR5^{lo}ICOS^{lo}$ Tfh, $CXCR5^{hi}ICOS^{hi}$ GC-Tfh, and $CXCR5^{lo}ICOS^{hi}$. While a majority of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells expressed programmed death-1 (PD-1), only a minor fraction of $CXCR5^{lo}ICOS^{lo}$ Tfh cells expressed PD-1 at very low density. $CXCR5^-ICOS^-$ $CD4^+$ T cells did not express PD-1 or CD45RO, thus were mostly composed of naïve $CD4^+$ T cells. A large fraction of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells did not express IL-7R. In contrast, $CXCR5^{lo}ICOS^{lo}$ Tfh cells expressed IL-7R together with CD45RO. Our study revealed that $CXCR5^{lo}ICOS^{lo}$ Tfh cells exclusively localized outside GCs. $CXCR5^{lo}ICOS^{lo}$ Tfh cells and GC-Tfh cells differentially help B cell subsets. While $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells were efficient at helping GC-B cells, and promoted their survival and Ig production. $CXCR5^{lo}ICOS^{lo}$ Tfh cells lacked the capacity to help GC-B cells due to FAS-L expression. In contrast, $CXCR5^{lo}ICOS^{lo}$ Tfh cells were more efficient than $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells at inducing naïve B cells to proliferate and

differentiate into antibody secreting cells via high production of IL-21. $CXCR5^{lo}ICOS^{lo}$ Tfh cells expressed *BCL6* and *PRDM1* transcripts at equivalent levels with $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells. Furthermore they helped B cells in a manner dependent on IL-21, IL-10, ICOS, and CD40L. $CXCR5^{lo}ICOS^{lo}$ Tfh cells robustly upregulated ICOS and PD-1 expression upon activation, thus sharing phenotype with $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells. Therefore, $CXCR5^{lo}ICOS^{lo}$ Tfh cells might represent extrafollicular helper cells engaged in inducing the differentiation of B cells into extrafollicular plasma cells. However, we cannot exclude the possibility that $CXCR5^{lo}ICOS^{lo}$ Tfh cells represent precursors of $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells. $CXCR5^{lo}ICOS^{lo}$ Tfh cells expressed lower amounts of Bcl-6 protein than $CXCR5^{hi}ICOS^{hi}$ GC-Tfh cells, which might reflect the fact that $CXCR5^{lo}ICOS^{lo}$ Tfh cells are in a transition stage of differentiating into mature GC-Tfh cells. Robust upregulation of ICOS and PD-1 upon interaction with B cells also supports this hypothesis. Alternatively, $CXCR5^{lo}ICOS^{lo}$ Tfh cells might constitute both extrafollicular helper cells and GC-Tfh precursors, and modify their balance according the microenvironment of secondary lymphoid organs. Further characterization of $CXCR5^{lo}ICOS^{lo}$ Tfh cells together with determination of their developmental mechanisms will bring significant insights to the pathogenesis of human autoimmune diseases, where extrafollicular B cell responses are involved. Such studies will be also beneficial for the design of novel vaccines against infectious diseases.

Second, we assessed the quality of Tfh responses in seasonal influenza vaccinations by using blood samples from healthy subjects before and after the administration of trivalent split vaccine. We found that the Administration of seasonal trivalent split influenza vaccines induces a transient increase of influenza specific

$CXCR3^+$ Tfh cells expressing ICOS in the blood at day 7 after vaccination which correlates with magnitude of global protective antibody responses. The emergence of $ICOS^+CXCR3^+$ Tfh cells in blood correlated with the increase of pre-existing antibodies, but not with the induction of primary antibody responses. Consistently, *ex vivo* isolated $ICOS^+CXCR3^+$ Tfh cells efficiently induced memory B cells, to differentiate into plasma cells that produce influenza specific antibodies. This observation was unexpected, as blood $CXCR3^+$ Tfh cells lack the capacity to help naïve B and memory B cells *in vitro*. The capacity of activated blood $CXCR3^+$ Tfh cells to provide help to B cells seems limited, because they lacked the capacity to help naïve B cells *in vitro*, while being capable of inducing memory B cells to differentiate into plasma cells producing influenza specific antibodies. Based on these observations, we cannot exclude that current influenza vaccine might be largely dependent on the $ICOS^+CXCR3^+$ Tfh cells that can help only memory B cells, which may explain their limited effectiveness in particular where primary antibody responses are required, such as immunization to young children or against H5N1 avian influenza viruses.

Last, we found that GC-Tfh cells in tonsils contain a small fraction of cells expressing CXCR3 (GC-Tfh1). GC-Tfh1 cells co-expressed Bcl-6 and T-bet, transcription factors associated with Tfh and Th1 cells respectively. GC-Tfh1 cells largely shared the phenotype and gene expression profiles with GC-Tfh cells. However, they lack the capacity to help GC-B cells. Furthermore, GC-Tfh1 cells suppress the survival and the growth of GC-B cells as well as the proliferation and the cytokine production of GC-Tfh cells. GC-Tfh1 cells displayed three features distinct from GC-Tfh cells: expression of surface FAS-L, secretion of large amount of IFN-γ and the lack of

CD40L expression. These three features contributed the suppressive capacity of GC-Tfh1 cells to inhibit the activity of GC-B cells and GC-Tfh cells as the blocking of FAS/FAS-L interactions and IFN-γ combined with supplementation of soluble CD40L transformed GC-Tfh1 cells to become efficient helpers of GC-B cells. GC-Tfh1 cells require cell-to-cell contact to suppress GC-Tfh activity. However, they are clearly distinct from Tregs and T follicular regulatory (Tfr) cells as they produce large amounts of IL-2 upon activation and lack the expression of Foxp3 and CD25. Thus, GC-Tfh1 cells represent a previously undefined subset of GC-Tfh cells, which express surface FAS-L and display suppressive roles in GC environment.

Collectively, the characterization of the phenotype and function of blood and tonsillar Tfh cell subsets have contributed significantly to identify new biomarkers associated with antibody responses in vaccinations and autoimmune diseases. However, much remains unknown regarding their ontogeny and *in vivo* roles. Addressing these questions is challenging in humans. Thus, integration of the data on blood and tonsil Tfh cells including their phenotype, *in vitro* functions, and the gene expression profiles, from healthy individuals and patients with different diseases obtained in a standardized way will provide a useful database to address these questions.

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