การศึกษาการแสดงออกของนอทชรีเซพเตอร์ในฟอลิคูล่าเฮลเปอร์ทีเซลล์และผลของการยับยั้งการส่งสัญญาณของนอทชต่อการทำงานของฟอลิคูล่าเฮลเปอร์ทีเซลล์

นางสาวหนึ่งหทัย อินรัญ

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EXPRESSION PROFILE OF NOTCH RECEPTORS IN TFH CELLS AND THE EFFECT OF INHIBITION OF NOTCH SIGNALING ON THE EFFECTOR FUNCTIONS OF TFH CELLS

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ฟอลิคูล่าเฮลเปอร์ทีเซลล์เป็นทีเซลล์กลุ่มใหม่ที่จัดอยู่ในกลุ่มของทีเซลล์ผู้ช่วย สามารถพบทีเซลล์ชนิดนี้ได้ในส่วนของ follicles ในอวัยวะที่เป็นแหล่งผลิตลิมโฟไซต์ เช่น ต่อมทอนซิล เป็นต้น ฟอลิคูล่าเฮลเปอร์ทีเซลล์มีความสำคัญในการกระตุ้นบีเซลล์ให้สร้างแอนติบอดีที่จำเพาะเพื่อต่อต้านเชื้อโรคหรือสิ่งแปลกปลอมจากภายนอกและช่วยบีเซลล์ให้เปลี่ยนไปเป็นพลาสมาเซลล์หรือบีเซลล์ความจำ ฟอลิคูล่าเฮลเปอร์ทีเซลล์สามารถแยกออกจากทีเซลล์ผู้ช่วยชนิดอื่นๆได้จากการแสดง ออกของยีน CXCR5 และ ICOS วิถีสัญญาณของนอทชเป็นวิถีสัญญาณของเซลล์ที่พบได้ทั่วไปในเซลล์ต่างๆของร่างกาย การส่งสัญญาณของนอทชมีความสำคัญในการควบคุมกระบวนการทำงานต่างๆของเซลล์ การศึกษาการแสดง ออกของยีนในฟอลิคูล่าเฮลเปอร์ทีเซลล์ด้วยวิธี microarray พบการแสดงออกของยีนที่เกี่ยวข้องกับวิถีสัญญาณของนอทชมีการแสดงออกสูงในเซลล์ชนิดนี้ อย่าไรก็ตามการศึกษาถึงการแสดงออกของนอชทรีเซพเตอร์และความสำคัญของการส่งสัญญาณของนอทชต่อการทำงานของฟอลิคูล่าเฮลเปอร์ทีเซลล์นั้นยังไม่มีผู้ศึกษา ในการศึกษาครั้งนี้จึงศึกษาการแสดงออกของนอทชรีเซพเตอร์และยีนเป้าหมายของนอทชในฟอลิคูล่าเฮลเปอร์ทีเซลล์ที่ถูกแยกมาจากชิ้นทอนซิล โดยศึกษาการแสดงออกยีนเหล่านี้ด้วยวิธี Quantitative real time RT-PCR โดยผลการศึกษาพบการแสดงออกของยีน *Notch1-3*, *DTX1* และ *Hes1* ในฟอลิคูล่าเฮลเปอร์ทีเซลล์แต่ไม่พบการแสดงออกของ *Notch4* ในเซลล์ชนิดนี้ ที่สำคัญพบการแสดงออกของยีน *Notch1*, *Notch3* และ *Hes1* มีการแสดงออกสูงในฟอลิคูล่าเฮลเปอร์ทีเซลล์ ในการศึกษาผลของการยับยั้งวิถีสัญญาณของนอทชต่อการทำงานของฟอลิคูล่าเฮลเปอร์ทีเซลล์โดยใช้ยา GSI พบการแสดงออกของยีน Bcl-6 ยีนซึ่งมีความสำคัญในการควบคุมการทำงานของฟอลิคูล่าเฮลเปอร์ทีเซลล์มีการแสดงออกต่ำอย่างมีนัยสำคัญในกลุ่มของเซลล์ที่ได้รับยา GSI จากนั้นจึงศึกษาความสามารถของฟอลิคูล่าเฮลเปอร์ทีเซลล์ในการช่วยบีเซลล์ผลิตแอนติบอดี้ โดยผลการศึกษาไม่พบความแตกต่างของการผลิต IgG ระหว่างกลุ่มเซลล์ที่ได้ รับยากับกลุ่มควบคุม จากผลของการศึกษาทั้งหมดแสดงให้เห็นว่าวิถีสัญญาณของนอทชเกี่ยวข้องกับการทำงานของฟอลิคูล่าเฮลเปอร์ทีเซลล์

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

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KEYWORDS : FOLLICULAR HELPER T CELL / NOTCH SIGNALING / NOTCH RECEPTOR

NUENGHATHAI INRUN : EXPRESSION PROFILE OF NOTCH RECEPTORS IN TFH CELLS AND THE EFFECT OF INHIBITION OF NOTCH SIGNALING ON THE EFFECTOR FUNCTIONS OF TFH CELLS. ADVISOR : ASSOC. PROF. NATTIYA HIRANKARN, M.D., Ph.D., CO-ADVISOR : ASSOC. PROF. TANAPAT PALAGA, Ph.D., 73 pp.

Follicular helper T (Tfh) cells, a newly characterized effector T helper cell subset, are mainly found in B cell follicles of secondary lymphoid organs. Tfh cells are effector cells which provide help for antibodies production and for B cell differentiation. Tfh subset can be distinguished from other helper T cell subsets by the use of CXCR5 and ICOS expression. Notch signaling pathway is a well conserved pathway operating in various organisms and functioning in controlling many cellular processes. Previous study on gene expression in Tfh cells by microarray analysis showed that these cells expressed Notch signaling related genes. However, the expression profiles of Notch receptors and the effect of inhibition of Notch signaling on the effector functions in Tfh cells have not been studied. In this study, we investigated the expression profiles of Notch receptors and its target genes in Tfh cells isolated from human tonsils. Expression of *Notch1-4* and two Notch target genes were investigated by quantitative RT-PCR. *Notch1-3*, *DTX1* and *Hes1* were expressed in Tfh cells but the expression of *Notch4* was not detectable. More importantly, *Notch1*, *Notch3* and *Hes1*, are found to be expressed significantly higher than the control non-Tfh population. Next, we investigated the effect of inhibition of Notch signaling on Tfh cells function using gamma secretase inhibitor (GSI). Expressions of *Bcl-6*, one of essential genes regulating Tfh functions were significant lower in GSI treated cells than the control. We next investigated the ability of Tfh cells subset to induce IgG secretion of B cells. We found no difference in IgG production between GSI treated Tfh and the control group. Taken together, these results strongly suggested that Notch signaling is involved in effector function of human Tfh.

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CHAPTER I

INTRODUCTION

Background information and rationale

Follicular B helper T cells as known as follicular helper T cells or Tfh cells are a recent member of T helper (Th) cell subsets that predominantly found in germinal center of B cell follicle in secondary lymphoid tissues such as lymph nodes, tonsils and spleens. Tfh cells were identified by high expression level of CXC chemokine receptor 5 (CXCR5), chemokine receptor that guide them to B-cell follicles. Tfh cells are distinguishing from other T helper cells such as Th1, Th2, Th17 and regulatory T (Treg) cells by several criteria, *i.e.* expression of chemokine receptors, cytokine production, localization and functions. The effector functions of Tfh cells are to provide help for B cell differentiation to long lived plasma cells or memory B cells in germinal center, help for antibody production and germinal center formation. At present, the markers of Tfh cells that have been reported are CXCR5, the localization markers of Tfh cells, inducible costimulatory molecules (ICOS) (not found in mice), Programmed cell death 1 (PD-1), B cell lymphoma 6 (Bcl6) transcription factor and interleukin 21 (IL-21) cytokine.

Notch signaling is a conserved signaling pathway playing an important role for cell to cell connection. Notch signaling is involved in many cellular processes such as cell proliferation, differentiation, cell lineage specification and stem cell preservation.

Notch signaling is initiated by interaction between receptors and ligands. Notch receptor is a heterodimeric transmembrane protein expressed on surface of signal receiving cells. In human, there are four Notch receptors (Notch1-4) and five ligands (Jagged1, Jagged2, Delta like-1, Delta like-3 and Delta like-4). The Notch receptors composed of two structures, extracellular and intracellular domain. The extracellular domains of Notch are important for ligand binding and dimerization of the receptors, while the intracellular domains are important for the signal transduction. The activation of Notch signaling directly drives transcriptions of Notch target genes, including HES protein family such as *HES1* and *HES5*.

The study on gene expression in $CXCR5^{hi}ICOS^{hi}CD4^{+}$ T cells showed that $CXCR5^{hi}ICOS^{hi}CD4^{+}$ T cells have a unique gene expression profile that can distinguish them from other Th cells subsets. One expression microarray analysis reported significant upregulated and downregulated genes on $CXCR5^{hi}ICOS^{hi}$ tonsillar $CD4^{+}$ T cells. Interestingly, it showed high expression levels of Notch1 and gene that is a downstream target of the Notch signaling cascade. This study suggested that Notch signaling may play a critical role in regulating effector functions of Tfh subset. At the present molecular mechanisms regulating Tfh functions are currently not well understood.

On the other hand, the extensive expression profiles of Notch receptors and the effect of inhibition of Notch signaling on the effector functions of Tfh cells have not been investigated. In this study, we investigated the expression profile of Notch receptors and

Notch signaling molecules in Tfh cells from human tonsils by quantitative real time RT-PCR. In addition, we aimed to study the role of Notch inhibition on Tfh functions. We investigated by used γ-secretase inhibitor (GSI) to inhibit Notch signaling and then we studied the role of Notch signaling on effectors function of Tfh cells by 1) examining the expression of *Bcl-6* and *IL-21* by quantitative real time RT-PCR and 2) assaying for IgG production when co-culture pretreated Tfh cells with GSI, with autologous B-cells by enzyme-linked immunosorbent assay (ELISA).

Research objective

1. To study expression profile of Notch receptors in Tfh cells
2. To investigate the effect of inhibition of Notch signaling on the effector functions of Tfh cells

Hypothesis

Notch signaling is involved in regulating effectors function of Follicular helper T cells.

Benefit

1. Enhance our understanding of Notch signaling on the effector function of Follicular helper T (Tfh) cells.
2. The identification of molecules involved in Tfh cells effector function provides opportunities for new therapeutic approaches.

CHAPTER II

LITERATURE REVIEW

Identification of Follicular helper T cells

The developments of effector and memory T cells from naïve cells have many steps and spatially controlled processes. Resting T helper (Th) cells are primed to become antigen specific T cells by contacting with dendritic cells in the T- cell zone of secondary lymphoid tissues and differentiate to specialized effector or memory T cells [1]. T helper cells are important in providing help to B cells for their differentiation and survival.

The original T helper cells are divided by chemokine receptor and their cytokines production into two groups; T helper 1 (Th1) and T helper 2 (Th2). Th1 mainly produced interleukin-2 (IL-2) and interferon γ (IFN-γ), which have a specific transcription factors T-bet or known as Tbx21. They are necessary immune component against intracellular pathogens. While Th2 secreted interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-9 (IL-9) and interleukin-13 (IL-13). They are essential immune component against extracellular pathogens; helminths and parasites and play an important role in humoral responses [2, 3]. At present, there are many T helper cell subsets that produce various cytokines and have a specific marker. For example, there is T helper 17 (Th17) that produces interleukin-17A and F (IL-17A/F), interleukin-21 (IL-21), interleukin-22 (IL-

22) and interleukin-26 (IL-26) and has an important role in autoimmunity. T helper 9 (Th9) produces interleukin-9 (IL-9). The regulatory T cell (Treg) mainly produces interleukin-10 (IL-10) and tumor necrosis factor-β (TGF-β) and is necessary for immune homeostasis and self-tolerance maintaining [4-7]. The recent Th cell subset, Follicular helper T (Tfh) cells mainly produces interleukin-21 (IL-21) [8, 9].

In the past, Th2 is the main source that provides help for B cells in the germinal center of secondary lymphoid tissue because Th2 produces a high amount of IL-4, an essential cytokine that is important for B-cell proliferation and class switching [10]. However in the absence of IL-4 condition as shown in IL-4 and Stat-6 deficiency mice, T cell help functions still exist in germinal center suggesting the role of other factors [11, 12]. Over 20 years ago follicle-associated $CD4^+$ T cells were recognized in human germinal centers of secondary lymphoid organs [13]. At present we have clear data that follicle-associated $CD4^+$ T helper cells population which involved in B cell help functions and germinal center formation is Follicular B helper T (Tfh) cells, which are distinct from other T helper cell subsets by several markers.

Follicular Helper T (Tfh) cells

Follicular B helper T cells or Follicular helper T cells or Tfh cells are the class of newly characterized effector T helper cells that found in B cell follicles of secondary lymphoid organs such as lymph nodes, Peyer's patches, tonsils and spleens. They are identified by their constitutive expression of the B cell follicle homing receptor CXCR5 (C-X-C motif receptor 5) [14]. The study on gene microarray analysis in human $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells showed that Tfh cells have a unique gene expression profile that can distinguish them from other Th cells subsets [15]. Tfh cells are distinguishable from other effector T cells subset such as Th1, Th2, Th17 or Tregs [15, 16] by several criteria, including expression pattern of chemokine receptor; CXCR5 that are used as the most convenient markers to identify this subset of cells, transcription factor; Bcl-6, cellular location, migration, cytokine stimulation; IL-21 and functions that summarized in **Figure 1** [17]

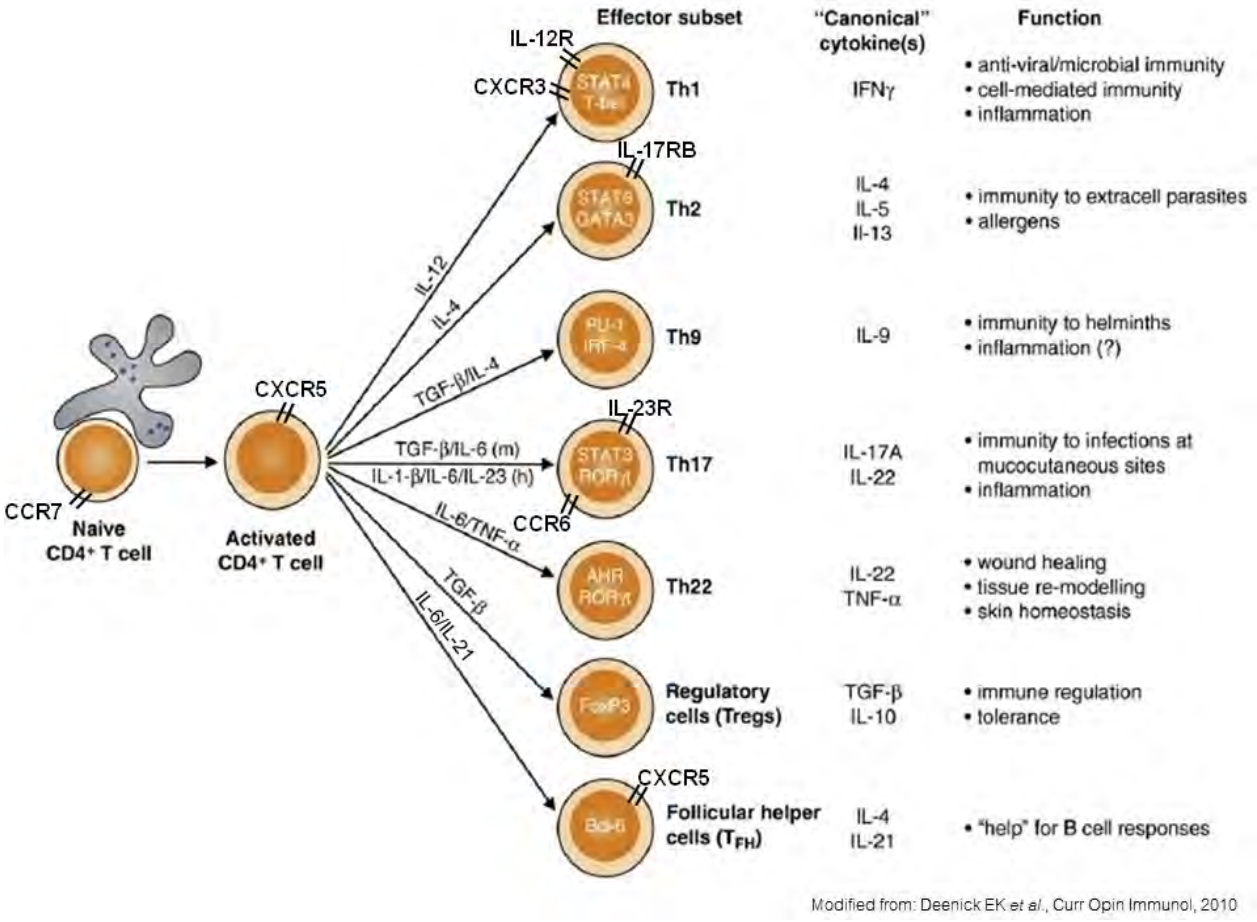


Figure 1 Effector T cells (T helper 1; Th1, T helper 2; Th2, T helper 9; Th9, T helper 22; Th22, regulatory T cell; Treg and follicular helper cells; Tfh) differentiation and expression of transcription factors, signature cytokines, chemokine receptors and their functions. (The transcription factors which are supposed to play a role in subset specific differentiation have been depicted here in the nucleus) [17].

Tfh cells in lymphoid tissues express markers demonstrative of activation, like other effector T cells such as CD69, CD95 and ICOS and express low levels of CCR7 and CD62L similar to Th1 and Th2 cells. Tfh cells have a high expression level of CXCR5, the localization markers of Tfh cells, Inducible costimulatory molecule (ICOS), B cell lymphoma 6 (Bcl6) transcription factor and interleukin 21 (IL-21) cytokine that were transient in other Th cell subsets but they were consistent in Tfh cells [15]. Moreover those genes were important for the function of Tfh cells. Currently, the common markers that are useful to identify human Tfh cells are CXCR5, ICOS, Bcl6 and IL-21.

Tfh cells have an important role in humoral immune response. The effector functions of Tfh cells are provision of help for high affinity antibodies production, class switching and for B cells differentiation into effector cells; long lived plasma cells and memory B cells in germinal center. Signature cytokine produced by $CXCR5^{+}$ T cells is IL-21, a cytokine which has been previously shown to associate with NKT cells [18], Th17 cells [19, 20] and Tfh cells (Figure 1). In addition, Tfh cells do not express a signature markers of Th1 (IFN-γ and T-bet) or Th2 (IL-4 and GATA-binding protein 3 (GATA3)). While Tfh cells express IL-21 like Th17 but they do not express IL-17, IL-17F, IL-22 or retinoid related orphan receptor-γt (ROR-γt) that are a specific markers of Th17.

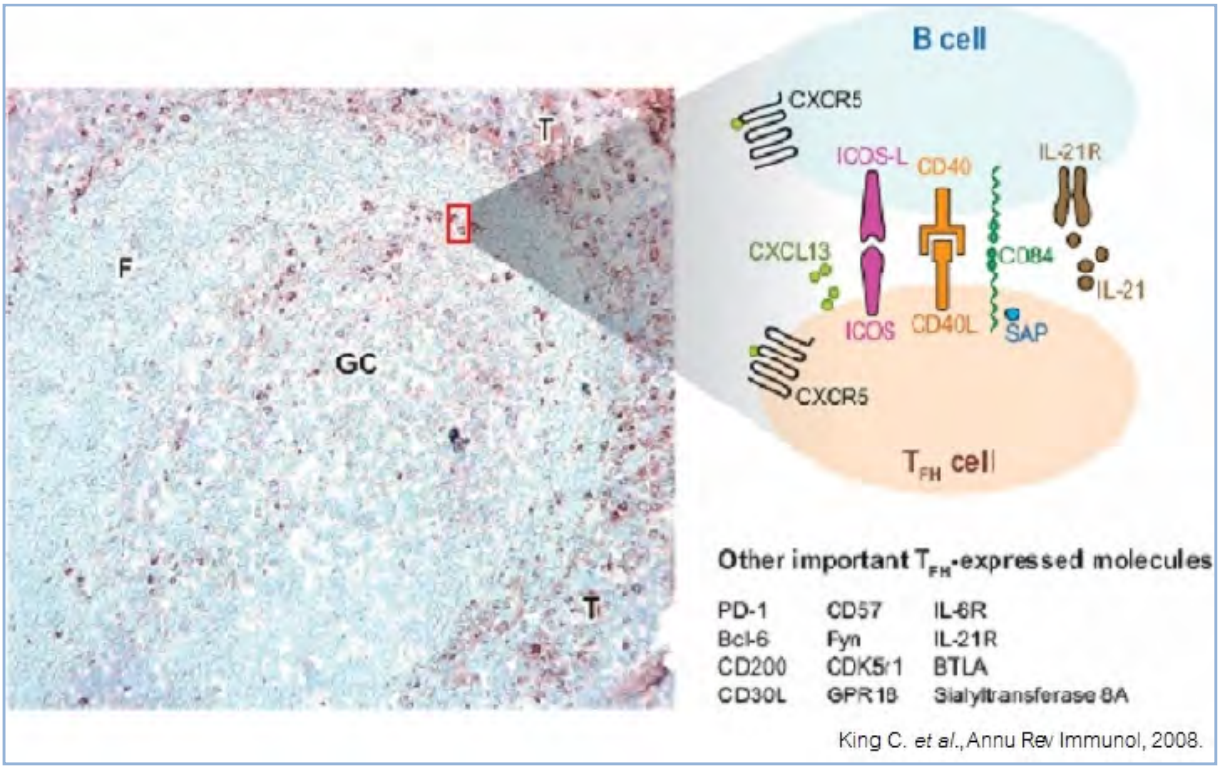


Figure 2 Important molecules for Follicular helper T (Tfh) cells function. **(Left)** The expression of IL-21 in human tonsil **(Right)** Some of the best characterized interacting molecules for Tfh cells and B cells. Listed in this figure are other important molecules expressed by Tfh cells that likely play a key role in the effector function of Tfh cells [21]. (GC: Germinal center, F: Follicle and T: T cell areas)

The essential molecules for Tfh cells

- CXC chemokine receptor 5 (CXCR5)

CXC chemokine receptor 5 [chemokine (C-X-C motif) receptor 5] or CXCR5 is a chemokine receptor expressing on mature and recirculating B cells. In addition, CXCR5 were found to be nearly of antigen undergoes $CD4^+$ T cells. CXCR5 is together with its ligand B-cell-attracting chemokine 1 (BCA-1/CXCL13) which expressed by follicular dendritic cells [21]. CXCR5 is mainly absent in naive $CD4^+$ T cells and $CD8^+$ T cells [22]. It plays an important role for B cell follicle formation [23] and the localization of B cells and T cells in the follicular lymphoid tissues. CXCR5 expression is irreversible when the T helper cells differentiation is complete. CXCR5 is highly express on the Tfh cells and can be used to distinguish them from other T cell subsets as shown in **Figure 1** [24]

- Inducible costimulatory molecule (ICOS)

ICOS or CD278 is a CD28-like costimulatory molecule which is important in a T-dependent antibody response and germinal center reaction. ICOS is a member of CD28 super-family which includes the costimulatory molecules; CD28, ICOS and coinhibitory molecules; CTLA4, PD1 and BTLA receptors [25]. Expression of ICOS is regulated during T cell activation and differentiation. ICOS is upregulated after receiving a signal during T cell activation. Importantly, it was highly expressed in Tfh cells. In addition, ICOS is expressed by other T cells as well, -particularly the Th2 cells. The ligand for

ICOS (ICOSL or Bh7) is expressed on antigen presenting cells including B cells and also in a range of non-lymphoid tissues [26]. Previous study showed high expression levels of ICOS in human tonsillar $CXCR5^{+}$T cells of germinal center [9]. In addition, recent study showed a defect in the maturation of B cells and numbers of Tfh cells were reduced in human and mice that have ICOS deficiency. These data indicated that ICOS is important for Tfh cells differentiation [27, 28].

- B cell lymphoma 6 (Bcl-6)

Bcl-6 or B cell CLL/lymphoma 6 is a transcriptional factor that expresses specifically in human and mouse Tfh cells [29]. Previous study showed that high expression level of ICOS is useful for the identification of human $CXCR5^{hi}ICOS^{hi}PD\text{-}1^{hi}$ Tfh cells, but not in mice [30]. Bcl-6 is a master regulator of Tfh cells and important for the germinal center formation [31]. T cells in mice with Bcl-6 deficiency showed an incomplete development of Tfh cell and germinal center reactions and altered production of other effectors T cell subsets. These data demonstrated that Bcl-6 is required for the generation of Tfh cells. Upregulatation of Bcl-6 expression on Tfh cells repressed the expression of selective transcription factors of Th1, Th2 and Th17. Furthermore, Bcl-6 expression repressed the expression of Blimp-1, a transcription factor which expressed in T cells zone of the lymphoid tissues. In addition the expression of Bcl-6 can be induced by IL-6 and IL-21 [29].

- Interleukin-21 (IL-21)

Interleukin-21 (IL-21) is a member of γ-chain family of cytokines, which includes IL-2, IL-4, IL-7, IL-9, IL-13 and IL-15 [32]. Initial studies have demonstrated that IL-21 has pleiotropic effects in proliferation, differentiation and effector functions of B cells, T cells, natural killer cells and dendritic cells [33]. IL-21 is expressed by antigen-stimulated peripheral T cells [34] and Tfh cells [21, 35]. IL-21 receptor (IL-21R) is expressed in many cell types [36] including B cells, T cells and NK cells [15, 34, 37, 38]. Tfh cells can produce large amount of IL-21. IL-21 is important for Tfh cells development and germinal center formation [35]. Moreover, IL-21 acts as autocrine in Tfh cells development [16]. Two recent studies have shown a critical role of IL-21 in Tfh cells and germinal center B cells generation [16, 35].

- Programmed cell death 1 (PD-1)

Programmed cell death 1 or PD-1 is a co-inhibitor in the CD28 family. PD-1 is an immunoreceptor which is important for interaction between T and B cells. Furthermore it is essential in regulating the adaptive immune response [39]. PD-1 expresses on B-cells, T cells and myeloid cells where as its ligands, B7-H1 (PDL-1) expresses on dendritic cells, macrophages, T cells and B cells. B7-DC (PDL-2) expresses on dendritic cells, macrophages and upregulates upon activation [40]. PD-1 is highly expressed on Tfh cells.

- Signaling lymphocytic activation molecule-associated protein (SAP)

Signaling lymphocytic activation molecule-associated protein, or SLAM associated protein, or SAP is small adaptor protein. It is a necessary signaling protein for lymphocyte activation molecule. The family of SLAM receptors are compose of SLAM (CD150), SLAMF2 (CD48), SLAMF3 (CD229 or Ly9), SLAMF4 (CD224 or 2B4), SLAMF5 (CD84), SLAMF6 (NTB-A or Ly108) and SLAMF7 (CD319 or CRACC). SAP was shown to be critical for T cells to help B cells produce normal antibody responses in both humans and mice [41, 42]. SAP is essential in Tfh cells mediated germinal center formation [43]. In addition, ICOS expression decreased and delayed in T cells from mouse that have SAP deficiency. In human and mouse Tfh cells, CD84 (SLAMF5) and Ly108 (SLAMF6) were highly expressed and therefore in vivo study showed that CD84 (SLAMF5) is necessary for the differentiation of Tfh cells and germinal center formation [44, 45].

Tfh cells and Autoimmune disease

Germinal center is an important site for high-affinity antibody production. Tfh cells are necessary for the regulation of autoantibodies production and germinal center formation. An abnormal selection of germinal center results in a production of autoantibodies and the development of autoimmune diseases. The aberrant of Tfh cells expansion results in production of autoantibodies and plays an important role in a lupus like phenotype as show in many lupus models. In mice homologous for the sanroque allele of Roquin develop a lupus like disease accompanied by increased number of Tfh cells and germinal center formation [46]. Furthermore, the development of lupus-like disease was dependent on the enhancement of germinal center formation while it could be decreased when Bcl-6 gene was deleted [47]. BXSB-Yaa mice have a high level of IL-21 DNA and protein serum that associated with disease severity. In the same way in human, a variation of IL-21 gene may be a risk factor for development of SLE [48].

Patients with systemic lupus erythematosus (SLE) have increased numbers of $CXCR5^{+}ICOS^{hi}PD\text{-}1^{hi}CD4^{+}$ T cells in the peripheral blood and it associated with disease severity, including severe kidney damage and higher titers of anti-double stranded DNA antibodies [49, 50].

Notch cascade

The Notch gene was discovered 90 years ago. It was first identified by Thomas Hunt Morgan and colleagues who described a partial loss of function that shown notches at drosophila melanogaster wing [51, 52]. The Notch signaling pathway is a cell signaling pathway that is highly conserved in many cellular organisms, including drosophila and mammalian cells as shown in **Table 1**. It is important for cell-cell communication, which involves in gene regulation that controls many cellular processes such as maintenance of stem cell, cell fate specification, differentiation, proliferation, function and apoptosis [53].

Table 1. The conserve components of Notch signaling molecules between Mammalian and Drosophilla [54].

Component	Mammals	*Drosophila*
Receptors	Notch 1–4	Notch
Ligands	Delta-like 1,3,4	Delta
	Jagged 1,2	Serrate
Processing molecules	Presenilin 1,2	Presenilin
	Metalloprotease	Metalloprotease
	Furin-like protease	Furin-like protease
Downstream transcription factors and coactivators	CSL (RBP-Jκ)	Su(H)
	Mastermind-like 1–3	Mastermind
Modulators	Lunatic, Radical, and Manic Fringe	Fringe
	Numb, Numb-like	Numb
	Deltex 1–3	Deltex
Target genes	Hes 1,5	Hairy/En(spl)
	HeyL	

Allman *et al.*, Immunological Reviews, 2002

Notch receptors and ligands

Drosophila have one Notch receptor (dNotch) and two ligands: serrate and delta while, in mammalian cells, there are four Notch receptors, *i.e.* Notch1, Notch2, Notch3 and Notch4 and five notch ligands as shown in **Figure 3**. They express on various cell types including antigen presenting cells. These five ligands are Jagged1, Jagged2, Delta like-1, Delta like-3 and Delta like-4 (Figure 3, right) [53]. The Notch structures commonly contain an N-terminal domain and DSL (Delta, Serrate and Lag). The Notch receptor is a transmembrane receptor protein that composes of extracellular and intracellular domains (Figure 3, left). The extracellular domains are important for ligand binding and dimerization of the receptors, while the intracellular domains are important for the signal transduction. The extracellular domain composed of 36 EGF-like repeats in Notch1 and Notch2, 34 in Notch3 and 29 in Notch4 which are essential for ligands binding and Lin12 repeats (LIN) or cysteine rich Notch. The intracellular domain of Notch composed of two protein-protein interaction; RAM domains and ANK repeats (six ankyrin), two nuclear localization signals (NLS), a transactivation domain (TAD) which is not present in Notch3 and Notch4, proline-glutamate-serine and threonine-rich (PEST) sequences.

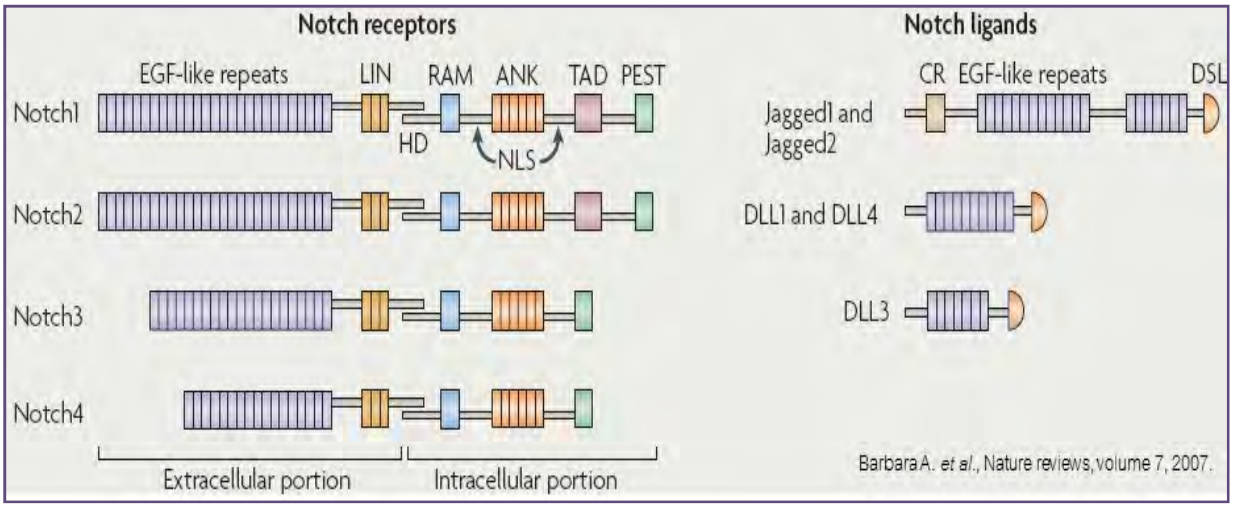


Figure 3 Structure of Notch receptors and ligands [55]. (**Left**) Mammalian Notch1-4 receptor contains extracellular and intracellular domains. The extracellular domain composes of multiple EGF-like repeats and LIN repeats. The intracellular domain consists of the membrane-proximal RBP-J-associated molecule (RAM) domain, nuclear localization sequences, ankyrin repeats (ANK) which essential for protein-protein interaction, a carboxy-terminal transactivation domain (TAD) which essential for transcription activation, and a PEST (proline/glutamate/serine and threonine-rich) motif which is a main regulated for Notch degradation. Notch3 and Notch4 receptors are shorter and lack of TAD domain. (**Right**) Five mammalian Notch ligands; Jagged1, Jagged2, Delta-like 1 (DLL1), DLL3 and DLL4. Notch ligands compose of DSL (Delta-Serrate-Lag2) domain and multiple EGF-like repeats. Cysteine-rich (CR) domain is a conserve region in Jagged1 and Jagged2.

Notch signaling pathway

When the Notch receptors are engaged by their ligands, the transmembrane domain of Notch is cleaved firstly, an ADAM (a disintegrin and metalloproteinase) or know as TACE (tumour necrosis factor α converting enzyme) family protease and the second cleavage by γ-secretase complex that containing presenilin, nicastrin, APH1 and PEN-2 proteins [56]. Upon the enzymatic cleavage, the intracellular domain localizes to the nucleus and bind to a transcription factor CSL (CBF1-suppressor of hairless-Lag1), which is a homologue to mouse RBP-J (recombination signal binding protein-J). Then the intracellular domain of Notch moves co-repressor and recruits co-activator to activate Notch target genes. The activation of Notch signaling directly drives a transcription of Notch target genes including helix-loop-helix transcription factors of the Hairy enhancer of split (HES) family such as HES-1 and HES-5 [57], HEY family, Nrarp (Notch-regulated ankyrin-repeat protein [58] and Deltex family such as Deltex-1 (**Figure 4**) [59].

Inhibition of γ -secretase or loss of presenilin or nicastrin can prevent Notch signaling by suppressing all of Notch receptor processing [60]. Therefore, we can use γ-secretase inhibitor to inhibit the Notch activation [61].

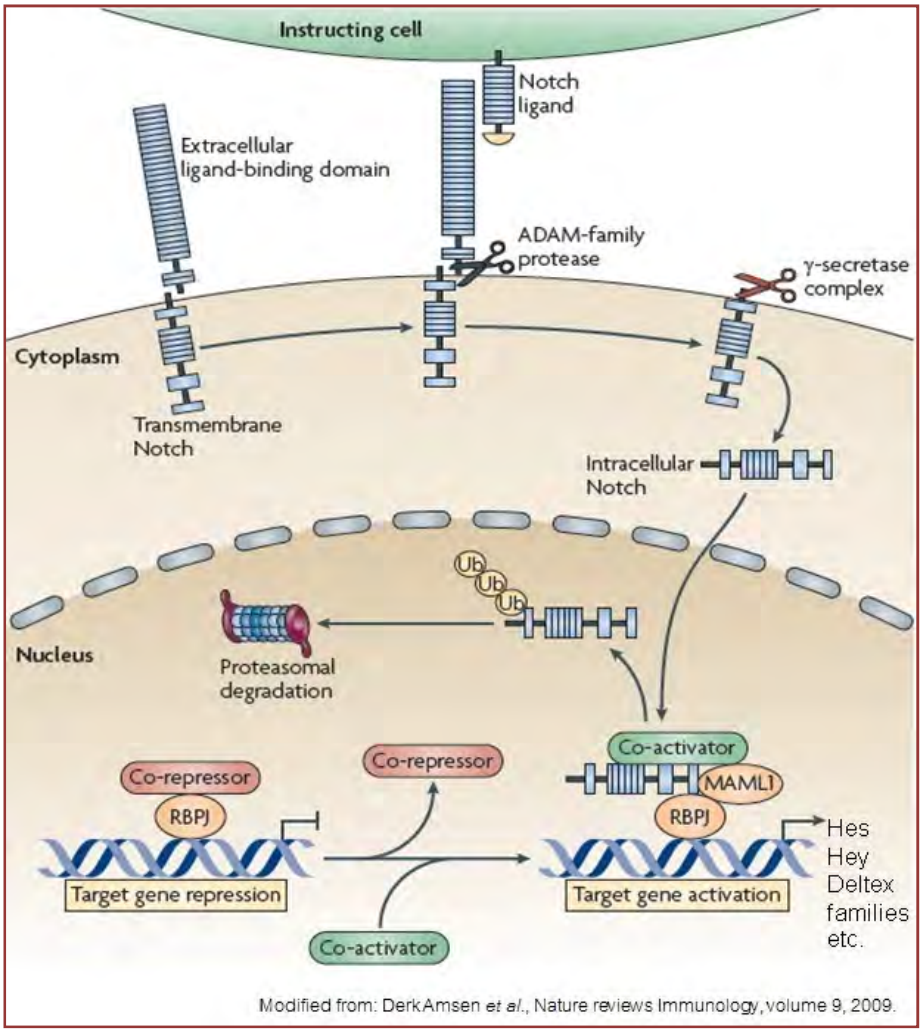


Figure 4 The Notch signaling pathway. Notch signaling is initiated by interaction of receptors and ligands. The extracellular region of Notch was cleaved by an ADAM family protease and next by a γ-secretase complex. This last cleavage makes Notch intracellular region releasing from the plasma membrane and translocating to the nucleus. In nucleus, intracellular region of Notch binds with RBPJ (CSL-CBF1-suppressor of hairless-Lag1) transcription factors that are bound to the promoters of Notch target genes. And then the intracellular of Notch convert the co-repressors to the co-activators to activate the expression of Notch target genes such as Hes, Hey and Deltex families [62].

Notch target genes

Notch signaling drive Notch target genes to various outcomes in many cellular and developmental processes. The most described Notch target genes in human are Hes1, Hes5 and Hey1 [63, 64]. Other Notch target genes are NRARP and Deltex-1, the negative regulators of Notch signaling.

HES (hairy and enhancer of split) family of basic helix-loop-helix, a highly conserve protein, identified in drosophila as a neurogenic gene that is important for the development and signaling of T-cells [65-67]. HEY (HES with YRPW motif) families are transcriptional factors of Notch cascade which function as transcriptional repressors. The activity of HEY family in peripheral T cells is continuing characterized [68].

In addition, Notch target genes which are important in cancer are c-myc [69-71], cyclinD-1 [72] and p-21 [73]. A number of additional genes have been reported containing NF-kB2 [74], Notch1 itself, Bcl-2 [75] and others.

Role of Notch ligands and receptors in peripheral T cells

Notch signaling controls many cell fate decisions and differentiation processes in development and function of T and B lymphocytes. To date, several groups have studied the role of Notch ligands and their receptors in T cell development [76, 77]. Notch1 is important for the selection between T and B cell lineage and plays a selective role in T cell lineage [78, 79]. The Notch1-Notch4 mRNA levels are significantly

increased following antigenic stimulation of the naive $CD4^+$ T cells [80]. It is proposed that all of Notch receptors (Notch1-4) can play an important role in activation of peripheral T cells. The cleaved activated form of Notch1 and the Notch target, HES-1 were upregulated in activated T cells, showing continuous activation of Notch signaling [80]. In addition, the different Notch receptors have specific effects on $CD4^+$ T cells such as a specific role of Notch3 in peripheral T cells [81]. In T helper 1 cells, Notch can controls IL-10 production by a STAT4-dependent process that converts pro-inflammatory Th1 cells into regulatory T cells [82]. Lastly, the inhibition of Notch signaling with γ-secretase inhibitors was shown to decrease the proliferation of $CD4^+$ and $CD8^+$ T cells and decrease the production of IL-2 and IFN-γ of peripheral T cells [80, 83]. Therefore, besides their role in lymphocyte development, notch ligands and receptors also play an important role in peripheral T cells by affecting their effector functions.

Notch signaling and Follicular helper T (Tfh) cells

Recent study compared the expression of $CXCR5^+$ T cells with other T cell subsets, Th1, Th2, effector memory and central memory T cells [15]. It was shown that $CXCR5^+$ T cells can be distinguished from those cells by several specific genes expression pattern such as a specific cytokine production which differ from Th1 and Th2. Furthermore, one study on the $CXCR5^+$ T cell subpopulations including

$CXCR5^{hi}ICOS^{hi}CD4^{+}$ T cells, $CXCR5^{low}ICOS^{int}CD4^{+}$ T cells and $CXCR5^{-}ICOS^{-/low}CD4^{+}$ T cells showed that $CXCR5^{hi}ICOS^{hi}$ $CD4^{+}$ T cells seem to be a terminal differentiation of T helper cells with a unique gene expression profile. The microarray expression analysis showed a specific gene set related to costimulatory molecules, cytokines, cytokine receptors, intracellular signaling, transcription factors and apoptosis. This microarray expression analysis reported a list of significant upregulated and downregulated genes on $CXCR5^{hi}ICOS^{hi}$ tonsillar and peripheral blood $CD4^{+}$ T cells as shown in **Figure 5** [84].

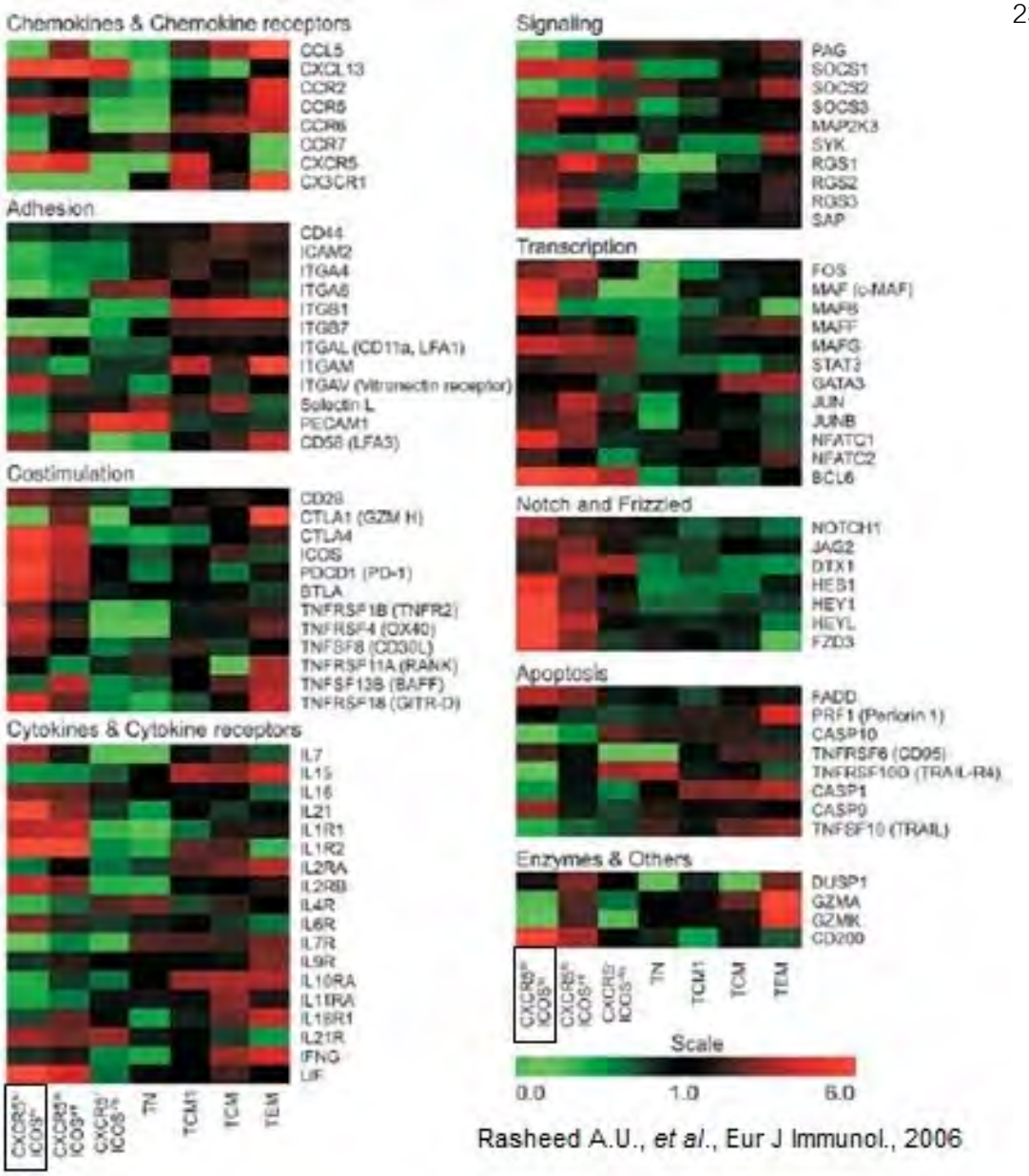


Figure 5 Molecular signatures of $CXCR5^{high}ICOS^{high}CD4^{+}$ Tfh cells [84]. The microarry analysis of mRNA expression levels of related genes in chemokines and chemokine receptors, adhesion, co-stimulation, cytokines and cytokine receptors, intracellular signaling, transcription factor, Notch and Frizzled molecules, apoptosis and enzymes on $CXCR5^{high}ICOS^{high}CD4^{+}$ T cells, $CXCR5^{low}ICOS^{intermediate}CD4^{+}$ T cells, $CXCR5^{-}ICOS^{-/low}CD4^{+}$ T cells, Naive T cell (TN), Central memory T cell (TCM), Effector memory T cell (TEM) populations.

Interestingly, it showed high expression levels of *Notch 1* and transcription factors that are a downstream target of the Notch signaling cascade such as *HES1*, *HEY1* and *HEYL*. In addition, the CXCR5^{hi}ICOS^{hi} Tfh cells differentiation may highly depend on the Notch and Wnt expression cascade suggested by the changes of Notch and Wnt expression molecules between CXCR5^{-}ICOS$^{-/lo}$ and CXCR5^{hi}ICOS^{hi} CD4^{+} T cells. These gene expression pattern changes may drive a differentiation and function of Tfh cells suggesting a role of Notch signaling in CXCR5^{hi}ICOS^{hi} Tfh subset, as shown in **Figure 6** [84].

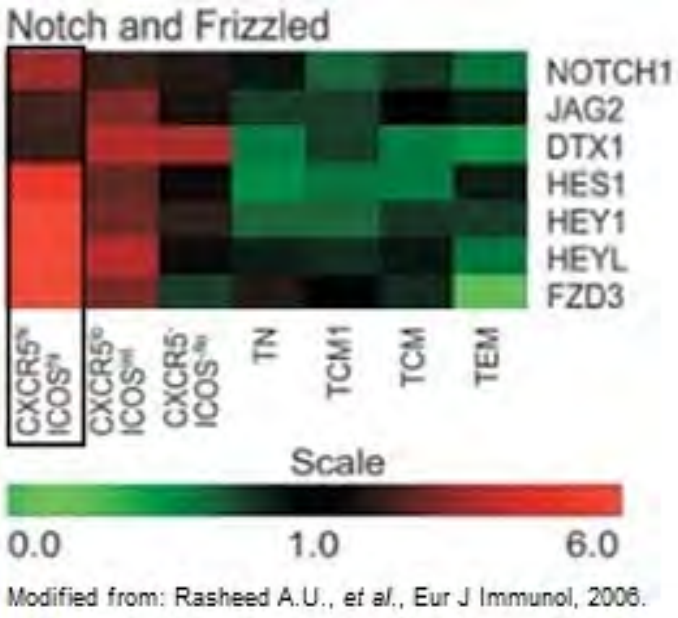


Figure 6 Expression patterns of Notch and Wnt signaling molecules in Tfh cells subset. Microarray analysis of human tonsillar and peripheral blood in different CD4^{+} T cells subsets. It was shown high expression level of Notch1 receptor and the target genes of Notch signaling pathway in CXCR5^{high}ICOS^{high}CD4^{+} Tfh cell subset [84].

CHAPTER III

MATERIALS AND METHODS

1. Samples

Human tonsil tissues (n=14) were collected from healthy individual who have hypertrophic tonsil and underwent tonsillectomy. All samples were obtained from King Chulalongkorn Memorial Hospital. Informed consent was obtained from all donors.

The inclusion criteria of this study are as follows:

- The donors who have hypertrophy or chronic inflammation tonsil and underwent tonsillectomy.
- Both sexes at all ages

The exclusion criteria of this study are as following:

- The donors who have an abnormal serology (Anti-HIV and Anti-HBs Ag: positive).
- The donors who have tonsillar abscess.
- The donors who have tonsillar tumor.

Table 2. Summary of total human tonsil tissues used in each experiment

Tonsil number	Experiment details
1	Set up for Tfh cells isolation and identification
2	Set up for Tfh cells and B cells co-culturing experiment
3	Set up for Bcl-6 and IL-21 mRNA expression
4	Expression profiles of molecules in the Notch signaling pathway
5	Expression profiles of molecules in the Notch signaling pathway
6	Expression profiles of molecules in the Notch signaling pathway
7	Expression profiles of molecules in the Notch signaling pathway
8	Expression profiles of molecules in the Notch signaling pathway
9	Effects of IL-CHO treatment on the expression of *Bcl-6* and *IL-21* mRNA in Tfh and non-Tfh cells
10	Effects of IL-CHO treatment on the expression of *Bcl-6* and *IL-21* mRNA in Tfh and non-Tfh cells
11	Effects of IL-CHO treatment on the expression of *Bcl-6* and *IL-21* mRNA in Tfh and non-Tfh cells
12	Effects of IL-CHO treatment on biological functions of Tfh in helping B cells for antibody production
13	Effects of IL-CHO treatment on biological functions of Tfh in helping B cells for antibody production
14	Effects of IL-CHO treatment on biological functions of Tfh in helping B cells for antibody production

2. Antibodies

Surface staining antibodies for Fluorescence activated cell sorting (FACS) were APC-mouse anti-human CD4, PE mouse anti-human CD278 (ICOS), Alexa Fluor®488 rat anti-human CXCR5 and PerCP anti-human CD19 (Leu™-12). All of the antibodies were purchased from BD Biosciences. Goat anti-human IgG (H+L), purified and peroxidase-mouse anti-human IgG antibodies for Enzyme-linked immunosorbent assay (ELISA) were obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA). Anti-CD3 and anti-CD28 antibodies for cells stimulation were purchased from BD Biosciences (BD Biosciences Pharmingen, San Diego, CA USA).

3. Cell Isolation

Human tonsils were isolated from freshly obtained tissues. Tonsillar cells were isolated by mechanical disruption. Cells were filtrated with labware cell strainers 40μm (BD Falcon). Tonsillar mononuclear cells were separated by Ficoll-Hypaque (Sigma Chemical Company, St. Louis, Missouri, USA) density gradient centrifugation at 2,200 rpm for 30 minutes. Cells were washed with RPMI media at 1,500 rpm centrifugation for 15 minutes. RPMI media contains 20μM Hepes, 7.5% sodium bicarbonate, MEM non-essential amino acids, penicillin and streptomycin. The cells were counted by hemocytometer with 0.4% of trypan blue. Mononuclear cells were stained with anti-CD4 APC, anti-CXCR5 Alexa Fluor®488, anti-CD278 (ICOS) PE and anti-CD19 PerCP

antibodies and incubated for 30 minutes at 4ºC in the dark. Cells were suspended with 10% fetal bovine serum (Gibco, Karlsruhe, Germany) in sterile 1X phosphate-buffered saline (PBS) and were sorted by FACSAria II (BD Biosciences). The $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells, $CXCR5^{lo}ICOS^{lo}CD4^{+}$ T cells and $CD19^{+}$ cells populations were defined as Tfh, non-Tfh (control) and B-cells, respectively.

4. **Inhibition of Notch activation**

γ-secretase inhibitor (GSI) was used to` inhibit Notch activation. $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells, $CXCR5^{lo}ICOS^{lo}CD4^{+}$T cells and/or B-cells were pretreated with 25 μM γ-secretase inhibitor IL-CHO or 1% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) control in 5%CO_2 incubator at 37ºC for 60 min before using for next experiment.

5. **Tfh cells culture**

Treated $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells or non-Tfh cells ($3x10^{5}$ cells) with γ -secretase inhibitor IL-CHO or DMSO were cultured in culture media that contained RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 20μM Hepes (Hyclone), 7.5% sodium bicarbonate (Invitrogen, Carlsbad, CA, USA), MEM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA). The Tfh cells and non-Tfh cells were incubated in 96 well plates in 5%CO_2 incubator at 37ºC for 24 hours. In vitro stimulation was performed

using anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) antibodies (BD Biosciences). The Tfh cells treated with DMSO were served as control.

6. Co-culture cells

The $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells treated with 25 μM γ-secretase inhibitor IL-CHO or 1%DMSO control were used for co-culturing with tonsillar B cells of the same donor in RPMI 1640 medium supplemented with 10%FBS, 20μM Hepes, 7.5% sodium bicarbonate, MEM non-essential amino acids, penicillin and streptomycin. B cells were isolated from individual human tonsil. Tonsillar mononuclear cells were labeled with a specific surface marker of B cell, anti-CD19 antibody. Then these cells were sorted by FACSAria II (BD Biosciences) same Tfh cells population. The $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells and B-cells were cultured at 1:1 ratio ($1x10^5$ cells: $1x10^5$ cells) in 96 well round bottom plates. In vitro stimulation was performing use anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) antibodies (BD Biosciences). Cells were co-cultured in 5%CO_2 incubator at 37ºC for 4, 7 and 10 days. Un-treated Tfh cells co-cultured with B cells were served as control. The supernatants collected from co-cultures were stored at -70ºC.

7. Enzyme-linked immunosorbent assay (ELISA)

IgG concentrations in the co-culture supernatants were determined by sandwich ELISA. First, we set up the ELISA condition by varying the goat anti-human IgG (primary

antibody), mouse peroxidase anti-human IgG (secondary antibody) and normal serum condition as shown in table 5. We varied goat anti-human IgG antibodies by diluting this antibody at 1, 1:10 and 1:100. We diluted mouse peroxidase anti-human IgG antibody with blocking solution into 1:1000, 1:2000, 1:4000 and 1:10000 to block non-specific reactivity. We diluted normal sera with 1XPBS into 1:100, 1:1000 and 1:10000. In this experiment, we used normal serum as positive control. Lastly, the most appropriated condition for ELISA for this experiment is 1:10 of goat anti-human IgG antibodies, 1:2000 of mouse peroxidase anti-human IgG antibody and 1:100 of normal serum as were labeled in **table 3.**

The 96 well plates were coated with 1:10 of goat anti-human IgG antibody at 4 ºC overnight. The 200 µl of blocking solution were added and incubated for 1 hour at room temperature in the dark to block non-specific reaction. Blocking solution contained 3% skim milk in 1X PBS and Tween20. Then 100 µl of the 1:100 co-culture supernatant were added to the plate and incubated for 2 hours at room temperature in the dark. The IgG concentration was determined by peroxidase-mouse anti-human IgG followed by 100 µl TMB (3,3'5,5'-tetramethyl benzidine) substrate. TMB substrate contained TMB dissolved in DMSO, TMB buffer and 30%H_2O_2. The 1M H_2SO_4 was used to stop the reaction. The washing buffer contained sterile 1X PBS solution and tween20. Normal human serum and 1X PBS was served as positive and negative controls, respectively.

Table 3. Antibodies and normal serum dilution for set up ELISA condition.

Mouse peroxidase anti-human IgG (secondary antibody) dilution	Normal serum dilution	Goat anti-human IgG (primary antibody) dilution					
		1	1	1:10	1:10	1:100	1:100
1:1000	negative	1.596	1.567	0.918	0.982	0.711	0.677
	1:100	1.571	1.54	1.516	1.495	1.319	1.383
	1:1000	1.482	1.489	1.058	1.305	0.741	0.708
	1:10000	1.395	1.335	1.001	0.832	0.607	0.566
1:2000	negative	1.361	1.238	0.493	0.495	0.257	0.146
	1:100	1.496	1.473	1.456	1.388	1.231	1.255
	1:1000	1.441	1.378	0.913	0.906	0.376	0.312
	1:10000	1.39	1.371	0.63	0.598	0.397	0.309
1:4000	negative	1.335	1.322	0.218	0.212	0.113	0.097
	1:100	1.516	1.543	1.023	1.068	0.839	1.082
	1:1000	1.45	1.373	0.574	0.508	0.151	0.219
	1:10000	1.234	1.228	0.265	0.298	0.127	0.104
1:8000	negative	1.057	1.052	0.173	0.172	0.103	0.108
	1:100	1.286	1.163	0.826	0.813	0.608	0.677
	1:1000	1.293	1.218	0.368	0.344	0.107	0.116
	1:10000	1.112	1.122	0.171	0.179	0.111	0.116

8. **RNA isolation and purification**

Total RNA was isolated from sorted $CXCR5^{hi}ICOS^{hi}CD4^{+}$Tfh cells and $CXCR5^{lo}ICOS^{lo}CD4^{+}$ T cells using the RNeasy mini kit (Qiagen, Chatworth, CA, USA). For some experiments when cells less than $5x10^{5}$ cells, total RNA was prepared using the RNeasy micro kit (Qiagen, Chatworth, CA, USA) according to the instruction of manufacturer. These kits combine with a silica membrane. The lysis buffer and 70% ethanol was added to provide correct for binding conditions. Then the sample was applied to an RNeasy Mini spin column or RNeasy MinElute spin column. In these columns, the total RNA binds to the membrane. And next, contaminants were washed away. Lastly, total RNA was eluted in 30 µl or 14 µl RNase free water and stored at -70ºC.

9. **Measurement of the Amount of Total RNA**

Two µl of each RNA sample was aliquoted for measuring the total RNA concentration. The amount and purity of the RNA sample was measured by NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, United States) at optical density 260 nm (OD260) and 280 nm (OD280).

10. **Complementary DNA (cDNA) synthesis**

Synthesis of single-strand complementary DNA (cDNA) from RNA extract of Tfh and non-Tfh cells was carried out using the total RNA of 0.25 µg (maximum volume of

RNA template was not exceeding 11.55 µl). Total RNA was reverse transcribed into cDNA by Reverse Transcriptase Reagent (Invitrogen, Carlsbad, CA, USA). The 18.45 µl of reverse transcription mastermix containing 3 µl of 10XRT buffer, 6.6 µl of 25mM $MgCl_2$, 2.0 µl of 10mM 4dNTP, 0.5 µl of 50µM Random hexamer, 0.6 µl of 20U of RNase inhibitor and 0.25 µl of 50U of superscript Reverse Transcriptase, 18.45 µl of mastermix was added into 0.25 µg (11.55 µl) RNA template. The reverse transcription was performed at 25ºC for 10 minutes, 48 ºC for 30 minutes and 95ºC for 5 minutes. The cDNA was kept at -20ºC until needed.

11. **Quantitative real time RT-PCR**

Quantitative real time RT-PCR was performed on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) using SYBR Green. The expression of the house keeping gene, β-*actin*, was used for Real time PCR normalization. Real time PCR primers were designed using the Primer Express Software Version 3.0. Real time PCR was performing used the indicated primers in **Table 4**. All primer pairs were designed to span across an intron-exon boundary to distinguish an amplification of genomic DNA. Each PCR reaction was set up for 25 µl reaction volume consist of 23 µl of real-time PCR mastermix that contained 12.5 µl of Maxima SYBR Green/ROX qPCR Master Mix (2x) (Fermentas), 0.3 µl of 0.3µM forward primer, 0.3 µl of 0.3µM reverse and 2 µl of cDNA template. The PCR amplification of *Notch1-3, Deltex1* and β-*actin*

included an initial denature temperature at 95ºC for 15 minutes followed by amplification step by denaturation at 95ºC for 15 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for 60 seconds repeated for 50 cycles. For *Notch4* and *Bcl-6*, an initial denaturing temperature was 95ºC for 10 minutes followed by the amplification step by denaturation at 95ºC for 15 seconds, annealing at 61ºC for 30 seconds and extension at 72ºC for 60 seconds repeated for 50 cycles. For *Hes-1*, an initial denaturing temperature was 95ºC for 15 minutes followed by the amplification step, denaturation at 95ºC for 15 seconds, annealing at 57ºC for 30 seconds and extension at 72ºC for 60 seconds repeated for 55 cycles. For *IL-21*, an initial denaturing temperature was 95ºC for 10 minutes then followed by amplification step, denaturation at 95ºC for 15 seconds, annealing at 60ºC for 30 seconds and extension at 72ºC for 60 seconds repeated for 50 cycles. After the PCR processes were completed, the real-time PCR results were automatically reported by an ABI Prism 7500 Real-Time PCR system.

Table 4. The sequences of real time PCR primers

Primers	Sequences	Product size
1. *Notch1*	fwd, 5'-CAGCCTGCACAACC AGACAGA-3' rev, 5'-TGAGTTGATGAGGTCCTCCAG-3'	298 bp
2. *Notch2*	fwd, 5'-TGAGTAGGCTCCATCCAGTC-3' rev, 5'-TGGTGTCAGGTAGGGATGCT-3'	530 bp
3. *Notch3*	fwd, 5'-CATCTGGTTGCTGCTGACAT-3' rev, 5'-TGCCTCATCCTCTTCAGTTG-3'	180 bp
4. *Notch4*	fwd, 5'-TGCACTGAGCCAAGGCATAG-3' rev, 5'-TCGACACAGAGGCCTCCATT-3'	60 bp
5. *Deltex-1*	fwd, 5'-CCCAGCTTGTGCCCTACATC-3' rev, 5'-TCGTAGGCGTTCTGGATGGT-3'	202 bp
6. *Hes-1*	fwd, 5'-AGCACAGAAAGTCATCAAAGCC-3' rev, 5'-TTCATGCACTCGCTGAAGCC-3'	259 bp
7. *Bcl-6*	fwd, 5'-GCCGGACACCAGGTTTTG-3' rev, 5'-AGGCCATTTTGTCTTCACCAA-3'	68 bp
8. *IL-21*	fwd, 5'-TCTGCCAGCTCCAGAAGATGT-3' rev, 5'-GGCCTTCTGAAAGCAGGAAA-3'	67 bp
9. β*-actin*	fwd, 5'-ACCAACTGGGACGACATGGAGAA-3' rev, 5'-GTGGTGGTGAAGCTGTAGCC-3'	380 bp

12. Statistical Analysis

The relative gene expression data were calculated and analyzed by using $2^{-\Delta\Delta_{CT}}$ method [85].

The $2^{-\Delta\Delta_{CT}}$ were calculated from,

$$\Delta C_T = C_{T,\text{Target gene}} - C_{T,\text{Internal control}}$$

$$\Delta\Delta C_T = (C_{T,\text{Target gene}} - C_{T,\text{Internal control}})_{\text{Time } x} - (C_{T,\text{Target}} - C_{T,\text{Internal control}})_{\text{Time 0}}.$$

For example, we calculated $2^{-\Delta\Delta_{CT}}$ for this study from first, we calculated ΔC_T of Tfh_{timex} from $C_{T(Tfh),Bcl\text{-}6} - C_{T(Tfh),\beta\text{-}actin}$ and ΔC_T of non-Tfh_{time0} from $C_{T(non\text{-}Tfh),Bcl\text{-}6} - C_{T(non\text{-}Tfh),\beta\text{-}actin}$. Then $\Delta\Delta C_T$ was calculated from ΔC_T of $Tfh_{\text{Time } x}$ - ΔC_T of non-$Tfh_{\text{Time 0}}$.

The statistical analysis was performed by using SPSS software version 15.0 (SPSS Inc, Chicago, IL). The non parametric test (Mann-Whitney U test) was used to compare data between groups. A *p*-value less than 0.05 were considered significant.

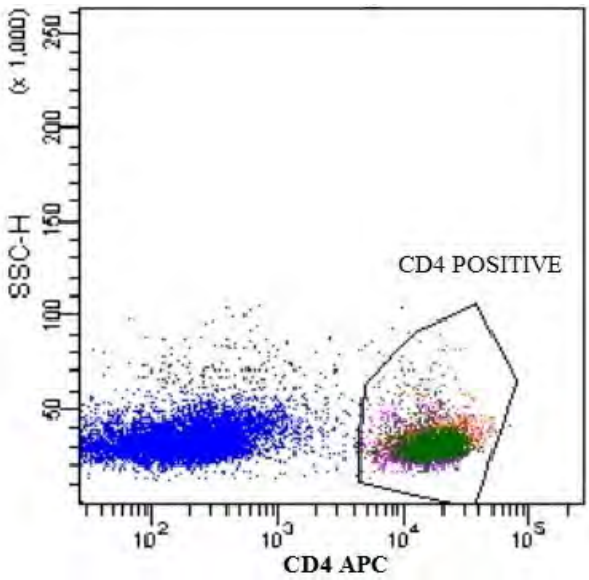
CHAPTER IV

RESULTS

1. Isolation and Identification of CXCR5^{hi}ICOS^{hi} CD4^{+} Tfh cells

Tonsillar cells were isolated from freshly obtained tonsil tissues of human individuals. Tosillar mononuclear cells were separated by Filcoll-Hypaque density gradient centrifugation. Human tonsillar CD4^{+} T cells were purified by fluorescence activated cell sorting (FACS) (**Figure 7A**). Human tonsillar Tfh populations were identified by the co-expression of Tfh markers, CXCR5 and ICOS, and analyzed by FACS. Surface staining pattern of CXCR5 and ICOS separated CD4^{+} T cells into two subsets; CXCR5^{hi}ICOS^{hi}CD4^{+} and CXCR5^{lo}ICOS^{lo}CD4^{+} cells as Tfh and non-Tfh cells, respectively (**Figure 7B**). CXCR5^{hi}ICOS^{hi}CD4^{+} subsets were used as Tfh for further study and CXCR5^{lo}ICOS^{lo}CD4^{+} non-Tfh cells population were use as the control population in all experiments. The number of the individual Tfh, non-Tfh and B cells were summary in Table 5 (n=14).

A.



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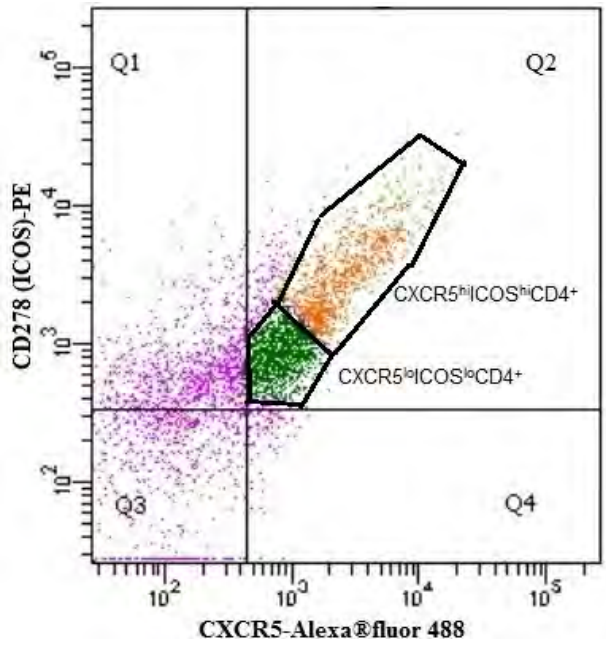


Figure 7 Profile of CXCR5 and ICOS surface staining of human tonsillar CD4$^+$ T cells. Tonsillar cells were surface labelled with CD4, CXCR5 and ICOS antibodies. (A) Human tonsillar cells were gated on the CD4$^+$ population. (B) Among the gated CD4$^+$ cells, the profile of CXCR5 and ICOS surface staining is shown. The human tonsillar CXCR5^{hi}ICOS^{hi}CD4$^+$ Tfh cells (orange) and CXCR5^{lo}ICOS^{lo}CD4$^+$ non-Tfh cells (green) population are gated for sorting.

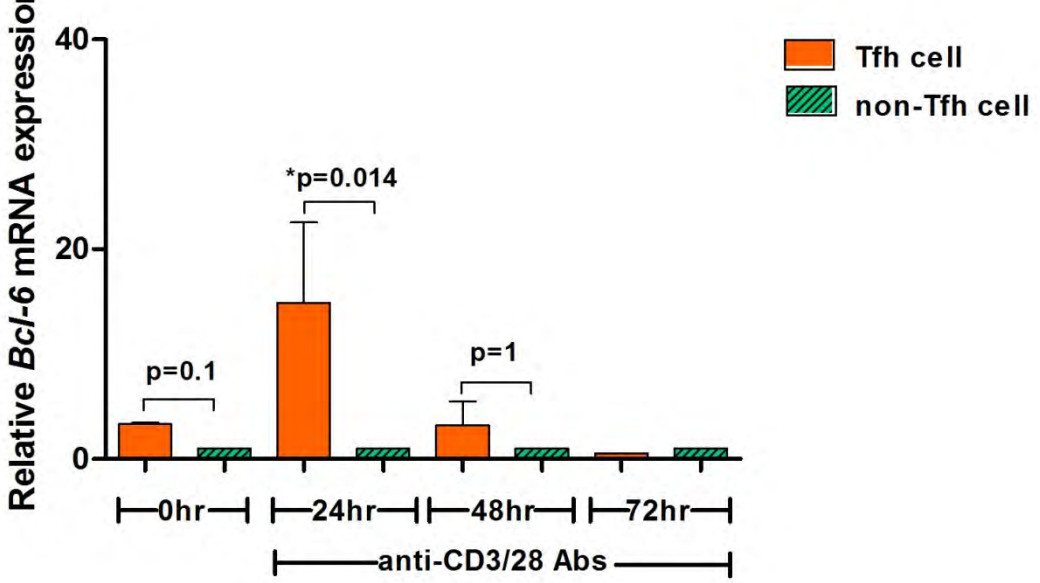
Table 5. Summary of the number of Tfh, non-Tfh and B cells from FACS

Tonsil number	Tfh cells number	% of Tfh from $CD4^+$ population	non-Tfh cells number	% of non-Tfh from $CD4^+$ population	B cells	% of B cells
1	-	-	-	-	-	-
2	$6.27x10^5$	15.7	$9.33x10^5$	36.6	$51.5 x10^5$	5.4
3	$13.1 x10^5$	47	$46 x10^5$	23.3	-	-
4	$12.7 x10^5$	39.1	$10.1 x10^5$	20.5	-	
5	$4.18 x10^5$	42.8	$10.5 x10^5$	22.7	-	-
6	$5.81 x10^5$	36.8	$9.95 x10^5$	16.5	-	-
7	$17 x10^5$	31.1	$19 x10^5$	22.1	-	-
8	$4.98 x10^5$	14.3	$21.5 x10^5$	36.1	-	-
9	$13.9 x10^5$	26	$11.9 x10^5$	29.5	-	-
10	$7.03 x10^5$	12.5	$30.1 x10^5$	54.7	-	-
11	$6.5 x10^5$	35.1	$6.7 x10^5$	19.9	-	-
12	$20.7 x10^5$	24.1	$2.37 x10^5$	24.9	$24 x10^5$	7.8
13	$11.4 x10^5$	39	$1.02 x10^5$	26.5	$94 x10^5$	58.2
14	$7.9 x10^5$	37.2	$7.97 x10^5$	23.6	$63.1 x10^5$	54.3

2. Phenotypic confirmation of the Tfh population

At present, the markers that have been reported for identification of Tfh cells are CXCR5, ICOS, Bcl-6 and IL-21. In this study, we confirmed the phenotype of $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells population from sorted human tonsillar cells by demonstively the *Bcl-6* and *IL-21* mRNA expression. Tfh and non-Tfh cells were stimulated with plate-bound anti-CD3/CD28 for 24, 48 and 72 hours. Total RNA was analyzed for the *Bcl-6* and *IL-21* mRNA expresssion. The relative expression of the two genes were analyzed by quantitative real-time RT-PCR. Expression level of β*-actin* was used as internal control. The results showed that the sorted human tonsillar $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells expressed high levels of both *Bcl-6* and *IL-21* mRNA than the control $CXCR5^{lo}ICOS^{lo}CD4^{+}$ non-Tfh population (Figure 8). At 24 hour after stimulation, both mRNA expression of *Bcl-6* and *IL-21* in Tfh cells were significantly higher than those from the non-tfh cells population (*p*-value = 0.014). Therefore, these results confirmed that sorted $CXCR5^{hi}ICOS^{hi}CD4^{+}$ T cells are Tfh cells which express specific markers resemble to the previously represented Tfh cells.

A.



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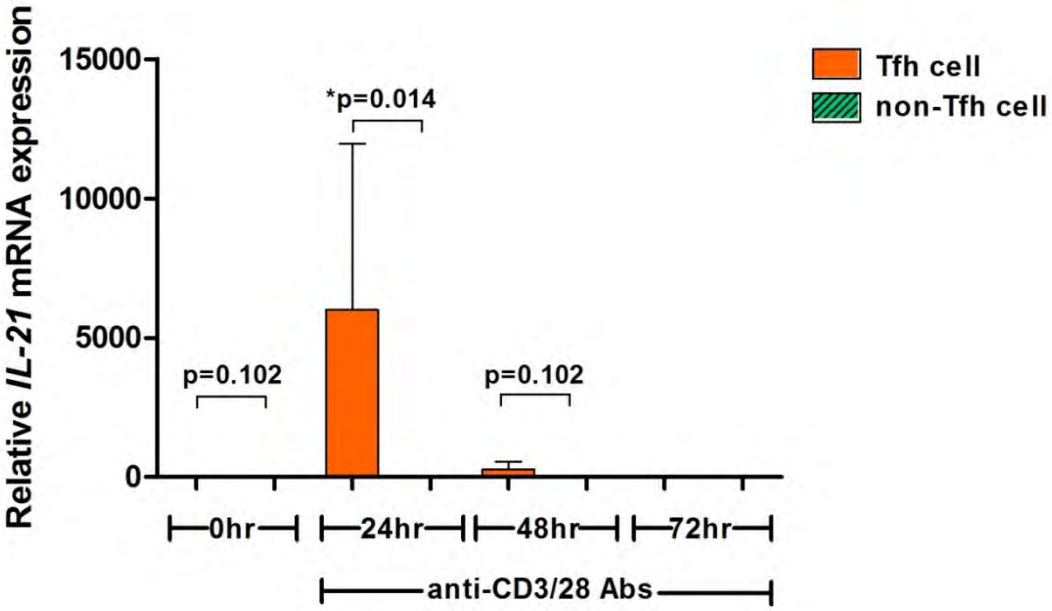


Figure 8 Expression of *Bcl-6* and *IL-21* mRNA in unstimulated and stimulated sorted human tonsillar Tfh cells population. Relative mRNA expression of (A) *Bcl-6* and (B) *IL-21* in sorted $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh and $CXCR5^{lo}ICOS^{lo}CD4^{+}$ non-Tfh cells population. RNA was extracted from these cells on 0, 24, 48 and 72 hours. Data shown are derived from four independent experiments.

3. Expression profiles of Notch receptors and the Notch target genes in stimulated $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells

To gain an insight into the involvement of Notch signaling pathway in regulating Tfh functions, mRNA expression of the molecules of the Notch signaling pathway, i.e. Notch receptors and the Notch target genes, were analyzed by comparing those of human tonsillar Tfh cells with non-Tfh cells. We analyzed mRNA expression of *Notch1-4* and two target genes of Notch signaling, *DTX1* and *Hes1*. The expression profiles of these Notch signaling molecules were analyzed by quantitative real-time RT-PCR. The mRNA levels of each the gene in non-Tfh cells were set as baseline. The results showed that *Notch1* and *Notch3,* and *Hes1* were expressed higher in $CXCR5^{hi}ICOS^{hi}$ $CD4^{+}$ Tfh cells while *Notch2* and *Deltex1* were expressed at the same level in both populations (**Figure 9).** The expression of *Notch4* was not detectable in both subsets of cells in this study (data not shown).

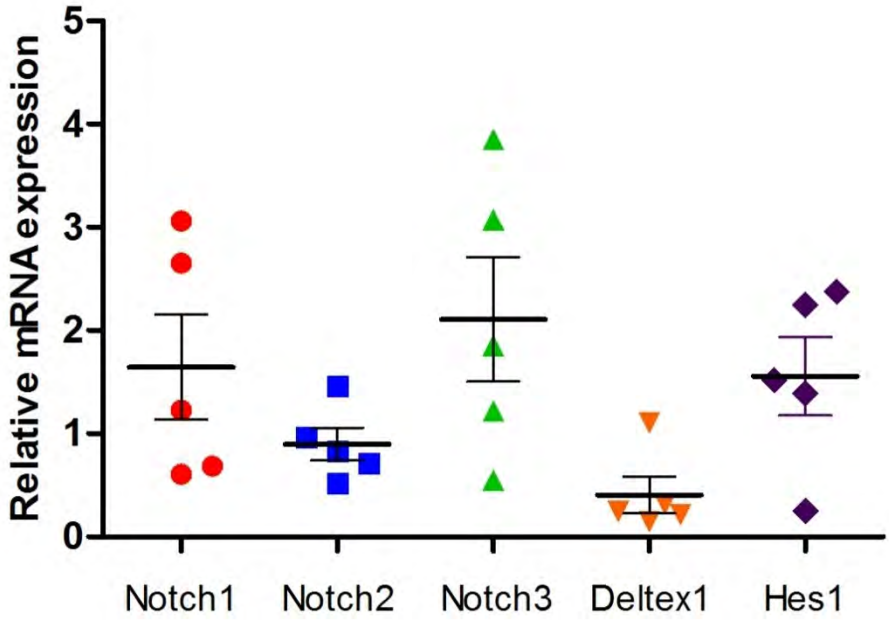


Figure 9 Expression profiles of mRNA of molecules in the Notch signaling pathway in sorted tonsillar Tfh. Total RNA was isolated from unstimulated sorted cells and the expression of mRNA of the Notch receptors (*Notch1-4*) and Notch target genes (*Hes-1* and *Deltex-1*) were analyzed by quantitative real time RT-PCR. Expression of each gene in the control $CXCR5^{lo}ICOS^{lo}CD4^{+}$ T non-Tfh cells was set as 1. Each point represents data obtained from each individual (n=5).

4. Effects of inhibition of Notch signaling by treatment with γ -secretase inhibitor on phenotypes of Tfh cells

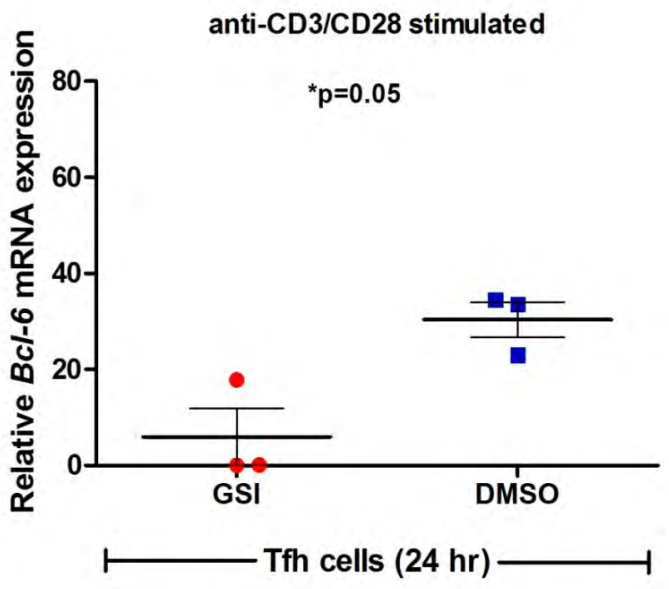
4.1 The effector functions of Tfh cells

Tfh cells have unique gene expression profiles that distinguish them from other T helper cell subsets. Bcl-6 and IL-21 have an essential role in Tfh cells functions. To investigate the role of Notch signaling in regulating Tfh functions, we took a pharmacological approach by inhibiting the Notch signaling and examined its effect on *Bcl-6* and *IL-21* mRNA expression. γ-secretase inhibitor (GSI) is a specific inhibitor of the γ -secretase enzymatic activity which is essential for cleavage of Notch receptors upon activation. This enzymatic activity is required for processing of all Notch receptors (Notch1-4).

Sorted Tfh or non-Tfh cells were pretreated with γ-secretase inhibitor, IL-CHO, or vehicle control DMSO before stimulating with plate-bound anti-CD3/CD28 antibodies for 24 hours. Total RNA was extracted from these cells and the expression of *Bcl-6* and *IL-21* mRNA were determined by quantitative real-time RT-PCR. The relative expression levels of mRNA of these genes were analyzed by comparing those in Tfh cells and non-Tfh cells population with or without treatment with γ-secretase inhibitor, IL-CHO. Cells pretreated with DMSO were used as control groups. The results showed that *Bcl-6* mRNA expression levels were significantly lower in IL-CHO-treated Tfh cells compared with those from DMSO-treated Tfh cells (p-value = 0.05 as shown in Figure 10).

Similarly, *IL-21* mRNA expression level was lower in IL-CHO-treated Tfh cells, compared with DMSO treated control, but the difference was not statistical significant (*p*-value = 0.275) (Figure 11). In non-Tfh cells population, both of *Bcl-6* and *IL-21* mRNA expression levels were not different between IL-CHO-treated or vehicle control groups as shown in Figure 10 and Figure 11, respectively. This result strongly suggested that Notch signaling regulates the expression of key genes which play important roles in differentiation and the effector functions of Tfh.

A.



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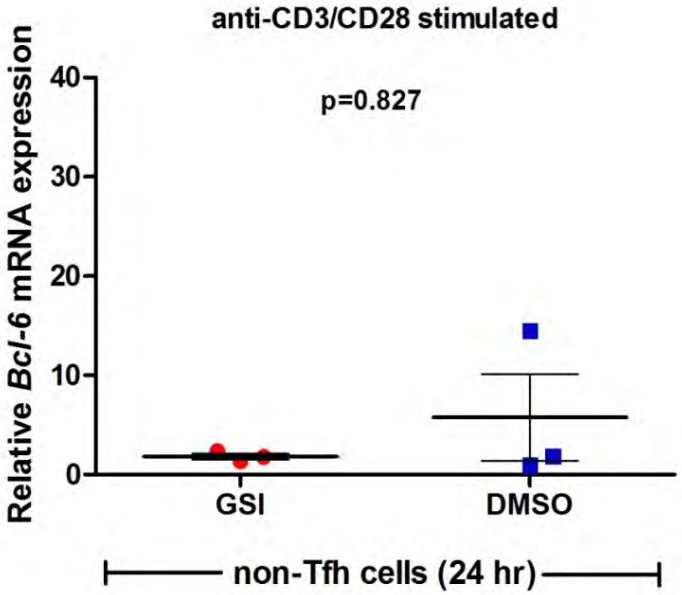
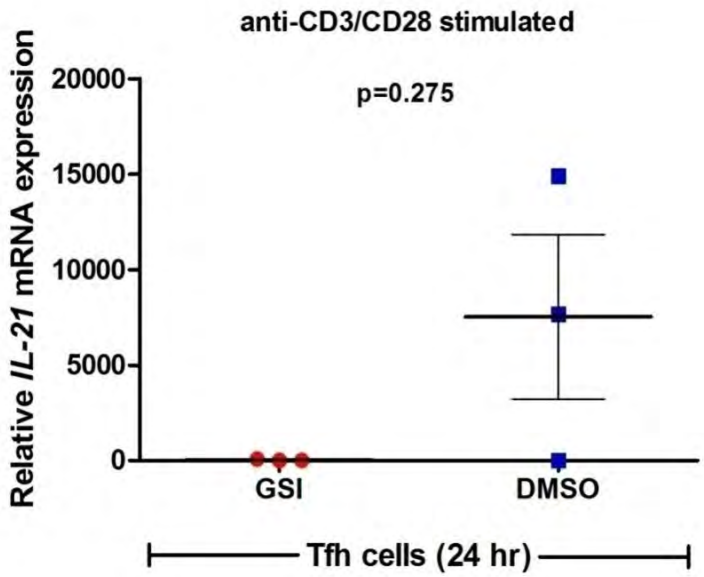


Figure 10 Effects of IL-CHO treatment on the expression of *Bcl-6* mRNA in Tfh and non-Tfh cells. Tfh and non-Tfh cells were pretreated with γ-secretase inhibitor IL-CHO or vehicle control DMSO before stimulating with plate-bound anti-CD3/CD28 for 24 hr. Relative mRNA expression of *Bcl-6* gene in (A.) Tfh and (B.) non-Tfh cells were analyzed by quantitative real-time RT-PCR. The mRNA levels in cells pretreated with DMSO were used as control group. Data derived from cells of three individuals.

A.



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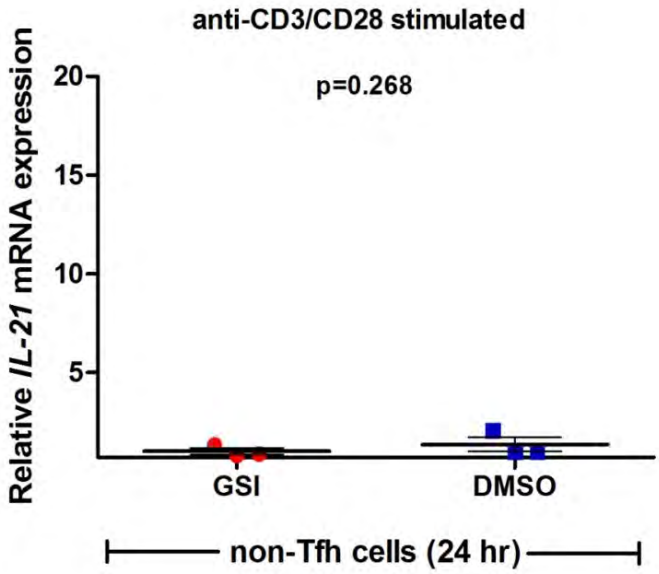


Figure 11 Effects of IL-CHO treatment on the expression of *IL-21* mRNA in Tfh and non-Tfh cells. Tfh and non-Tfh cells were pretreated with γ-secretase inhibitor IL-CHO or vehicle control DMSO before stimulating with plate-bound anti-CD3/CD28 for 24 hr. Relative mRNA expression of *IL-21* gene in (A.) Tfh and (B.) non-Tfh cells were analyzed by quantitative real-time RT-PCR. The mRNA levels in cells pretreated with DMSO were used as control group. Data derived from cells of three individuals.

4.2 Effects of IL-CHO treatment on biological functions of Tfh in helping B cells for antibody production

Tfh cells are known to support immunoglobulin (Ig) secretion by germinal center B cells. This is one of the important biological functions of Tfh cells. In this study, we established the T/B co-culture system to study the functions of Tfh as helpers for Ig production by B cells. Sorted Tfh or non-Tfh cells were co-cultured with sorted B cells in the presence of plate-bound anti-CD3/CD28 antibodies. The most appropriated condition for ELISA for this experiment is 1:10 of goat anti-human IgG antibodies, 1:2000 of mouse peroxidase anti-human IgG antibody and 1:100 of normal serum.

To perform the experiment, we co-cultured $1x10^5$ of Tfh or non-Tfh cells with an equal number of B cells from the same donor. Tfh or non-Tfh cells with B cells were cultured for 4, 7 and 10 days. B cells cultured in the absence of T cells served as control. Detection of IgG production by this ELSA condition was shown in **Figure 12**. As shown in Figure 12, the amount of IgG secretion of B cells alone on day 4, 7 and 10 was lower than in the co-culturing groups, although they were not statistically significant. These results suggested that T cells (both Tfh and nonTfh) promoted help to B cell for IgG production.

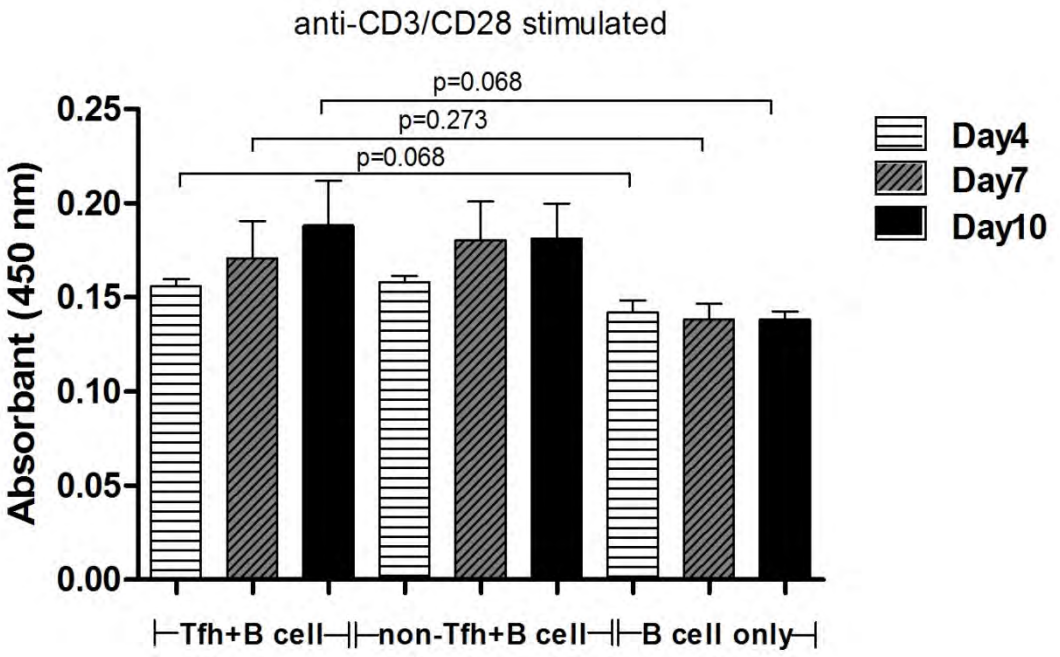


Figure 12 IgG secretions in the co-culture supernatant of Tfh or non-Tfh cells with B cells. Tfh and non-Tfh cells were cultured with B cells from the same donor for 4, 7 and 10 days. B cells cultured in the absence of T cells were used as control. Stimulation was performing used anti-CD3/CD28 antibodies. The amount of IgG secretion in the cell culture supernatants were determined by ELISA.

To investigate the role of Notch signaling pathway in this biological function ofTfh, IL-CHO were used to pre-treat sorted T cells (Tfh and non-Tfh) before co-culturing. The amounts of antibody in the co-culture supernatants were measured by ELISA. $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells or $CXCR5^{lo}ICOS^{lo}CD4^{+}$ non-Tfh cells were derived from the same donor with B cells. Tfh or non-Tfh cells were pretreated with 25μM IL-CHO or DMSO control. These cells were used for co-cultured with B cells for 4, 7 and 10 days. B cells cultured in the absence of T cells were used as control. Stimulation was

performing used anti-CD3/CD28 antibodies. When amount of IgG in the co-culturing groups with Tfh with or without IL-CHO treatment were compared, it was shown no difference of IgG secretion in the co-culture supernatant of B cells with Tfh with and without IL-CHO treatment all of duration (day 4, 7 and 10), *p*-value = 0.3 as shown in **Figure 13**. Same Tfh cells, IgG secretion in the co-culture supernatant of B cells with non-Tfh with and without IL-CHO treatment were not different between these groups all of duration (*p*-value = 0.1 as shown in Figure 13).

A.

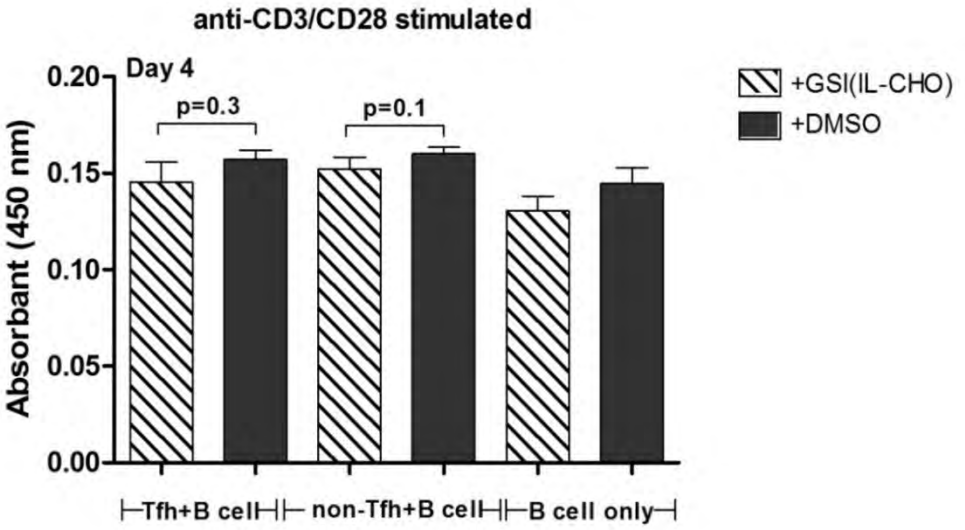
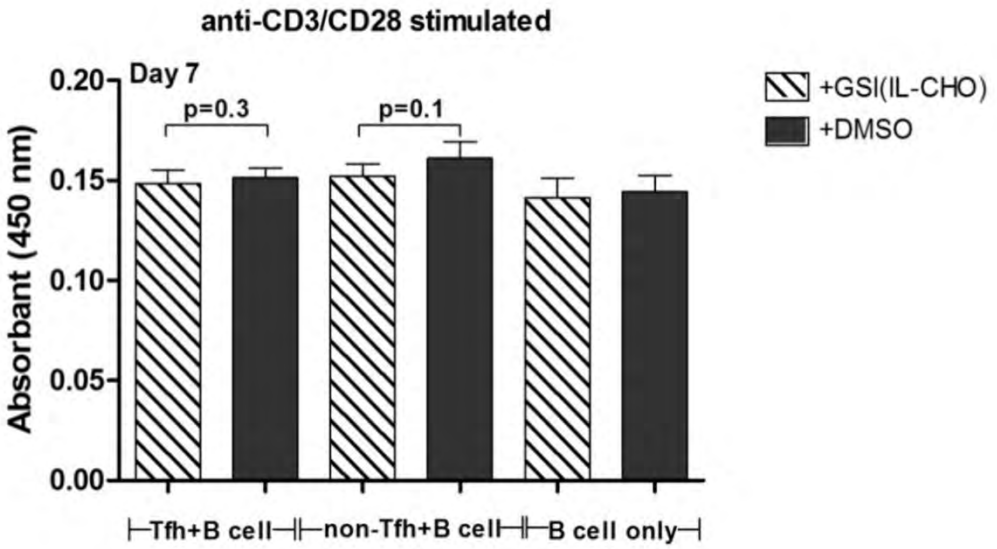


Figure 13 Effects of IL-CHO treatment on the helper functions of Tfh for immunoglobulin production. $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh and $CXCR5^{lo}ICOS^{lo}CD4^{+}$ non-Tfh cells pretreated with γ -secretase inhibitor IL-CHO or vehicle control DMSO and stimulated with anti-CD3/CD28 antibodies were co-cultured with B cells from the same donor for (A) 4 days, (B) 7 days and (C) 10 days. The amounts of IgG in the culture supernatants were determined by ELISA. Data shown are derived from three individuals.

B.



C.

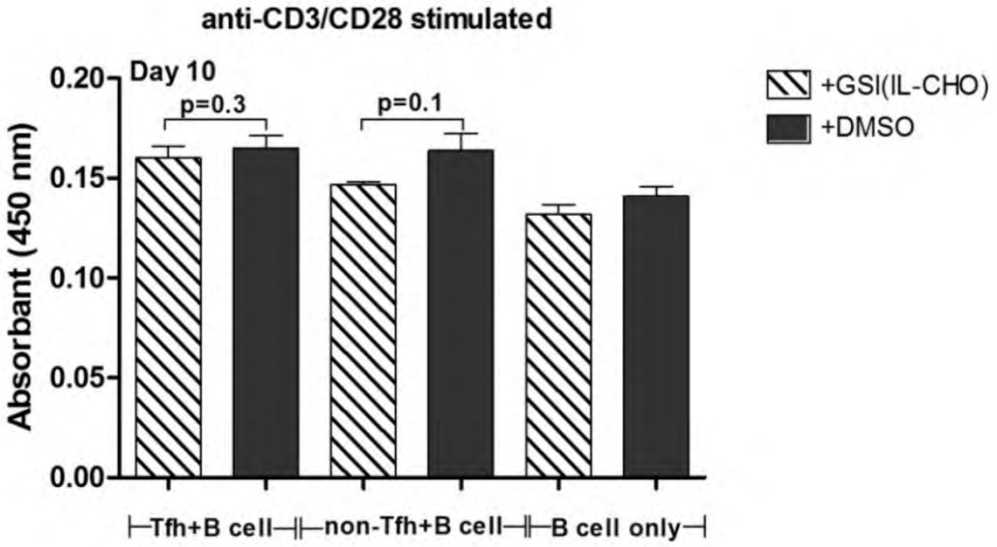


Figure 13 Continued

CHAPTER V

DISCUSSION

At present, human Tfh cells were defined by the expression of CXCR5, which guides them into B cell follicles of secondary lymphoid organs. Furthermore, markers that distinguish them from other Th subsets are ICOS, a transcription factor Bcl-6 and cytokine IL-21, which are essential for their functions. Here, we successfully isolated and identified human $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells and $CXCR5^{lo}ICOS^{lo}CD4^{+}$ non-Tfh cells from human tonsils. The human tonsillar Tfh and non-Tfh cells population were identified by the expression of CXCR5 and ICOS expression levels. We verified the sorted Tfh cells population by mRNA expression levels of *Bcl-6* and *IL-21*. We found that both mRNA expressions of *Bcl-6* and *IL-21* were expressed in Tfh at higher level than in the non-Tfh cells population. These results indicated that sorted human tonsillar $CXCR5^{hi}ICOS^{hi}CD4^{+}$ T cells were Tfh cells.

Ata-Ur Rasheed *et al*. reported a large number of genes which up-regulated and down-regulated in peripheral and tonsillar $CXCR5^{hi}ICOS^{hi}$ Tfh cells [84]. This large scale microarray analysis showed sets of genes that are important for cell migration, adhesion, signaling, transcription, differentiation and apoptosis. Interestingly, in the signaling gene set, there were high expression levels of *Notch 1*, and the downstream target of the Notch cascade such as *Hes1*. The Notch signaling is a well conserved signaling

pathway operating in various organisms and functioning in controlling many cellular processes, including differentiation and function of helper T cells. Since there was no other reports yet to address the involvement of Notch signaling in Tfh differentiation and function, we undertook this investigation in this study. We investigated the expression profiles of Notch receptors and Notch target genes in $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells and found *Notch1-3, DTX-1* and *Hes-1* were expressed in $CXCR5^{hi}ICOS^{hi}CD4^{+}$ Tfh cells but the expression of *Notch4* was not detectable. Interestingly, the expression of *Notch1*, *Notch3* and *Hes-1*, one of Notch signaling target molecule, are found to be expressed significantly higher than those in non-Tfh cells. These results are consistent with the microarray data that link Notch signaling and Tfh cells. Interestingly, we additionally reported for the first time that Notch3 was also significantly expressed at higher level in Tfh cells subset. Our result confirmed that Notch signaling is operating in Tfh cells. Notch signalling might play a role in the develpment of Tfh cells similar to its role in other types of T cells [55]. However, besides its role in T cell development, Notch has been reported to play a part in effector function of T cell as well. For example, Notch signaling directly regultes *IL-4* expression in Th2 cells and T-bet expression in Th1 cells [86, 87] . Therefore, we hypothesized that Notch might play a role in regulating effector functions in Tfh cells subset which is the main aim of this study.

Tfh cells have specific genes that are essential for their functions, one of these genes are *Bcl-6* and *IL-21*. Bcl-6, a transcription factor of Tfh cells, is selectively expressed in mice and human Tfh cells. It is considered a master regulator of Tfh cells

differentiation [14, 21]. Bcl-6 is a transcriptional repressor and by suppressing various microRNA expressions, it induces Tfh differentiation [88-90]. IL-21 cytokine is highly produced by Tfh cells along with other helper T cells. Lately, two groups have reported the role of IL-21 in Tfh cells and germinal center B cell generation [16, 35]. Therefore, in this study, we investigated the effect of inhibition of Notch signaling on expressions of these two markers related to the Tfh cells functions. We pretreated Tfh cells with γ - secretase inhibitor (GSI), the Notch signaling inhibitor, which inhibit the processing of all Notch receptors. Using this inhibitor, therefore, can circumvent the problem of functional redundancy among receptors. Interestingly, *Bcl-6* mRNA expression level was lower in GSI treated Tfh cells than control DMSO treated group. Although it was not statistically different, the *IL-21* mRNA expression level was also lower in IL-CHO-treated Tfh cells, compared with DMSO treated control (*p*-value = 0.275). If we analyzed the result from each tonsillar tissue, we found that the level of IL-21 mRNA was lower in 2 indivuals but was upregualted in 1 individual. The reasons for these conflicting results are currently not known but it may derive from the genetic backgrounds of each individual or other unknown confounding factors. More samples might have to be performed in the future to get clearer results. Both *Bcl-6* and *IL-21* mRNA expression levels were not different between non-Tfh with and without IL-CHO treatment groups. In summary, our findings strongly suggest that Notch signaling regulate *Bcl-6* genes and it may contribute to Tfh functions. However, our inhibitor inhibits processing of all Notch receptors and we did not know if Notch1 or Notch3 is the main receptor responsible for Bcl-6 regulation.

Future study using specific siRNA will be needed to clarify this problem. And for clarify that Notch is directly regulate Bcl-6 transcription factor or IL-21 gene, the chromatin immunoprecipitation (ChIP) assay are needed.

Tfh cells are known to have an important role in humoral immunity. It has the ability to effectively induce B cells for antibodies production [8, 9]. Recent study reported the ability of Tfh to help for antibodies production corresponds with high expression levels of CXCR5 and ICOS [84]. In this study, we found that the IgG production in Tfh cells co-culture with B cells from the same donor were higher than culturing B cells alone confirming previous report that Tfh cells could help B cells for antibody production. However, this IgG production was detected in very low level in our study. When the role of Notch signaling on ability to promote IgG production of B cells by Tfh cells was investigated, we found very low IgG production in the cells culture supernatants in both GSI treated cells and those control groups. There was no difference in IgG secretion between Tfh with γ-secretase inhibitor, IL-CHO and DMSO treatment groups. A very low IgG production of the co-culturing cells may be cause from 1) γ-secretase inhibitor (GSI) that may have effect with B cells. As known, Notch signaling is an essential signaling that are important for many cellular processes and affect in many cells type including B cells. In this experiment, Tfh, non-Tfh and B cells were pretreated with GSI or DMSO before used for co-culturing. All of these cells were not wash before bring them for co-culturing so GSI may be have an effect for inhibit

Notch sinaling of B cells. 2) Viability of sorting cells, the long period of soring time may have an effect to these cells and in this experiment all of these cells were not check for viability after a cell sorting process so these cells may be death.

However, this experiment can be further improved by either 1) increasing cell numbers in the co-culture cells condition, 2) add a common antigen in co-culturing cells, 3) check viability of cells after a cell sorting process or 4) adjust better condition for ELISA.

CHAPTER VI

CONCLUSION

We successfully isolated Tfh population ($CXCR5^{hi}ICOS^{hi}CD4^{+}$Tfh cells) from human tonsils by using two distinctive markers, CXCR5 and ICOS. In addition, we confirmed the phenotype of Tfh population by the expression of Bcl-6 and IL-21. Importantly, we found high expression levels of *Notch1-3* and target gene of Notch signaling, *Hes1* in human tonsillar Tfh cells population when compared with the control non-Tfh cells. The role of Notch signaling in the effector function of human Tfh cells was investigated by using a γ-secretase inhibitor for blocking the Notch signaling. First, we investigated role of Notch signaling in Tfh cells function by studied mRNA expression of *Bcl-6* and *IL-21* in GSI treated Tfh cells. We found *Bcl-6* mRNA was significantly downregulated in GSI treated Tfh compared with the DMSO treated control. We next compared the ability of Tfh cells subset to help induce IgG secretion of B cells. IgG secretion in the co-culture supernatants of GSI treated Tfh cells were lower than DMSO control in all durations tested. All of these results indicate the role of Notch signaling in regulating effector functions of Tfh cells. Future analysis into the involvement of Notch signaling required in Tfh cells function using new technologies, including small interfering RNA-based gene knockdown and gene knockout, are needed.

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APPENDICES

APPENDIX A

Reagents for Tfh cells isolation

1. 1XPBS buffer

Na_2HPO_4	4.88	g
$NaH_2PO_4.H_2O$	1.54	g
NaCl	3.04	g
MilliQ water	1 litter	

Adjust pH to 7.2-7.4; add milliQ water up to 1 liter. The solution was mixed and sterilizes by autoclaving at 121 ºC for 15 min. Store the solution at 4 ºC.

2. RPMI medium

Powder RPMI 1640	1 pack
20μM Hepes solution	10 ml
7.5% sodium bicarbonate	10 ml
MEM non-essential amino acids solution	10 ml
Penicillin-Streptomycin	10 ml
MilliQ water	960 ml

The milliQ water was sterilized by autoclaving at 121 ºC for 15 min. Dissolve powder RPMI 1640 1 pack, 20μM Hepes 10 ml, 7.5% sodium bicarbonate 10 ml, MEM non-essential amino acids 10 ml and Penicillin-Streptomycin 10 ml in 960 ml of sterile milliQ water. The solution was mixed and sterilized by autoclaving at 121 ºC for 15 min. Store the solution at 4 ºC.

3. **Culture media** (R10 medium ;10%FBS in RPMI medium)

Powder RPMI 1640	1 pack
20μM Hepes	10 ml
7.5% sodium bicarbonate	10 ml
MEM non-essential amino acids	10 ml
Penicillin and Streptomycin	10 ml
MilliQ water	960 ml
Fetal bovine serum (FBS)	100 ml

The milliQ water was sterilized by autoclaving at 121 ºC for 15 min. Dissolve powder RPMI 1640 1 pack, 20μM Hepes 10 ml, 7.5% sodium bicarbonate 10 ml, MEM non-essential amino acids 10 ml and Penicillin-Streptomycin 10 ml in 960 ml of sterile milliQ water. The solution was mixed and sterilized by autoclaving at 121 ºC for 15 min. Add 100 ml of fetal bovine serum in RPMI buffer. Store the solution at 4 ºC.

4. **1M Hepes solution**

Hepes powder	23.83	g

Adjust volume up to 100 ml with distilled water, sterile with filter 0.2 µm and store at 4ºC.

5. **Fetal bovine serum (FBS)**

Heat-inactivate fetal bovine serum at 56ºC for 30 minutes and store at -20ºC.

6. **Ficoll-Hypaque solution**

Store Ficoll-Hypaque solution in the dark at 4ºC.

7. **1% Trypan blue**

Trypan blue	0.3	g
MilliQ water	30	ml

Dissolve trypan blue 0.3 g with milliQ water 30 ml. The solution was mixed and stored at 37 ºC.

APPENDIX B

Reagents for Tfh cells sorting

1. **Cell sorting solution** (10%FBS in PBS solution)

1XPBS	90 ml
Fetal bovine serum (FBS)	10 ml

Add fetal bovine serum 10 ml in 1XPBS solution 90 ml. The solution was mixed and store at 4Cº.

2. **Flow buffer solution** (1%FBS in 1XPBS solution)

1XPBS	99 ml
Fetal bovine serum (FBS)	1 ml

Add fetal bovine serum 1 ml in 1XPBS solution 99 ml. The solution was mixed and store at 4Cº.

APPENDIX C

Reagents for ELISA

1. **1XPBST solution**

1xPBS	500 ml
Tween20	250 µl

Add Tween20 250 µl in 1XPBS 500ml. The solution was mixed and store at room temperature.

2. **Blocking solution**

3%skim milk in 1XPBST solution.

3. **TMB buffer** (Potassium citrate tri basic monohydrate = K_3citric x $1H_2O$)

Citric acid	19.69 g
Potassium citrate	33.25 g
Citric acid solution	57 ml
MilliQ water	Q.S. 500 ml

Dissolve citric acid 19.69 g with milliQ water 400 ml. Then add potassium citrate 33.25 g and next add citric acid solution 57 ml or when pH = 4. Next add more

milliQ water up to total 500 ml. The solution was mixed and sterilized by autoclaving at 121 ºC for 15 min. Store the solution at 4 ºC.

4. H_2O_2+TMB (3,3',5,5'-tetramethyl benzidine) substrate

TMB	2.5 mg
DMSO	250 µl
TMB buffer	9.9 ml
H_2O_2	3.4 µl

Dissolve TMB 2.5 mg in 250 µl of DMSO solution. Then add TMB buffer 9.9 ml and H_2O_2 3.4 µl and then mix the solution.

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