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REGULATIONS OF *BCL6* EXPRESSION BY NOTCH SIGNALING IN HUMAN FOLLICULAR HELPER T CELL

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วิถีสัญญาณน็อทช์เป็นวิถีที่มีมีการแสดงออกในทุกสิ่งมีชีวิตที่มีหลายเซลล์ มีความสำคัญในการควบคุมการแสดงออกของยีนที่เกี่ยวข้องกับการเจริญ และการทำงานของเซลล์ในเซลล์ต่างๆ รวมถึงทีเซลล์กลุ่มผู้ช่วย ฟอลลิคูลาร์เฮลเปอร์ทีเซลล์ (TFH) เป็นหนึ่งในกลุ่มทีเซลล์ผู้ช่วยที่มีการจำแนกจากกลุ่มอื่นๆโดยใช้ลักษณะที่มีการแสดงออก $CXCR5^{hi}ICOS^{hi}CD4^{+}$ บนผิวเซลล์ และมีการแสดงออกของยีน *BCL6* ซึ่งเป็นยีนที่จำเป็นต่อการเปลี่ยนสภาพและรักษาสภาพของTFH TFHสามารถพบได้ในอวัยวะภูมิคุ้มกันทุติยภูมิ เช่น ต่อมทอนซิล ต่อมน้ำเหลือง และม้าม หน้าที่ของ TFH คือ กระตุ้นให้มีการสร้าง germinal center และช่วยบีเซลล์ให้พัฒนาไปเป็นพลาสมาเซลล์หรือเมมโมรีบีเซลล์ และหลั่งแอนติบอดี มีการศึกษาก่อนหน้านี้ได้รายงานว่า พบการแสดงออกของยีนที่เกี่ยวข้องกับวิถีสัญญาณน็อทช์ใน TFH ของมนุษย์ นอกจากนี้ในหนูที่ถูกทำให้ไม่มีการแสดงออกของน็อทช์1และน็อทช์2 ที่ถูกเหนี่ยวนำให้สร้างภูมิคุ้มกันโดยไข่ของ *Schistosoma mansoni* มีจำนวนของ TFH ลดลง เมื่อเทียบกับหนูสายพันธ์ปกติ แต่การควบคุมการเปลี่ยนสภาพของ TFH โดยวิถีสัญญาณน็อทช์ยังไม่มีการพิสูจน์ชัดเจน ดังนั้น งานวิจัยชิ้นนี้มีเป้าหมายเพื่อศึกษาการแสดงออกของวิถีสัญญาณน็อทช์ในการควบคุมการแสดงออกของ *BCL6* ใน TFH ของมนุษย์ โดยแยกเซลล์มาจากต่อมทอนซิลโดยใช้ลักษณะการแสดงออก $CXCR5^{hi}ICOS^{hi}CD4^{+}$ เป็นเครื่องหมาย ซึ่ง TFH ที่แยกได้นั้นมีการแสดงของยีน *BCL6* และ *IL21* รวมถึงน็อทช์รีเซปเตอร์และลิแกนด์ ในระดับสูงกว่ากลุ่มที่ไม่ใช่ TFH นอกจากนี้ยังพบการแสดงออกของโปรตีนน็อทช์1ที่ถูกกัดด้วยเอนไซน์ ซึ่งบ่งชี้ว่าวิถีสัญญาณน็อทช์มีการกระตุ้นในTFH แต่พบว่าการแสดงออกระดับยีนของน็อทช์รีเซปเตอร์และลิแกนด์ลดลงเมื่อ TFH ถูกกระตุ้นโดย CD3/CD28 แอนติบอดี ซึ่งอาจเป็นผลจาก IL-2 ซึ่งเป็นตัวควบคุมเชิงลบของ TFH อีกทั้งพบตำแหน่งโปรตีนของ RBP-Jκ ซึ่งเป็นโปรตีนที่สำคัญของวิถัสัญญาณน็อทช์ในบริเวณ *BCL6* โปรโมเตอร์ ที่ 849 คู่เบสต้นน้ำจากตำแหน่งเริ่มต้นการถอดรหัส ซึ่งพิสูจน์ว่ามีการจับบน *BCL6* โปรโมเตอร์ของน็อทช์1 โดยเทคนิค ChIP และผลการศึกษาพบว่าน็อทช์1 ไปจับบริเวณ *BCL6* โปรโมเตอร์ของ TFH ดังนั้น วิถีสัญญาณน็อทช์มีความสำคัญในการควบคุมการแสดงออกของ *BCL6* ใน TFH

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

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ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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LEELAWALEE RUNGRUANG: REGULATIONS OF *BCL6* EXPRESSION BY NOTCH SIGNALING IN HUMAN FOLLICULAR HELPER T CELL. ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., CO-ADVISOR: PROF. NATTIYA HIRANKARN, M.D. Ph.D., 80 pp.

The highly conserved Notch signaling pathway operates in multi-cellular organisms. It plays important roles in gene expression regulation that controls many cellular processes, including differentiation, proliferation and activation of T helper cells. Follicular helper T cell (TFH), identified as $CXCR5^{hi}ICOS^{hi}$ $CD4^{+}$ subset T cell, is one of the $CD4^{+}$ T helper cells which express *BCL6* as a critical transcription regulator. This cell subset mainly localizes in the germinal center (GC) of secondary lymphoid tissues such as tonsils, lymph nodes and spleen. Their functions are to help maintain the formation of GC, and to regulate differentiation of B cells and production of antibodies. Previous study reported the unique transcriptomic patterns of genes related to the Notch signaling pathway in human TFH. Moreover, in mice with *Notch1/Notch2* double-deficient $CD4^{+}$ T cells immunized with *Schistosoma mansoni* eggs, numbers of TFH decreased and TFH development was impaired. These previous studies suggesting a role of Notch signaling in differentiation and/or functions of TFH. However, how Notch signaling regulates TFH differentiation was not well documented. Therefore, this study proposed to investigate the involvement of Notch signaling pathway in the regulations of *BCL*6 expression in human TFH. TFH from freshly dissected tonsils were sorted using $CD4^{+}CXCR5^{hi}ICOS^{hi}$ as marker. The expression of *BCL6* and *IL21* mRNA were confirmed by quantitative PCR (qPCR) to be higher in these sorted cell population. Moreover, cleaved Notch1 was found in freshly isolated TFH. The expression profiles of mRNA of Notch receptors and their ligands were higher in freshly isolated TFH, compared with nonTFH ($CD4^{+}CXCR5^{lo}ICOS^{lo}$). However, expression of Notch receptors were down-regulated after *in vitro* stimulation using anti-CD3/CD28 antibodies, possibly by negative feedback of IL-2. Promoter analysis of *BCL6* gene identified a putative RBP-Jκ recognition site at -849 bp upstream of transcription starting site. Therefore, the direct association of Notch1 on the *BCL6* promoter of TFH was investigated by Chromatin immunoprecipitation (ChIP) using anti-Notch1 antibody. ChIP analysis revealed specific association of Notch1 with *BCL6* promoter at position 849 base pairs upstream of transcription starting site. Taken together, this result reveals a critical role of Notch signaling in direct regulation of *BCL6* expression.

Field of Study: Medical Microbiology
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I
INTRODUCTION

Background and rationale

Follicular helper T cells (TFH) belong to a subset of helper T cells which is important for humoral immunity. They can be found in germinal center (GC), where isotype switching and affinity maturation of antibodies occur, in the secondary lymphoid organs. Their functions are to sustain B cells to undergo differentiation into plasma cells and produce antibody. TFH identified by expression of surface markers $CD4^{+}CXCR5^{hi}ICOS^{hi}$. They have *BCL6* as a master transcription regulators which is necessary for differentiation. IL-21 is an essential cytokine of TFH that affects B cells survival and development. Dysregulation of TFH contributes to defective humoral immune response.

Notch signaling regulates many cellular processes including activation, proliferation, cell fate decisions and apoptosis. Notch signaling is a highly conserved pathway which operate in various cell types and between vertebrate and mammal. To date, there are 4 different mammalian Notch receptors and five Notch ligands. Previous studies have been reported the regulation of Notch in T helper cells differentiation and their effector functions. Notch is expressed at a low level in naïve CD4 T cells and colocalizes with CD4 upon stimulation. In addition, evidence shows that Notch controls the development of naïve CD4 T cell into T helper1 (Th1) or T helper 2 (Th2) subset depending on the interaction between their receptors and ligands. Remarkably, researchers reported the association of Notch intracellular domain, a part of Notch protein which migrates to nucleus where it regulates the expression of target gene, on

Gata3, *ROR$_\gamma$t* and *Foxp3* promotor, which are master transcription regulators of Th 2, Th 17 and regulatory T cells (Treg), respectively.

The role of Notch in human TFH have been studied by microarray analysis. It was reported that the expression of Notch signaling molecules and Notch target gene were higher in TFH than other T helper subsets. In addition, decreasing in TFH numbers was found in mice with Notch1 and Notch2 targeted deletion. This defect correlates with impaired germinal center formation and B cell development were reported. However, the involvement of Notch in regulation of TFH have not been fully formally addressed. Therefore, this study aims to investigate the role of Notch in human TFH by directly regulating the transcription factor *BCL6*.

Research objectives

1. To investigate the expression profiles of Notch receptors and their ligands in human freshly isolated and stimulated TFH
2. To investigate direct association of Notch receptor on *BCL6* promoter of human TFH

Hypothesis

Notch signaling regulates expression of transcription factor BCL6 by directly association with promoter of *BCL6*

Benefit

The basic knowledge on the involvement of Notch signaling in controlling *BCL6* expression in TFH may help better design of therapy targeting humoral immune response.

CHAPTER II

LITERATURE REVIEW

Follicular helper T cells

Follicular helper T cells (TFH) are helper T cells subset have been discovered in the past decade. They are localize in Germinal center (GC) of secondary lymphoid organs such as lymph nodes, spleen, and tonsils. Human TFH were characterized by expression of *BCL6,* a critical transcriptional regulator for TFH differentiation (5, 6), and expression of surface markers CD4, chemokine receptor 5 (CXCR5), and inducible costimulator (ICOS) or program death1 (PD1) (7). Whereas in mice, TFH were characterized by expression of surface markers CD4, CXCR5 and PD1 (3, 8). TFH can produce many cytokines such as IL-4, IL-10 and importantly IL-21. Signature IL-21 cytokine influences germinal center formation, B cell development and also maintains TFH themselves (9). Their function is necessary for humoral immune response. TFH stimulate B cells to produce immunoglobulins in response to infection. Uncontrolled TFH or defective in TFH can cause pathogenesis related to immune diseases (3, 10, 11).

Phenotypes and characteristics of TFH

Expression of surface marker molecules

Human TFH identified by using the expression of surface markers $CD4^{+}CXCR5^{hi}ICOS^{hi}$ but in some studies, $CD4^{+}CXCR5^{hi}PD1^{hi}$ was used instead. TFH express high level of CXCR5, a homing receptor responding to B cell attracting chemokine-1 (BCA-1/CXCL13) which is secreted by follicular dendritic cells in germinal center. In addition to CXCR5, TFH also highly expresses ICOS which is

defined as marker of activated T cells, whereas PD1 is an inhibitory receptor molecule (12, 13).

B cell lymphoma 6 (BCL6)

BCL6 is a master transcription regulatory essential for TFH development. Naïve T cell upregulates *BCL6* upon receiving IL-6 signal from follicular dendritic cells. After that, they downregulate C-C chemokine receptor type 7 (CCR7) while upregulate CXCR5 to migrate into germinal center (14). *BLIMP1* is a transcription factor antagonist to *BCL6*. Helper T cells that express *BLIMP1* develop to Th1, Th2, Th17 or other helper cell lineages but not TFH (5, 15).

IL - 21

IL-21 is a major cytokine secreted by TFH. It has pleiotropic effects on proliferation, activation and differentiation in many cell types i.e. natural killer cells, dendritic cells, B and T lymphocytes (13). In B cells and TFH, IL-21 from TFH controls the formation of germinal center, regulates plasma cells and memory B cells development. Moreover, IL-21 is an essential autocrine for the regulating survival of TFH (8).

Costimulatory receptors

Costimulatory receptors are important for communication between TFH and B cells. Incomplete of costimulatory signal results in dysregulation of TFH and moreover impairment of humoral immunity. Important costimulatory receptors expressed on TFH are signaling lymphocytic activation molecule-associated protein or SAP, CD40L and CD28, which are complementary for CD84, CD40 and CD86 that are expressed on B cell surfaces, respectively (3, 16).

TFH effector function

TFH is a helper cells subset that important in humoral immune response. Their functions are to maintenance GC B cells and help B cells to differentiate into antibody-secreting plasma cells and memory B cells (12, 17). Without help from TFH via co-stimulatory receptors, such as CD40L, SAP and ICOS, and IL-21 cytokine, B cells are unable to produce antibody and may die by apoptosis. In contrast, B cells that have high affinity to T cell dependent antigen (Ag) and receive complete signaling from TFH can develop into plasma cells and produce antibody (Figure1) (3).

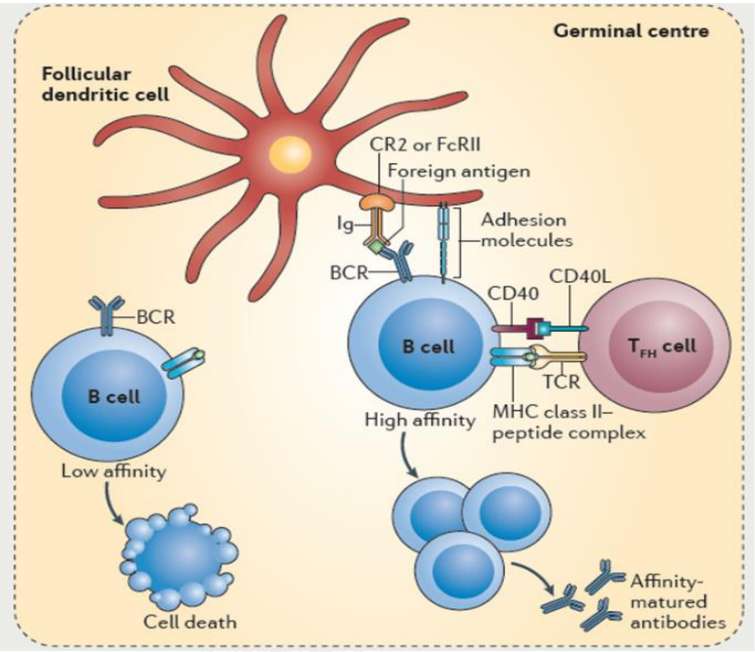


Figure 1 Schematic diagram of interaction between TFH and GC B cells in germinal center

When infection occurs, follicular DC presents T-dependent Ag to GC B cell. Stimulated GC B cell internalize and process Ag and then presents peptide for TFH. After that, with help provided from costimulatory molecules and cytokine IL-21 of TFH, GC B cell differentiates into plasma cells and produce antibody. In the absence of signaling from TFH or low affinity to Ag of GC B cell, GC B cells will die by apoptosis (3).

Role of TFH in diseases

Dysregulation of TFH functions has been found associated with incompetent immune response. Because the functions of TFH are important for immune response, their defects can result in autoimmune diseases and immunodeficiencies as described below (18, 19).

Autoimmunity

Aberration of TFH has been found in many autoimmune disorders such as systemic lupus erythematosus (SLE), Myasthenia gravis, Rheumatoid arthritis, Sjogren's syndrome including autoimmune thyroiditis diseases, Graves's disease or Hashimoto thyroiditis. All of these diseases have high concentration of circulating TFH correlates with elevated levels of serum autoantibodies (3, 11, 20, 21). For example, many previous studies reported the association of TFH in pathogenesis of SLE in both mice and human (22, 23). In mouse model of lupus, researchers demonstrated that increasing of circulating TFH numbers induces the spontaneous formation of germinal center, and autoantibody production which contributes to severity of SLE disease (3, 22). Remarkably, SLE patients have circulating TFH correlating with highly level of autoantibodies which are anti-nuclear antibody (ANA) and anti-double strand DNA. Indeed, SLE patients had higher level of *IL-21* expression than that of healthy controls (3, 23, 24).

Immunodeficiency

Some diseases of immunodeficiency are due to the genetic defects in TFH function and/or differentiation. Well-known common diseases are X-linked lymphoproliferative disease (XLP), hyper IgM syndrome and common variable immunodeficiency (CVID) (3, 10). All of the mutated genes are linked to incomplete

signaling from receptors-ligands on the surface between TFH and B cells. XLP is a primary immunodeficiency that is the result of mutation in gene encoding SAP (3, 25). Mutation in gene encoding CD40L and ICOS causing Hyper IgM syndrome and CVID (26, 27), respectively. So, incomplete signal of costimulatory molecules from the TFH prevent B cells from producing effective antibodies. Furthermore, the mutation of either *CD40L* or *ICOS* results in not only functional but also developmental defects of TFH. In contrast, TFH develop normally but cannot help B cell activation in SAP mutant animal (19, 28, 29).

Negative regulation of TFH

Negative regulation is essential to control TFH activity and prevent autoimmune diseases. To date, there are 2 negative regulators of TFH were reported, transcription factors Foxp1 and IL-2 signaling (30). Foxp1 is a member of transcription factor of the forkhead box (Fox) family. Upregulation of Foxp1 via T cell receptor (TCR) stimulation inhibits TFH cell differentiation. In addition, Foxp1 directly inhibits *IL21* and indirectly limits *ICOS* expression (31). IL-2 signaling negatively regulates TFH in many studies (32, 33). Researcher demonstrated that excessive IL-2 signaling upregulates the expression of *BLIMP1*, an antagonist of *BCL6*, and suppresses the expression of *BCL6* via transcription factor STAT5 (33). Another study reported limitation of TFH development by IL-2 signaling *in vivo* (32). They found that in influenza virus infected mice treated with IL-2 cytokine, decreasing in TFH numbers were found to correlate with impairment of GC B cell development, compared with untreated control (32).

Notch signaling pathway

Notch signaling is an important signaling pathway for communication between cells. It controls development, proliferation, cell fate specification and even in cell death (2). Notch was first characterized in *Drosophila melanogaster* which play a role in wing margin development (34). Notch signaling is a highly conserved pathway form *Drosophila* to human (4). *Notch* gene are encodes type 1 transmembrane protein presented on cell surface. *Drosophila* Notch receptor and the four mammalian Notch receptors (Notch 1, 2, 3 and 4) compose of two main parts, extracellular and intracellular domains. Notch extracellular domain (NECD), required for ligand binding, consisting of multiple epidermal growth factor-like (EGF) repeats and three LIN Notch (LNR) repeats. Notch intracellular domain (NICD) contains a RAM, ankyrin (ANK) repeats which are flanked by two nuclear localization signals (NLS), a transcriptional activation domain (TAD), and a C-terminal Pro Glu Ser Thr (PEST) domain. *Drosophila* Notch ligands are belong to Delta and Serrate. There are five mammalian ligands. Three of them are Delta-like family (Dll1, Dll3 and Dll4). Others are Jagged 1 and Jagged 2. Extracellular domain of ligands contain EGF-like repeats and N-terminal DSL (Delta, Serrate, and Lag-2) domain. Serrate, Jagged 1 and Jagged 2 consist of an additional cysteine-rich (CR) domain between the EGF-like repeats and the cell membrane (2, 35). The structure of Notch receptors and their ligands shown in Figure 2. Interaction between Notch receptors expressed on the surface of a cell and Notch ligands expressed on the surface of adjacent cells leads to activation of the Notch signaling.

Notch does not directly bind to DNA but instead binds to CSL (CBF1, Suppressor of Hairless, Lag-1), a sequence-specific DNA-binding transcription factors,

to regulate gene expression. This protein is downstream of canonical Notch signaling cascade, necessary for NICD binding to DNA. In the steady state, CSL binds to DNA and represses transcriptional expression of Notch target genes. In contrast, when Notch is activated and translocated to the nucleus, CSL binds to NICD and recruits transcription activating complex to turn on the gene expression (35, 36). The consensus recognition sequences for CSL binding has been reported as the following 5'-CGTGGGAA-3' and 5'-TGGGAA-3' (37). Many studies have been reported the association of Notch on promoter. For example, binding of Notch on *IL6* promoter of macrophage cell line was reported (38). Wongchana et al. found the association of NICD with CSL at the position between 67 and 60 bp upstream of the *IL6* transcription promoter starting site (38).

Activation of Notch signaling cascade require the interaction of receptors with ligands. Subsequently, Notch is cleaved by two proteolytic enzymes, disintegrin and metalloproteinase domain-containing protein (ADAM) family and γ-secretase. These cleavages release NICD to translocate from plasma membrane to the nucleus and binds to CSL. After that transcription repressor complexes are replaced by transcription co-activators including mastermind proteins (MAML1-3) and p300 (4). This NICD-CSL and co-activators complexes regulates downstream target genes such as *HES1, MYC, p21 and HEY* to expression (2, 4) (Figure 3). Notch signaling can be inhibited using pharmacological drugs which are γ--secretase inhibitors (GSI). The effects of drugs are inhibition of the cleavage of NICD by enzyme γ-secretase. As a result, NICD cannot translocate into nucleus. Several studies used GSI such as IL-CHO and DAPT to prevent Notch activation (39-43).

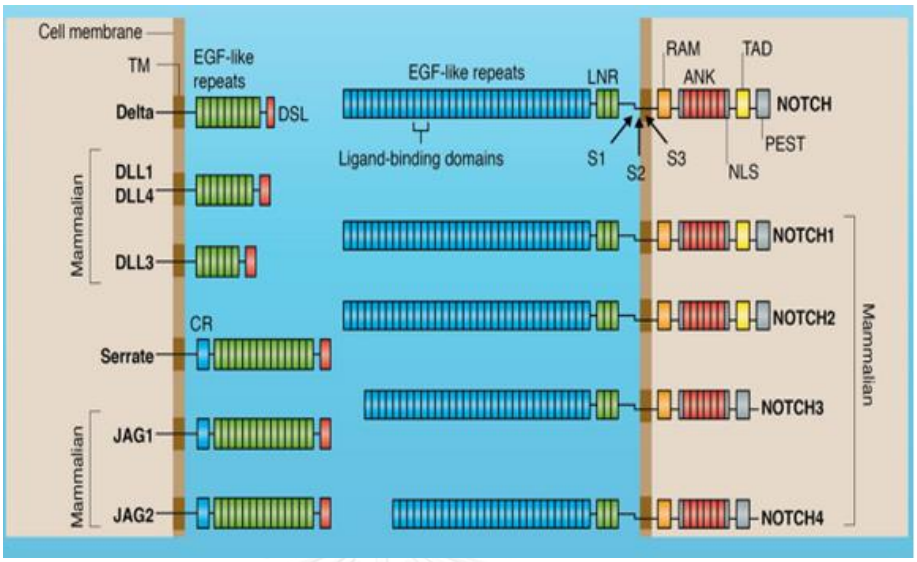


Figure 2 Structures of Notch receptors and ligands

Drosophila and Mammalian Notch receptors are compose of two main parts: extracellular and intracellular domain. NECD consisting of EGF multiple repeats and three LIN repeats. NICD contains of a RAM, ANK repeats flanked by two nuclear NLS, TAD and PEST domain. Extracellular domain of *Drosophila* and Mammalian Notch ligands contain EGF-like repeats and N-terminal DSL domain. Serrate, Jagged 1 and Jagged 2 consist of an additional cysteine-rich (CR) domain (2).

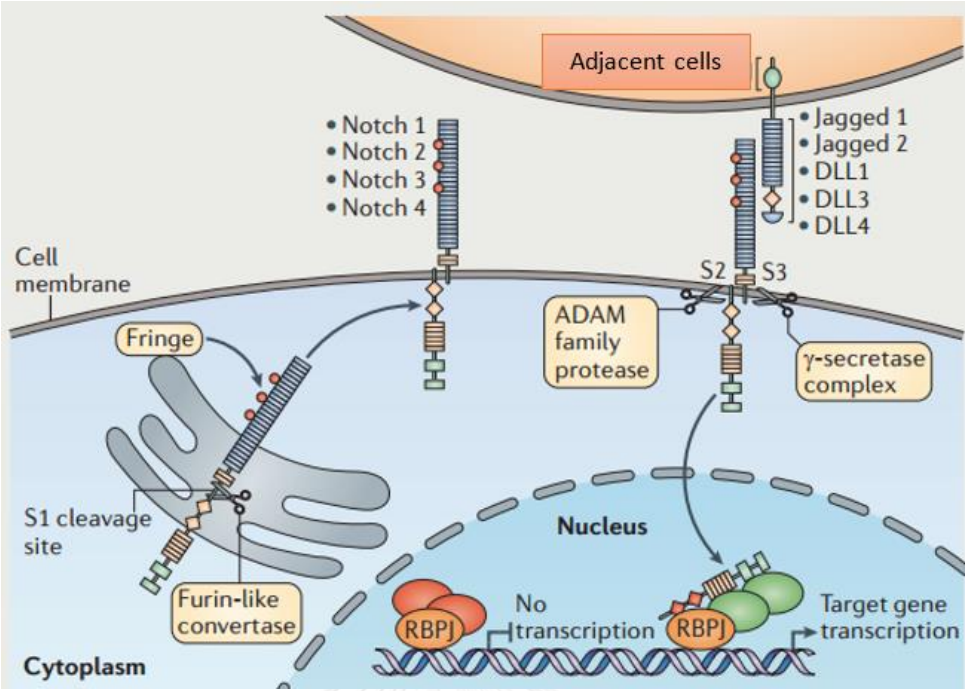


Figure 3 Notch signaling pathway

Notch signaling is initiated by interaction between Notch receptors and its ligand from adjacent cells. Notch receptors are cleaved by two enzymes, a metalloproteinase ADAM family and γ-secretase. Cleavage of Notch result in releasing of NICD which migrates to the nucleus and binds to RBPJk, also known as CSL. NICD-CSL complexes recruit Mastermind-like proteins (MAML1-3) and p300 which are co-activators. These transcription complex turn on target gene expression. In the absence of NICD, CSL form complexes with co-repressors and acts as transcriptional repressor (4).

Role of Notch signaling in T cells

Notch signaling have been well known to be involved in the control of T cell differentiation, proliferation and activation. (44, 45). Notch1 is required for thymocytes differentiation in early phase and important for lymphocytes lineage commitment. Common lymphoid progenitors develop to early thymic progenitors by expressing Notch1 which interact with Dll4 from thymic epithelial cells (4, 45). Notch1 is reported to be involves in T cell activation. Activation of Notch signaling can be induced by T cell stimulation with anti-CD3/CD28 antibodies. Blockage of Notch cleavage using pharmacological inhibitor result in decrease in IL-2 and IFN-γ production (41). Cytokine level in the T cells - dendritic cells co-culture in the presence of OVA peptide and gamma secretase inhibitor (GSI) was detected, compared with DMSO treated control. They reported that production of IL-10, IFN-γ and IL-17 was decreased in T cells treated with Notch inhibitor (46). These results imply that Notch can be activated in T cells via TCR signaling and its activation promotes the proliferation and cytokine production (41, 46).

Role of Notch signaling in helper T cells

Until now, several helper T cell subsets such as Th1, Th2, regulatory T cells (Treg) and TFH have been discovered (47). Master transcription regulator is necessary for helper T cells to differentiate. Upregulation of master transcription regulator in naïve $CD4^+$ T cells depend on cytokine environments and antigen stimulation. Indeed, naïve $CD4^+$ T cells differentiate into Th1, Th2, Th17, Treg and TFH when they upregulate *T-bet*, *Gata3, RORγt, Foxp3* and *BCL6* expression, respectively (21, 48).

The functions of effector helper T cells are different among subsets. Th1, one of the major subsets, plays an important role in cellular response to intracellular pathogen such as *Mycobacterium* spp. They secret large amount of IFN-γ and IL-2 cytokines. They can activate macrophages to kill intracellular microbial and also help cytotoxic T cells to kill infected cells. Overactivation of Th1 can contribute to several autoimmune disorders such as multiple sclerosis and rheumatoid arthritis (48, 49). Th2 are important for the clearance of extracellular pathogens and parasites (49). Major cytokines of Th2 are IL-4, IL-5 and IL-10. IL-4 stimulates *Gata3* expression in Th2 and also IgE production of B cells, which activates mast cells to release histamine. Therefore, besides cellular immune response, Th2 can mediate allergy and hypersensitivity (49-51). Researcher have been reported the association of Notch signaling in Th1 and Th2. The expression and interaction of Notch receptors and ligands on naïve T cell surface drive cell fate specification. For example, binding of Dll family such as Dll1 to Notch3 or Notch1 leads to upregution of *T-bet*, a master transcription regulator for Th1 (40, 52). Whereas interaction of Jagged family with Notch1 or Notch2 lead to Th2 differentiation (4, 53). However, the precise interactions between these receptors-ligands remains to be determined**.**

Effector function of Th17 is to promote immunity against extracellular bacteria and fungi infection. IL-17, a preferential cytokine produced by Th17, recruits neutrophils and macrophages to various infected tissues and stimulates the releasing of antimicrobial peptides (54). Excessive amount of IL-17 and aberrant Th17 activation play a pivotal role in pathogenesis of chronic inflammatory disorders and autoimmune diseases (55).

In contrast to other helper T cell subsets, Treg functions are mainly in order to suppress the immune system. Their cytokines affect in several types of effector immune cell. For examples, IL-10 can inhibit the activation of Th1 and Th2. IL-35 promotes Treg development while suppresses Th17 and Th1 proliferation. TGF-β inhibits the proliferation and activation of macrophages and monocytes (56). Inactivation of Treg cells results in autoimmune diseases or excessive immune response (57).

The association between NCID and promotor of *Gata3*, *ROR$_\gamma$t*, and *Foxp3* promoter have been reported in Th2, Th17 and Treg, respectively (43, 58, 59). Notch is also found to bind to *IL17* promoter of Th17 and this binding disappeared in GSI treated-Th17 (43). The binding of NICD on the promotor suggests the regulation of Notch signaling in gene expression at the transcriptional level. Hence, these results imply that notch regulate the development/differentiation and the function of Th2, Th 17 and Treg. The association of NCID on *T-bet* promotor has not been elucidated. However, there was a report that Notch can upregulate the *T-bet* expression in Th1 (40).

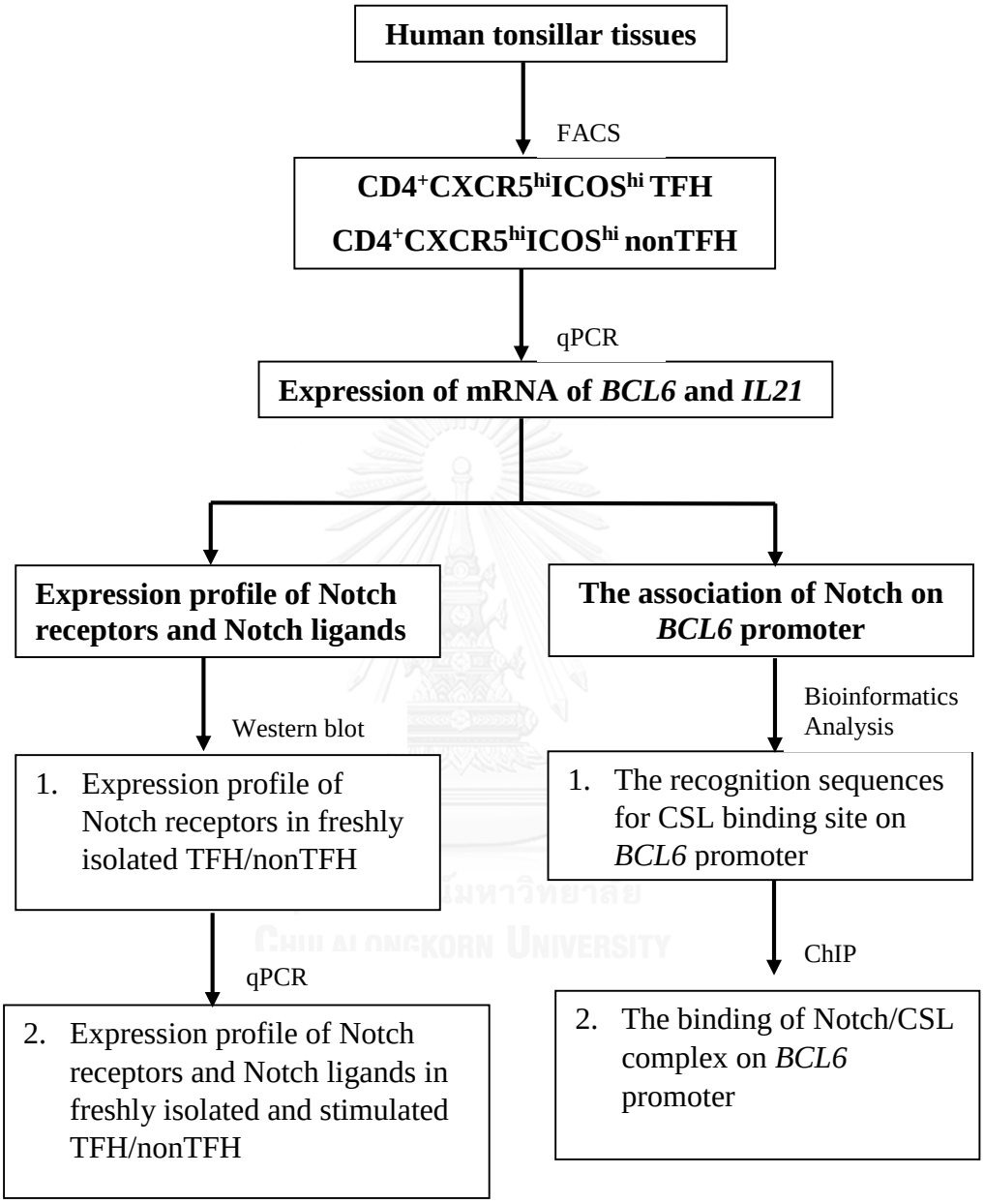
Role of notch signaling in regulation of TFH

Previous study by Rasheed et al. performed the comparative analysis of gene expression involved in cellular processes such as signaling, activation, differentiation and apoptosis of human tonsillar and peripheral blood CD4 T cells. (7). They reported that gene related to Notch signaling pathway are upregulated in the tonsillar CD4 T cell subsets, compared to peripheral blood CD4 T cell subsets. Interestingly, $CXCR5^{hi}ICOS^{hi}CD4^{+}$ T cells exhibited a unique high expression pattern of proteins related to the Notch signaling pathway.

Furthermore, Notch signaling regulating TFH in mice were also reported (60). T cell–specific deletion of Notch1 and Notch2 ($N1/N2^{-/-}$) in C57BL/6 mice immunized with or infected with parasites exhibited impairment in differentiation of $CD4^{+}CXCR5^{hi}PD1^{hi}$ TFH. In addition, impaired TFH differentiation correlated with strongly reduced numbers of GC B cells and the absence of high-affinity antibody (60). $N1/N2^{-/-}$ TFH cell not only defected in differentiation, but also in function. The level of mRNA expression of *BCL6* and *IL21* in TFH, nonTFH and non activated cells subsets of $N1/N2^{-/-}$ mice immunized with *S. mansoni* egg were compared. $N1/N2^{-/-}$ TFH had lower expression of both genes compared with normal mice. Diminished *IL21* expression can be explained deficiency in germinal center development and the absence of high-affinity antibody in $N1/N2^{-/-}$mice.

Moreover, previous study of Inrun et al. reported the expression profiles of Notch receptors and Notch target gene in human TFH. This study reported the higher expression of mRNA of *Notch1, Notch3* and *HES1,* a target gene of Notch signaling pathway, in freshly isolated TFH, compared with nonTFH (1). Importantly, inhibition of Notch cleaved by GSI in TFH resulted in decreasing of *BCL6* expression, compared with untreated control (1). All of previous reports suggested the involvement of Notch signaling in regulation of TFH. However the direct evidence linking Notch and BCL6 is lacking. Hence, in this study, we aimed to directly determine the role of Notch in TFH on *BCL6* expression, using Chromatin immunoprecipitation (ChIP) approach.

Methodology Overview



CHAPTER III

MATERIAL AND METHOD

3.1 Samples

Human tonsil tissues were collected from individuals who had tonsillar hypertrophy and undergone tonsillectomy. All samples were obtained from department of Otolaryngology, King Chulalongkorn Memorial Hospital. This project was ethically approved from Institutional review board (IRB), faculty of Medicine, Chulalongkorn University (IRB.No.539/55). The clinical indication for tonsillectomy are shown in Table 1. The donor who have chronic infection caused by hepatitis B virus (HBV) or human immunodeficiency virus (HIV), tonsillar abscess, neoplasia and genetic disorders were excluded from this study.

Table 1 The indication for tonsillectomy

Indication for tonsillectomy	
obstruction	1. Tonsillar hyperplasia 2. Sleep apnea 3. Failure to thrive 4. Col pulmonale 5. Swallowing abnormality
Neoplasia	Suspected neoplasia
Infection indication	• Recurrent infection 1. 3 episodes/year each 3 years 2. 5 episodes/year each 2 years 3. 7 episodes/year in a year • Peritonsilla abscess • Halitosis • Obstructive symptom

3.2 Antibody for cell sorting and cell stimulation

APC-anti-CD4, Alexa- Flour®488-anti-CXCR5 and PerCP-anti-CD278 (ICOS) antibodies (BD Biosciences, USA) were used to stain the mononuclear cells derived from tonsils. These antibodies are specific for surface markers that can separate TFH and nonTFH from others. Anti CD-3 and CD-28 antibody were used to stimulate the cells. (BD Biosciences, USA)

3.3 Isolation of TFH and nonTFH

Cells were isolated from fresh tonsils by mechanical disruption using blenders. Tonsillar cells were washed with RPMI (Hyclone) and then filtered with the 40µm cell strainer (BD falcon, USA). After that, tonsillar mononuclear cells were collected by gradient density method. In brief, tonsillar cells in RPMI suspension were slowly added to Ficoll-Hypaque (Sigma, USA) and then centrifuged at 2000 rpm for 30 minutes at room temperature (Becton, USA). The mononuclear cells appeared as white ring above ficoll-hypaque. The collected mononuclear cells were washed with sterile phosphate buffer saline (PBS) (Sigma) at 1500 rpm centrifugation for 5 minutes. 10 µl of Cells suspension were mixed with 90 µl of 0.4% trypan blue and counted by hemocytometer to calculate the total cell numbers. Cells were labelled with anti-CD4, anti-CXCR5 and anti- ICOS antibodies for 30 minutes in dark at 4°c. After that, the labeled cells were washed with ice cold PBS and centrifuged at 6000g 4°C for 5 minutes. Following the washing step, 10% Fetal Bovine Serum (FBS)/PBS were added to the labelled cells. Subsequently, cells were transferred to polystyrene round-bottom tube (BD falcon, USA). Cells were sorted by Fluorescence activated cell sorting (FACS) using FACSAriaII (BD Biosciences CA, USA). Sorted cell were suspended in 10%FBS in

PBS. TFH and nonTFH were defined as $CD4^{+}CXCR5^{hi}ICOS^{hi}$ cells and $CD4^{+}CXCR5^{lo}ICOS^{lo}$ cells, respectively.

3.4 Cell culture and stimulation

24-well plate (NuncTM, Thermoscientific, USA) were coated with 1 µg/ml anti CD-3 and CD-28 at 4°C overnight. Before using, plate was washed with sterile PBS for 3 times to remove unbounded antibodies. TFH/nonTFH were cultured in plate bounded with anti CD-3and CD-28 in completed RPMI media (Appendix 1). The plate was incubated at 37°C in 5% carbon dioxide incubator (Thermo electron, USA) for 24 hours. Cells were harvested and washed with sterile PBS then centrifuged at 5000 rpm for 5 minutes. Supernatant was removed. Cells pellet were stabilized in RNA later (Ambion, USA) and kept at -80 °c.

3.5 Western Blot

3.5.1 Protein Extraction and quantitation

Total protein from freshly isolated TFH/nonTFH were extracted by using buffer A and buffer B (Appendix 3). First, sorted TFH/nonTFH were washed with ice cold PBS 1 ml and 200 µl of buffer A followed by centrifuged at 8000rpm 4°C for 10 minutes, respectively. Buffer A was removed and 30 µl of buffer B which is lysis buffer to extract protein was added to each sample. Samples were centrifuged at 10000 rpm 4°C for 10 minutes then transferred the supernatant to a new 1.5 ml eppendorf (epf) tube (Axygen scientific, USA). Total protein were kept at -80°c. The concentration of total protein were detected by Bicinchoninic acid ™ (BCA) protein assay. Bovine albumin serum (BSA), were used as standard protein at concentration 1, 0.5, 0.25, 0.125, 0.063, and 0.031µg/µl, and extracted protein from TFH/nonTFH in dilution 1 µl:10 µl of sterile deionized water were added to each well in 96-well microtiter plate

(NuncTM, Thermoscientific, USA). Each well was added by 200µl of working reagent which was composed of reagent A and B (BCA protein assay reagent, Thermo scientific). The plate was incubated for 30 minutes at 37°C followed by an absorbance measurement at 540 nm using microplate reader anthos2010 (Biochrom, United Kingdom)

3.5.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer

SDS-PAGE gel was performed following the formulas shown in Appendix 6. Protein samples (µg) were mixed with 2XLaemmli buffer (Appendix 8) in an equal volume then heated at 100°C for 5 minutes on Thermomixer (Eppendorf, Germany). Heated protein samples and prestained molecular weight markers (Fermentas, Canada) were loaded into the gel that was immersed in the running buffer (Appendix 7). The sample were separated at 100 volts constantly for at least 120 minutes using Protein III system (Biorad, USA) instrument. After gel electrophoresis was finished, stacking gel were cut. Separating gel and six pieces of Whatman filter paper were equilibrated in transfer buffer (Appendix 9) for 5 minutes. Polyvinylidene fluoride (PVDF) membrane (GE Healthcare, USA) were soaked in absolute methanol then rinsed with deionized water twice before using. After that, three pieces of filtered papers were placed in semi-dry transfer Trans-Blot®SD (Biorad, USA). PVDF and separating gel were placed on filter papers, respectively. Lastly, three pieces of filter papers were placed on the top and air bubbles were eliminated by gentle rolling a glass tube. The semi-dry transfer was carried out under the condition using a constant current at 90mA for 90 min.

3.5.3 Antibody probing

After protein transfer was completed, the PVDF membrane were blocked with blocking solution (Appendix 4) twice for 5 minutes each on Labnet Rocker 25 (Labnet Internationational Inc, USA). Primary antibodies for probing were rabbit anti claved Notch1 (Cell signal Technology, USA), rabbit anti Notch1 (Santa cruze biotechnology, USA), rabbit anti cleaved Notch2, rabbit anti Notch2, rabbit anti Notch3 (Cell signal Technology, USA) and anti β-actin (Millipore, USA). PVDF membrane was probed with each primary antibody on rocker at least 1 hour before incubate at 4°C overnight. Primary antibody was removed then probed PVDF membrane was washed with washing solution (Appendix 5) for 5 minutes twice and 15 minutes twice. After that, membrane was incubated with each secondary antibody for an hour on rocker. Goat anti rabbit (GαR) and goat anti mouse (GαM) conjugated horse-radish peroxidase (HRP) (Amersham Bioscience, UK) are secondary antibodies that used in this study. After incubation, secondary antibody was removed. The membrane was washed with washing solution for 5 minutes twice and 15 minutes for three times. Primary and secondary antibodies were diluted in blocking solution before using as described in the Table 2.

Table 2 Working dilution of primary and secondary antibodies

Primary antibody	Working dilution	Secondary antibody	Working dilution
Cleaved Notch1	1:1000	Goat anti Rabbit IgG	1:2000
Notch1	1:2000	Goat anti Rabbit IgG	1:4000
Cleaved Notch2	1:2000	Goat anti Rabbit IgG	1:4000
Notch2	1:2000	Goat anti Rabbit IgG	1:4000
Notch3	1:1000	Goat anti Rabbit IgG	1:4000
β-actin	1:10000	Goat anti Mouse IgG	1:4000

3.5.4 Signal detection by chemiluminescence and autoradiography

The substrates for detection were composed of solution A and B as shown in Appendix 10. Briefly, solution A and B were freshly prepared, mixed and immediately probed to the membrane. After probing for 1 minute, membrane was covered with the plastic wrap and placed in Hypercassette (Amershan Bioscience, UK). Then High performance Chemiluminescence Film: Amershan Hyperfilm^{TM} ECL X-Ray film (Amershan Bioscience, UK) was used.

3.6 RNA Extraction and purification

Total RNA was extracted from freshly sorted/stimulated TFH and nonTFH using QIAGEN RNeasy mini kit (Hilden, Germany). According to the instruction of manufacturer, cells were disrupted with RLT buffer and homogenized by pipetting. Samples were added with 70% ethanol and then transferred to the RNeasy MinElute® spin column. This column is silica-membranes that permit RNA binding. Total RNA was washed with RW1 and RPE buffer, respectively. For the elution step, 30 µl of RNase free water was added to the column then centrifuged at 8000g for 1 minute. Total RNA was preserved at -80°c.

3.7 RNA Quantification

RNA sample concentration was measured using Nanodrop 1000 Spectrophotometer (Thermoscientific, USA). To determine concentration and check purity of RNA, 2 µl of RNA samples were subjected to absorbance measurement at 260 (OD_{260}) nm and 280 nm (OD_{280}) in the spectrophotometer. The concentration of RNA was calculated from OD_{260} reading (1.0 is equivalent to about 40 µg/ml of RNA). The purity of RNA was evaluated from a ratio of OD_{260}/ OD_{280} (Range for appropriate quality were 1.8-2.0).

3.8 cDNA synthesis

The reverse transcription of RNA to single-strand complementaryDNA (cDNA) was performed by using reagents of QIAGEN Quantitect® probePCR kit (Hilden, Germany). From manufacturer's protocol, reagent for convert RNA to cDNA was consisted of 10XRT buffer, 25mM MgCl2, 10mM 4dNTP, 50µM random hexamer, 20U of RNase inhibitor, and 50U of superscript Reverse Transcriptase. Mixed reagent was added to RNA. The RT-PCR program was set as following; 25°C for 10 minutes, 48 °C for 30 minutes and 95 °C for 5 minutes by PCRSprint (Thermo Scientific Hybaid, Rockford, U.S.A.).The cDNA were kept at -20°C.

3.9 Quantitative Polymerase chain reaction (qPCR)

Gene expression was measured by semi-quantitative RT- PCR using MJ miniOpticon (Bio-Rad, CA, U.S.A.). This qPCR amplification was performed with 1x IQ™SYBR®Green Supermix (BioRad, USA), 0.3µM of Forward (Fwd) and Reverse (Rev) primers, Rnase free water and 2µl of cDNA. Reaction without cDNA were used as negative control. The condition for qPCR amplification was initially denaturing at 95°C for 5 minutes, then amplification step follow as denaturation at 94°C for 30

seconds, annealing, and extension at 72°C for 30seconds. Temperature of annealing step was varied in each gene for 30seconds. The relative expression level of each gene was normalized to that of a house keeping gene *GAPDH.* The fold change of mRNA expression were calculated from level of mRNA expression of TFH compared with nonTFH. Primer sequences, product size and annealing temperature were summarized in Table 3.

Table 3 The nucleotide sequences of primers, product size (base pairs, bp) and annealing temperature for qPCR

Primer	Sequences	Product size (bp)	Annealing temperature (°c)
Notch1	Fwd 5'-CAGCCTGCACAACCAGACAGA-3' Rev 5'-TGAGTTGATGAGGTCCTCCAG-3'	280	55.5
Notch2	Fwd 5'-TGAGTAGGCTCCATCCAGTC-3' Rev 5'-TGGTGTCAGGTAGGCATGCT-3'	530	55.5
Notch3	Fwd 5'-TCTTGCTGCTGGTCATTCTC -3' Rev 5'-TGCCTCATCCTCTTCAGTTG -3'	485	56
Notch4	Fwd 5'-CACTGAGCCAAGGCATAGAC -3' Rev 5'-ATCTCCACCTCACACCACTG -3'	472	56
Dlk1	Fwd 5'-CCACGCAGATCAAGAACACC-3' Rev 5'-TTGCTATGACGCACTCATCC-3'	336	58
Dlk3	Fwd 5'-TTCCCTACCCTTCCTCGATT -3' Rev 5'-ATGGCAGGTAGCTCAAAACG-3'	133	57
Dlk4	Fwd 5'-GCGAGAAGAAAGTGGACAGG -3' Rev 5'-ACAGTAGGTGCCCGTGAATC-3'	166	57
Jagged1	Fwd 5'-TCCCGTGAAGCCTTTGTTAC -3' Rev 5'-AAGGGGTGCGGTATATTTCC-3'	104	55
Jagged2	Fwd 5'-AGCTGGAACGAGACGAGTGT-3' Rev 5'-TCTTGCCACCAAAGTCATCA -3'	222	60
BCL6	Fwd 5'-GCCGGACACCAGGTTTTG-3' Rev 5'-AGGCCATTTTGTCTTCACCAA -3'	68	61
IL21	Fwd 5'-TCTGCCAGCTCCAGAAGATGT -3' Rev 5'-GGCCTTCTGAAAGCAGGAAA -3'	67	62
IL2	Fwd 5'-ATGTACAGGATGCAACTCCTGTCTT -3' Rev 5'-GTTAGTGTTGAGATGATGCTTTGAC -3'	458	55
GAPDH	Fwd 5'-ACCACAGTCCATGCCATC -3' Rev 5'-TCCACCACCCTGTTGCTG -3'	452	60

3.10 Statistical analysis

The data was calculated the level of significant using paired and unpaired T-test. P-value less than 0.05 was statistically significance.

3.11 Chromatin Immunoprecipitation

3.11.1 Crosslinking protein-DNA binding

Freshly isolated TFH and nonTFH were fixed with 37% Formaldehyde (final concentration of formaldehyde in samples was 1%) to preserve the crosslinking of protein and DNA. After that, 10X Glycine (Appendix 13) was added at final concentration 0.125M to prevent over fixation. Cell suspension was centrifuged at 5000 rpm 4°C for 5 minutes followed by washing cells in 1 ml of ice cold PBS which contain 1x Protease inhibitor. Cells were lysed in 250 µl of SDS lysis buffer (Appendix 14) preparing to sonication. This step can be kept at -80°c.

3.11.2 Sonication to shear DNA

Before going to sonication step, remove one 10µl of each samples as unsheared genomics DNA and save at 4°c. Condition for sonication was set at 25% amplitude, pulse on 10 seconds and pulse off 20 seconds for 36 cycles using Vibrawcell™ sonicator (Sonics, USA). Samples in epf tube were sonicated on ice cold water. After sonication, Supernatant was collected from centrifugation at 12000g 4°C for 10 min and kept at -80°c. 10 µl of supernatant of sheared samples were removed before frozen to check the length of smear DNA. Each of 10 µl of aliquot unsheared and sheared samples were added with 40 µl of sterile water and 2 µl of 5M NaCl. Samples were incubated at 65°C for at least 4 hours to a night. After this step, samples can be stored at -20°C for a night. After 65°C incubation, 1µl of RNaseA was added to samples and incubated in 37°C water baht (member, USA) for 30minutes. Subsequently, samples

were added with 2µl of 0.5M EDTA, 4µl of 1M Tris-HCL and 1µl of Proteinase K and then incubated at 45°C for 1-2 hours. 25 µl of samples were mixed with 5µl of 30% glycerol (Appendix 28) before loading on a 1.5% agarose gel with a 100bp DNA marker. Gel were run in 0.5X TAE buffer (Appendix 26) at 100 volt 40 min using I-mupid (Eurogentec, Belgium). The gel was stained for 10 minutes with ethidium bromide then soaked with tap water. The length of sheared DNA in gel were detected by Documentation and Quantityone 4.4.1 instrument (Biorad, USA). The optimal of sheared DNA length were 200-1000 bps.

3.11.3 Immunoprecipitation (IP) of crosslinked Protein/DNA

Sheared samples were added with dilution buffer (Appendix 19) to 500 µl volume. ChIP-Grade Protein G agarose bead (cell signaling, USA) were added to samples and incubated at 4°C for 1 hour with rotation using mini rotator Bio RS-24 (Biosan, Latvia). After that, protein G beads were pelleted by centrifugation at 3000g 4°C for 1 minute. The supernatant was transferred to new 1.5 ml Eppendorf (epf) tubes. Samples were divided into 3 epf tubes. One tube with 10 µl of supernatant was set as an input and saved at 4°c. The others were 200 µl of supernatant that prepared for IP reactions. 2 µg of Notch1(C-20) Rabbit polyclonal IgG (cell signaling, USA) and Normal Rabbit IgG (Santa cruze, USA) were added to each tube of IP reactions and incubated at 4°Cwith rotation overnight. After that, protein G agarose beads were added to the samples with rotation for 1 hour at 4°c. After pellet beads by centrifugation at 3000 g 4°C for 1 minute, supernatant was removed. Then beads were washed by resuspending in 500 ml of each ice cold buffer and incubating for 5 min on a rotating platform followed by brief centrifugation at 3000g, at 4°C for 5 minutes and careful removal of the supernatant fractions. Ice cold buffer that used for washing were low

salt immune complex, high salt immune complex and Licl immune complex wash buffer followed by TE buffer (repeat twice). (All of ice cold buffer were shown in Appendix 20, 21, 22, and 23, respectively).

3.11.4 Elution of protein/DNA complexes

Elution buffer (Appendix 24) was prepared at 200 µl per 1 reaction. Input tube was added with 200 µl of elution buffer. IP tubes were added with 100 µl of elution buffer and incubated for 15 minutes at 25°C. After that, IP tubes were centrifuged at 3000g, 4°C for a minute. The supernatant was transferred to the new epf tubes. Elution buffer 100 µl was added again to the old IP tubes and incubation step were repeated to collect the supernatant at total volume 200 µl.

3.11.5 Reverse crosslinking of protein/DNA complexes

All tubes (Input and IP) were added with 8µl of 5M NaCl and incubated for at least 4 hours to overnight at 65°c. Each tube was added with 1µl RNaseA and incubated for 30 minutes at 37°c. Followed by 4µl of 0.5M EDTA, 8 µl of 1M Tris-HCL and 1 µl of Proteinase K were added to samples then incubated at 45°C for 1-2 hours.

3.11.6 DNA purification

The extraction and purification of reverse crosslinked DNA samples were performed by using QIAamp®DNA blood mini kit (Qiagen, USA). In brief, samples were added with proteinase K and buffer AL then incubated at 56°C for 10 minutes. After that, Absolute ethanol was added to samples and transferred to the column in collection tube. After centrifugation the collection tube at 8000 rpm for 1 minute, AW1 was added to collection tube and centrifuged again as described above. The collection tube was changed to a new one. The column was washed again with AW2 and

centrifuged at 14000 rpm for 2 minutes and moved from the collection tube to a new 1.5 ml epf tube. At least 25 µl of AE buffer was added to the column. After that, samples were incubated for 10 minutes at 25°C then centrifuged at 10000 rpm for 2 minutes. The column was removed. The total DNA was preserved at -20°c.

3.11.7 Detection the binding site on promoter by PCR

The CSL binding site of *BCL6* promoter were detected by PCR using Life Express Thermal Cycler bioer instrument (Bioer, China). 5 µl of DNA were mixed with 0.2 µM Fwd and Rev primers, human hypure water and 1X Maxima Hot start Green PCR master mix (Thermoscientific, USA). The primers that used were Fwd 5' -ACCTTGAAACGAGCCTCGAA-3' and Rev 5'-TGCTGAAGTGTGTCTCTCCTG-3'. The PCR amplification was programmed as follow; initial denaturation at 94°C for 5 minutes, denaturation at 94°C for a minute, annealing at 60°C for a minute, extension at 72°C for a minute. Denaturation to extension step were repeated totally 49 cycles. The PCR product was analyzed on an agarose gel (2%) using gel electrophoresis at 100 volt for 30 minutes. The amplified band were detected by Documentation and Quantityone 4.4.1 after staining the gel with ethidium bromide. The product size is 277 bp.

3.11.8 External control

Jurkat was used as external control. Sonication of Jurkat was set at 24% amplitude, pulse on 10 seconds and pulse off 20 seconds for 18 cycles. *HES1* promotor was detected by using primer Fwd' CCCTGGCTCCAAAAGAAATAGAC and Rev' CGAACGGCTCGTGTGAAACTTC (Alexei et al., 2013). This gene was amplified by PCR program as follow: initial denaturation, denaturation, annealing and extension at

95°C for 5 minutes, 95°C for 30 seconds, 62°C for 30 seconds and 72°C for 30 seconds, respectively. Denaturation to extension step were repeated totally 45 cycles. The size of product is 284 bp.

CHAPTER IV

RESULTS

4.1 Isolation of TFH and nonTFH from freshly dissected human tonsils

To confirm the characteristic of TFH isolated using the protocol described by Inrun (shown in Figure 4) (1), TFH and nonTFH were isolated and subjected for analysis of gene expression. Using the gating strategy as described in Figure 4, tonsillar $CD4^{+}CXCR5+ICOS+$ T cell were subdivided into 2 populations, i.e. $CD4^{+}ICOS^{hi}CXCR5^{hi}$ TFH and $CD4^{+}ICOS^{lo}CXCR5^{lo}$ nonTFH. This gating strategy may fail to identify 3 cell subsets as described by Rasheed et al. (7). Therefore, in this study, a more careful gating strategy was employed. As shown in Figure 5, population of tonsillar mononuclear cells were gated for $CD4^{+}$ T cells (L2) and the L2 subset was divided into 3 groups based on the level of ICOS and CXCR5 expression. With this gating strategy, tonsillar $CD4^{+}$ T cell can be devided to 3 popuations; $CD4^{+}ICOS^{hi}CXCR5^{hi}$, $CD4^{+}ICOS^{int}CXCR5^{int}$ and $CD4^{+}ICOS^{lo}CXCR5^{lo}$ as shown in Figure5. Cells in the L3 and L5 subset were used as TFH and nonTFH, respectively, for further study. Therefore, this gating strategy was employed for this study which is different from that of Inrun and may yield different results.

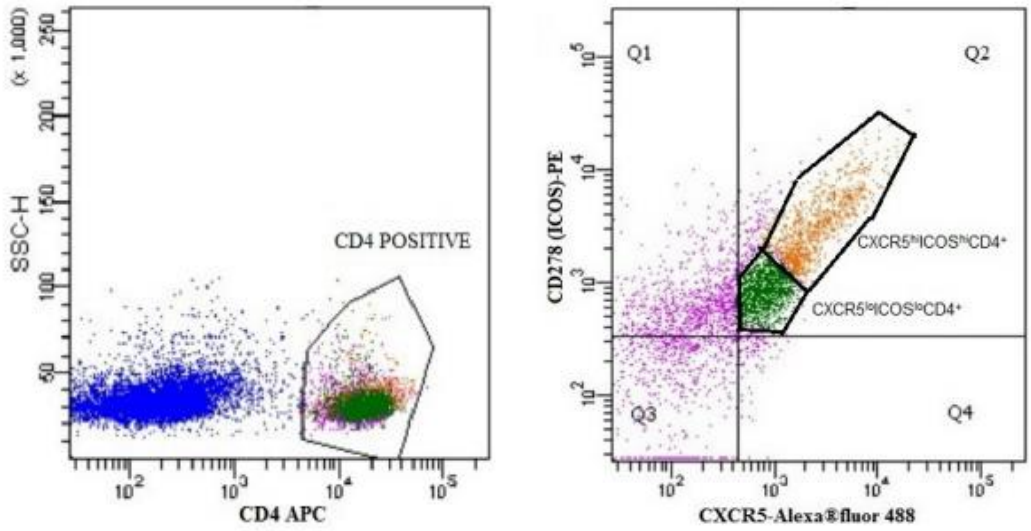


Figure 4 Gating strategy to identify human tonsillar CD4+ cells as described by Inrun (1)

Human mononuclear cells were labelled with anti-CD4, CXCR5 and ICOS antibodies. Cells were gated on $CD4^+$ population. Within the $CD4^+$ subset, cells were further gated into 2 populations by the expression of ICOS and CXCR5. $CD4^+ICOS^{hi}CXCR5^{hi}$ were determined as TFH (orange) and $CD4^+ICOS^{lo}CXCR5^{lo}$ were used as nonTFH control (green).

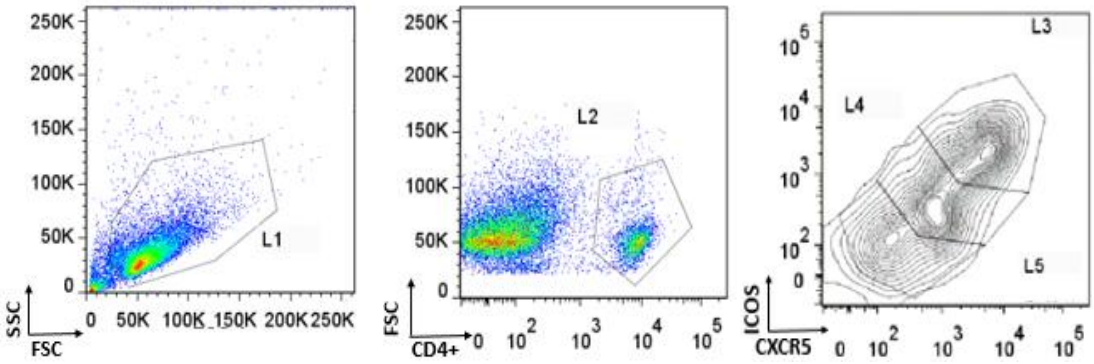


Figure 5 Gating strategy to identify human tonsillar $CD4^+$ cells used in this study

Human tonsillar $CD4^+$ T cells subset (L2) were gated from mononuclear cells (L1). $CD4^+$ T cells subset can be divided into 3 subsets. TFH and nonTFH were identified as subsets of $CD4^+ICOS^{hi}CXCR5^{hi}$ (L3) and $CD4^+ICOS^{lo}CXCR5^{lo}$ (L5), respectively. Population of L4 were $CD4^+ICOS^{int}CXCR5^{int}$ which were not used in this study.

4.2 Expression of signature genes, *BCL6* and *IL21* in freshly isolated TFH and nonTFH

Using the gating strategy described previously, TFH and nonTFH were sorted by cell sorter. The expression of *BCL6* and *IL21* mRNA were detected to confirm the identity of sorted TFH. *BCL6*, a critical transcription regulator, is important for differentiation and function of TFH. IL-21 is a signature and necessary cytokine to promote survival and also functions of TFH. Total RNA from freshly isolated TFH and nonTFH were converted to cDNA and the relative mRNA level of *BCL6* and *IL21* were determined by quantitative realtime PCR (qPCR). The relative mRNA expression of each gene was normalized to that of a house keeping gene *GAPDH*. The level of mRNA in the nonTFH was set as baseline. The results of *BCL6* and *IL21* mRNA expression are shown in Figure6 and Figure7, respectively. In three individuals, all TFH expressed significantly higher level of *BCL6* and *IL21* than in nonTFH. These results confirmed the identity and phenotypes of TFH as gated and sorted by the strategy described above. Therefore, the population of $CD4^{+}ICOS^{hi}CXCR5^{hi}$ (L3) as TFH and $CD4^{+}ICOS^{lo}CXCR5^{lo}$ (L5) as nonTFH were used further in this study.

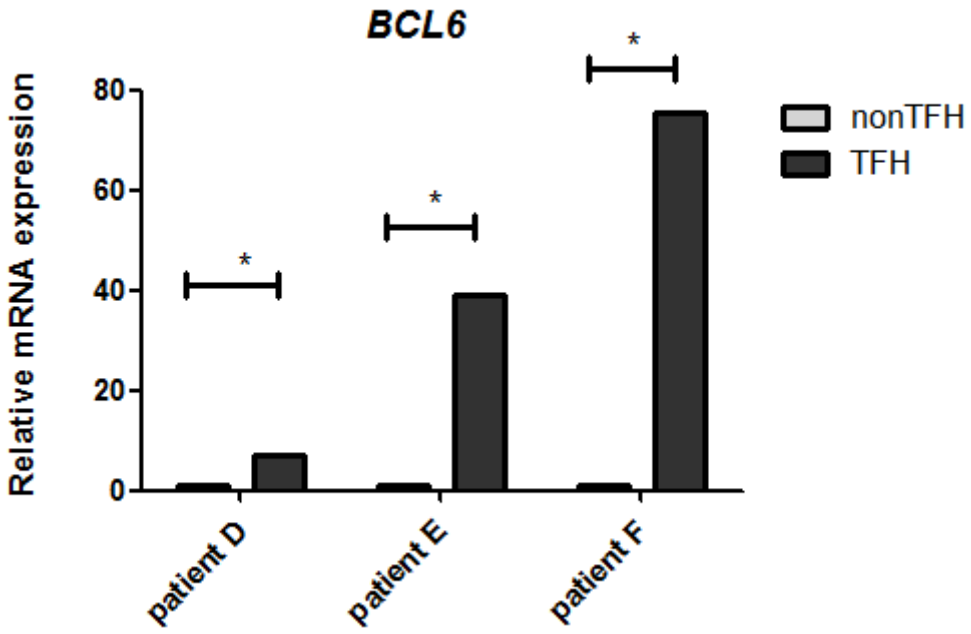


Figure 6 Expression of BCL6 mRNA in freshly isolated TFH and nonTFH

Expression of *BCL6* mRNA were detected to confirm the signature gene expression of freshly isolated human TFH. Data shown the level of mRNA expression of *BCL6* in TFH were higher than nonTFH. *GAPDH* was used as housekeeping gene. The patient D, E and F represent different individual (n=3). Each experiment performed intripicate. (* considered statistically significant p-value < 0.05)

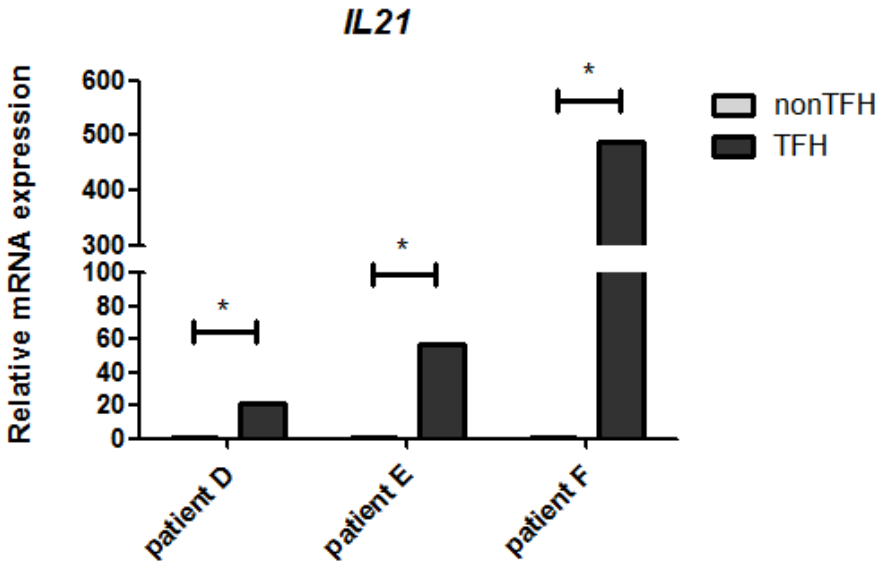


Figure 7 Expression of *IL21* mRNA in freshly isolated TFH and nonTFH

IL21 mRNA expression was detected to confirm the signature cytokine expression of freshly isolated human TFH. Data shown level of mRNA expression of *IL21* in TFH was higher than nonTFH. *GAPDH* was used as housekeeping gene. The patient D, E and F represent different individual (n=3). Each experiment performed intripicate. (* considered statistically significant p-value < 0.05)

4.3 The expression of Notch receptors in freshly isolated TFH and nonTFH

Previous report investigated the expression of Notch receptors in freshly isolated TFH at the transcriptional level and it showed that the relative level of mRNA of *Notch1* and *Notch3* was higher in TFH, compared to the nonTFH (1). Therfore, the expression of Notch receptors were confirmed in this study by Western blot. TFH isolated using method described by Inrun and the method adopated in this study were compraed. The results are shown in Figure 8 and Figure 9 . The level of cleaved Notch1 (val 1744), Notch1, cleaved Notch2 and Notch2 differed among individuals tested. Different gating protocol did not yield clear difference. This may be due to difference in genetic background and other factors such as age, gender or disease setting of individual. Furthermore, no clear difference was found at the level of all protein tested between TFH and nonTFH. These results suggest that at the time of isolation where cells have already committed to TFH, there is no differnce in the level of Notch receptors. The appearance of cleaved Notch1 suggesting that Notch signaling is activated in both TFH and nonTFH.

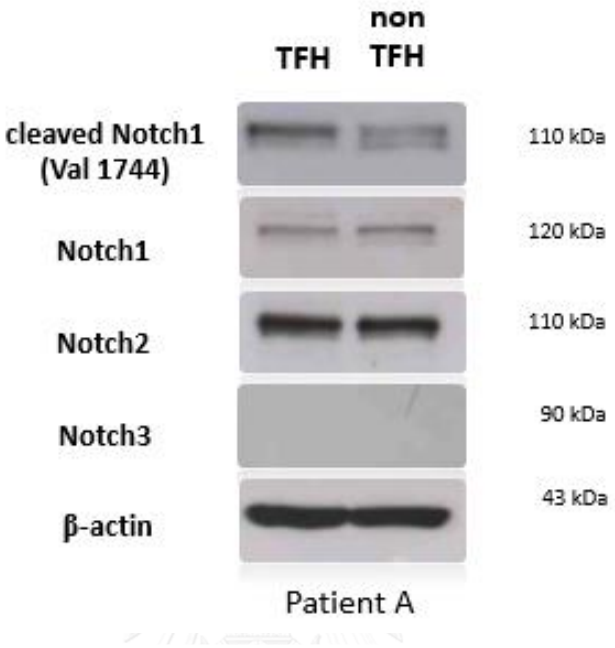


Figure 8 Expression profiles of Notch receptors in freshly isolated TFH and nonTFH using gating strategy described by Inrun (1)

Cleaved Notch1, Notch1, Notch2 and Notch3 in freshly isolate TFH and nonTFH were detected by Western blot. β-actin was used as internal loading control.

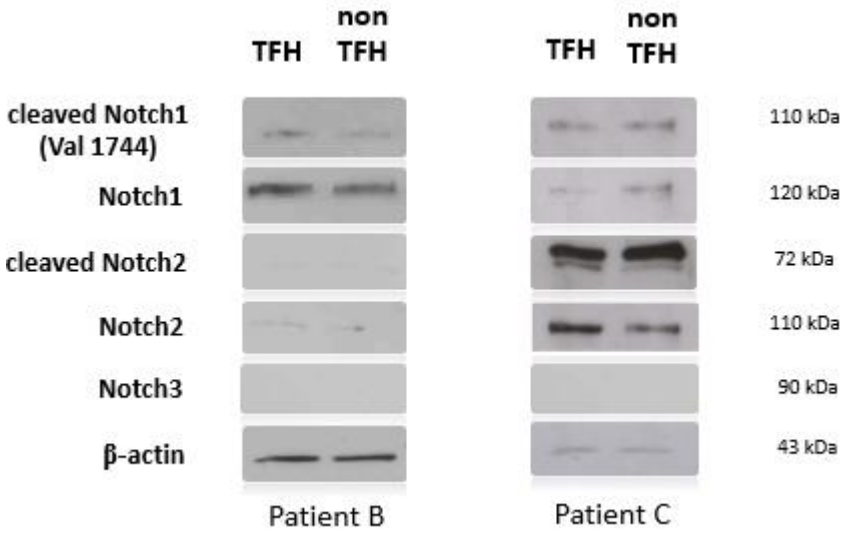


Figure 9 Expression profiles of Notch receptors in freshly isolated TFH and nonTFH using gating strategy described in this study

Expression profiles of cleaved Notch1, Notch1, claved Notch2, Notch2 and Notch3 in freshly isolate TFH and nonTFH were detected by western blot. β-actin was served as internal loading control.

4.4 The mRNA Expression of Notch receptors and Notch ligands in freshly isolated TFH

To investigate the involment in regulation of TFH by the Notch signaling, mRNA expression of Notch signaling molecules, i.e. Notch receptors (Notch1-4) and Notch ligands (Dlk 1,3,4 and Jagged 1-2) were analyzed by qPCR. The results of Notch receptors and Notch ligands are shown in Figure 10 and Figure 11, respectively. From these results, isolated TFH had higer level of mRNA expression of all Notch receptors and theirs ligands than nonTFH. Furthermore, *Notch1* and *Jagged2* are the two genes that clearly expressed in TFH more than nonTFH. Therefore, these results suggest that the Notch signaling pathway,especially *Notch1* and *Jagged2* may involved in the regulation of TFH.

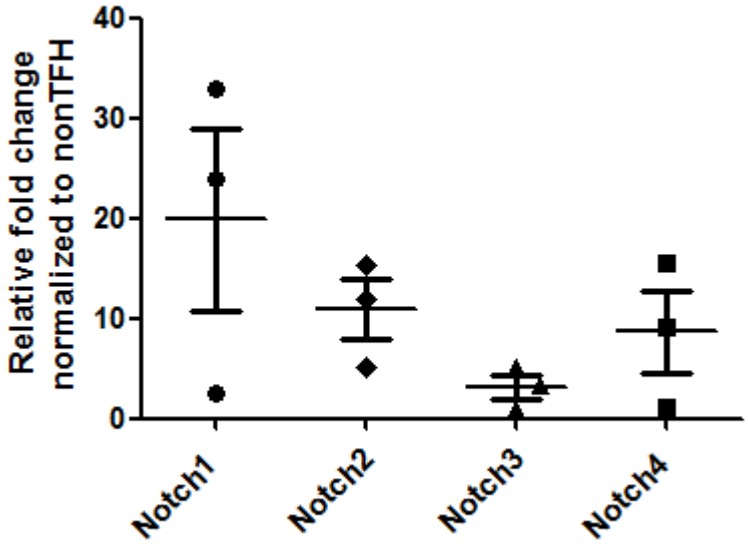


Figure 10 Expression profiles of mRNA of notch receptors in freshly isolated TFH

Expression of mRNA of Notch receptors (Notch 1-4) were measured in freshly isolated TFH. *GAPDH* was used as housekeeping gene. The relative fold change was calculated using the level of expression in TFH which was normalized to that of nonTFH. Each point represents data obtained from each individual (n=3).

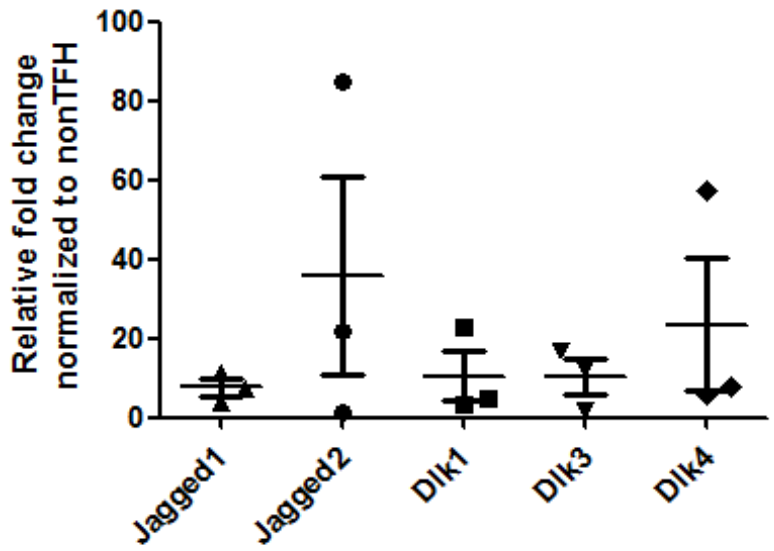


Figure 11 Expression profiles of mRNA of Notch ligands in freshly isolated TFH

Expression of mRNA of Notch ligands (Jagged 1, Jagged 2, Dlk1, Dlk3 and Dlk4) were measured in freshly isolated TFH. *GAPDH* was used as housekeeping gene. The relative fold change was calculated using the level of expression in TFH which was normalized to that of nonTFH. Each point represents data obtained from each individual (n=3).

4.5 The expression of mRNA of Notch receptors, Notch ligands, *BCL6* and *IL21* *in vitro* stimulated TFH

To investigate whether the Notch signaling operates during activation of TFH, the expression of Notch receptors and ligands were analyzed and compared between freshly isolated and *in vitro* stimulated TFH by qPCR. TFH and nonTFH were stimulated with plate bound anti-CD3/CD28 antibodies for 24 hours. Total RNA were collected and converted to cDNA to detect the level of mRNA expression.The relative fold changes of mRNA level were analyzed by comparing those in TFH normalized to nonTFH and were compared between unstimulated and stimulated conditions. The results are shown in Figure 12 and Figure 13. The results revealed that the expression of mRNA of all Notch receptors and ligands were down-regulated when TFH were stimulated with anti CD3/CD28 antibodies *in vitro*. Moreover, the expression of mRNA of *BCL6* and *IL21* were also down-regulate (Figure 14). These unexpected results are not caused by cell death because the clonal expansion which is a morphological activation of T cells appeared in stmulated TFH under microscopy (data not shown). To investigate the possiblity that IL-2, a known negative regulator of TFH, induces downregulation of *IL21* and *BCL6* mRNA, the qPCR was performed and the relative level of *IL2* was determined as shown in Figure 15. The result demonstrated that the level of *IL2* mRNA was higher in stimulated TFH compared to unstimulated TFH. These results help explain the effect of stimulation on expression of *BCL6* and *IL21* in TFH. Therefore, freshly isolated TFH were used to investigate the association of Notch1 on *BCL6* promoter by chromatin immunoprecipitation (ChIP).

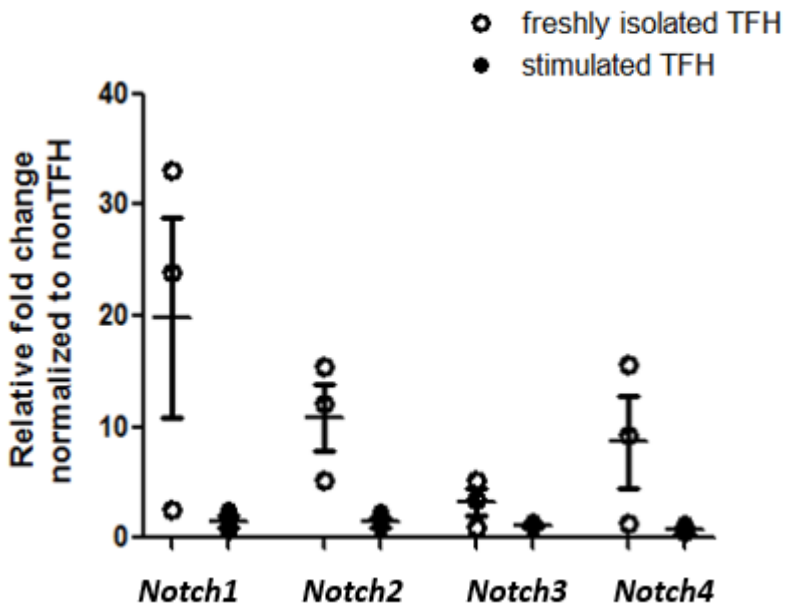


Figure 12 Expression profiles of mRNA of notch receptors in freshly isolated TFH and stimulated TFH

Expression of mRNA of notch receptors *(Notch1-4*) were measured in freshly isolated TFH and stimulated TFH. *GAPDH* was used as housekeeping gene. The relative fold change was calculated using level of expression in TFH normalized to nonTFH in each condition. Each point represents data obtained from each individual (n=3).

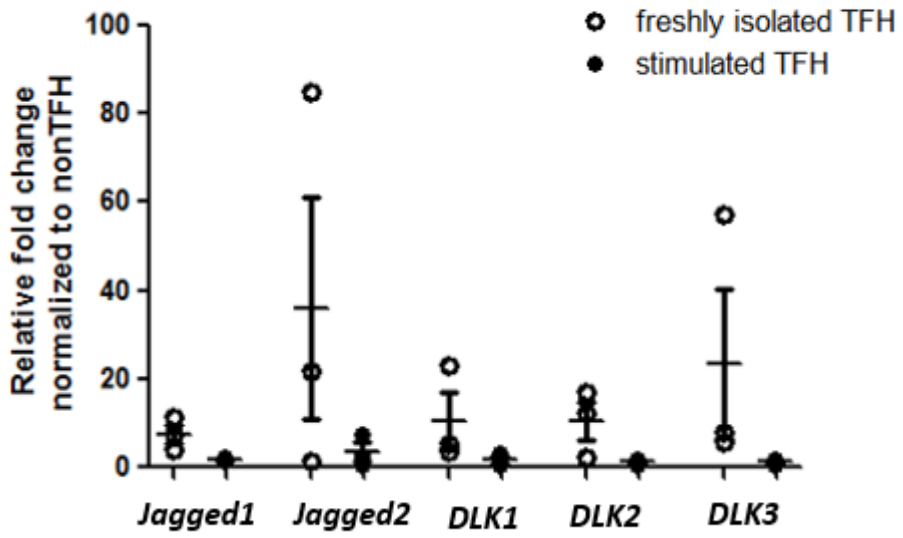


Figure 13 Expression profiles of mRNA of notch ligands in freshly isolated TFH and stimulated TFH

Expression of mRNA of notch ligands (*Jagged 1, Jagged 2, Dlk1, Dlk3* and *Dlk4)* were measured in freshly isolated TFH and stimulated TFH. *GAPDH* was used as housekeeping gene. The relative fold change was calculated using level of expression in TFH normalized to nonTFH in each condition. Each point represents data obtained from each patient (n=3).

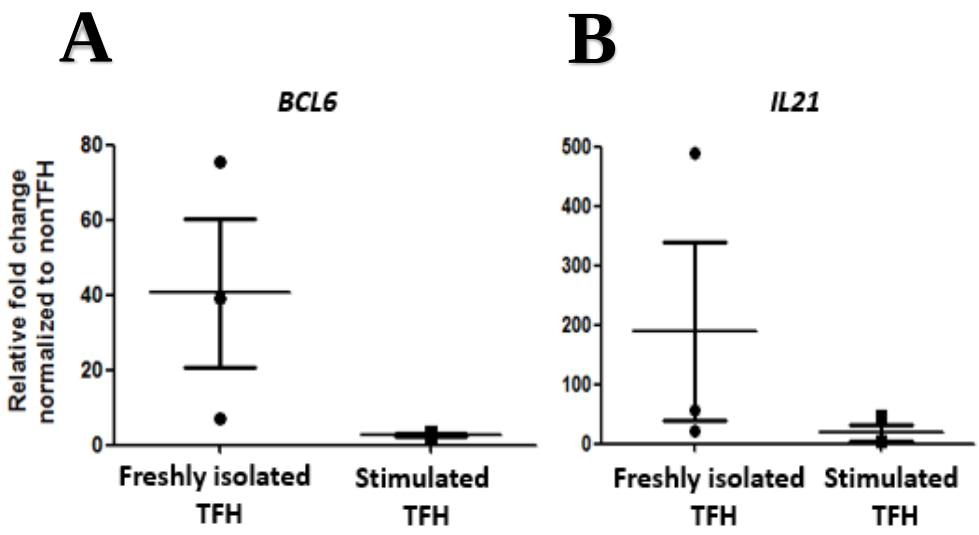


Figure 14 Expression of mRNA of *BCL6* and *IL21* in freshly isolated and stimulated TFH

The level of mRNA of *BCL6* (A) and *IL21* (B) expression were measured before and after stimulation of TFH with anti-CD3/CD28 antibodies for 24 hours. *GAPDH* was used as housekeeping gene. The relative fold change was calculated using the level of expression in TFH which was normalized to that of nonTFH. Each point represents data obtained from each patient (n=3).

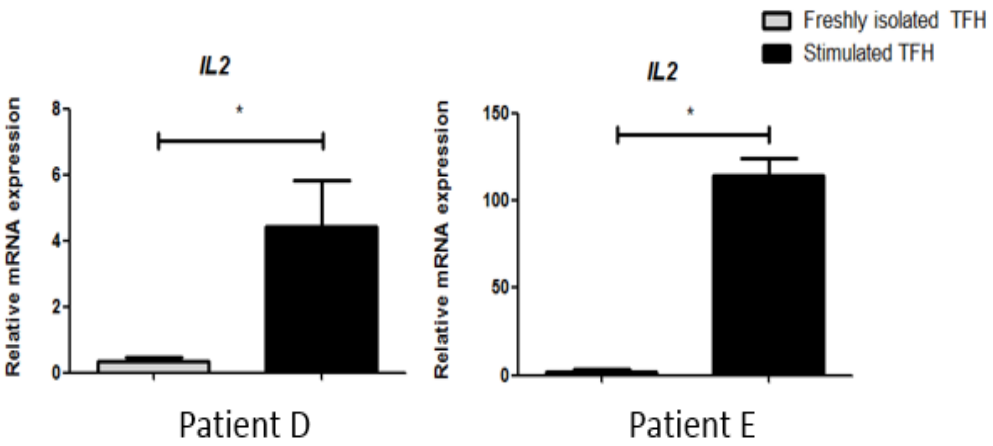


Figure 15 Expression of IL2 mRNA in freshly isolated TFH and in vitro stimulated TFH

The level of mRNA expression of *IL2* was determined in freshly isolated and in *vitro* stimulated TFH. The relative mRNA expression were calculated from the level of mRNA expression of *IL2* in freshly isolated TFH compared with that of stimulated TFH. The mRNA expression of *IL2* in freshly isolated TFH was used as baseline, *GAPHD* was used as housekeeping gene. (* considered statistically significant p-value < 0.05)

4.6 Direct association of Notch1 on *BCL6* promoter

4.6.1 Putative binding site of CSL on human *BCL6* promoter

CSL is a DNA-binding protein which can bind specifically to NICD and regulate gene expresstion. Tin et al. reported the consensus sequences for CSL binding on DNA by electrophoretic mobility shift assay (EMSA) and found that the high frequencies of recognition sequences are as follow: 5'-CGTGGGAA-3' and 5'-TGGGAA-3'(37).

Human *BCL6* locus is located on chromosome 3. Promotor sequence analysis revealed that there is a upstram complementary sequence of recognition motif for CSL binding at approximately upstream 849 bps upstream from the transcription starting site (TSS) as shown in Figure 16.

The association of transcription factor on the human *BCL6* promoter which was analyzed by ChIP-seg have been reported (UCSC Genome Bioinformatics). The transcription factors such as STAT1, IRF1, STAT3 and RCOR1 that bind near the CSL binding sites are shown in Figure 17.

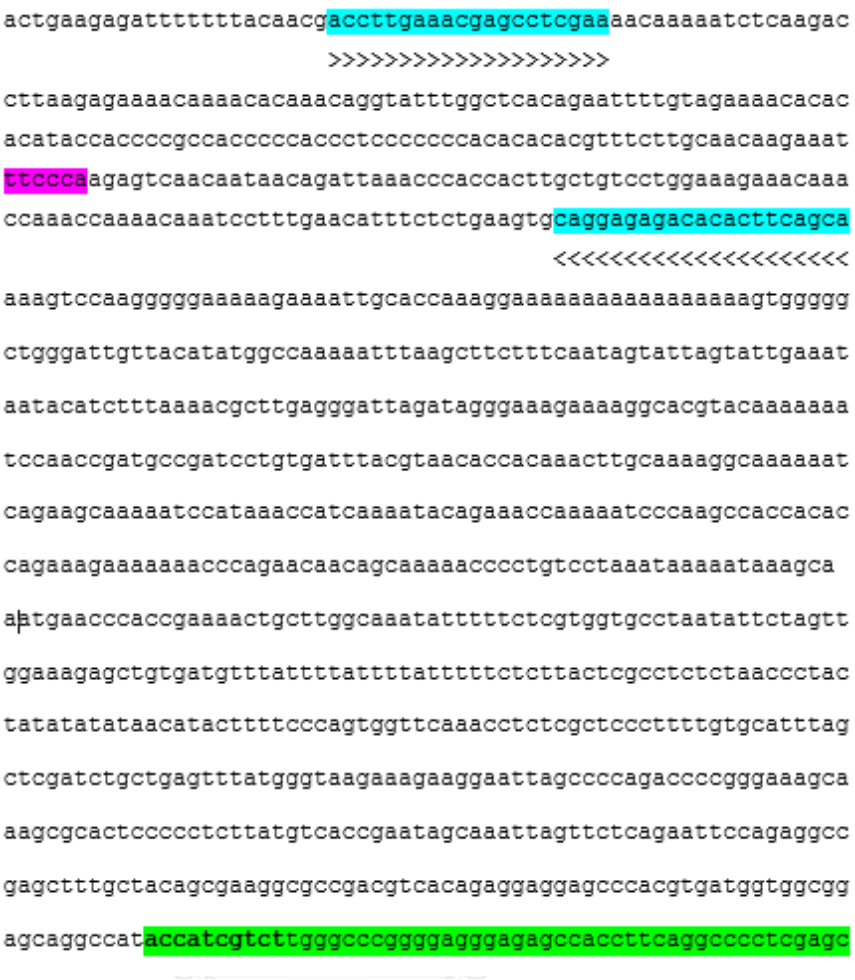


Figure 16 Nucleotide sequences of human BCL6 gene reported from NCBI

Homo sapiens *BCL6* gene have been reported a full length of nucleotide sequences in NCBI as Reference NG_007149.1. The potential CSL binding site are highlighted in pink and the transcription starting site are highlighted in green. The specific sequences for primer (Fwd 5'-accttgaaacgagcctcgaa -3' and Rev 5'-tgctgaagtgtgtctctcctg-3') binding sites are highlighted in blue.

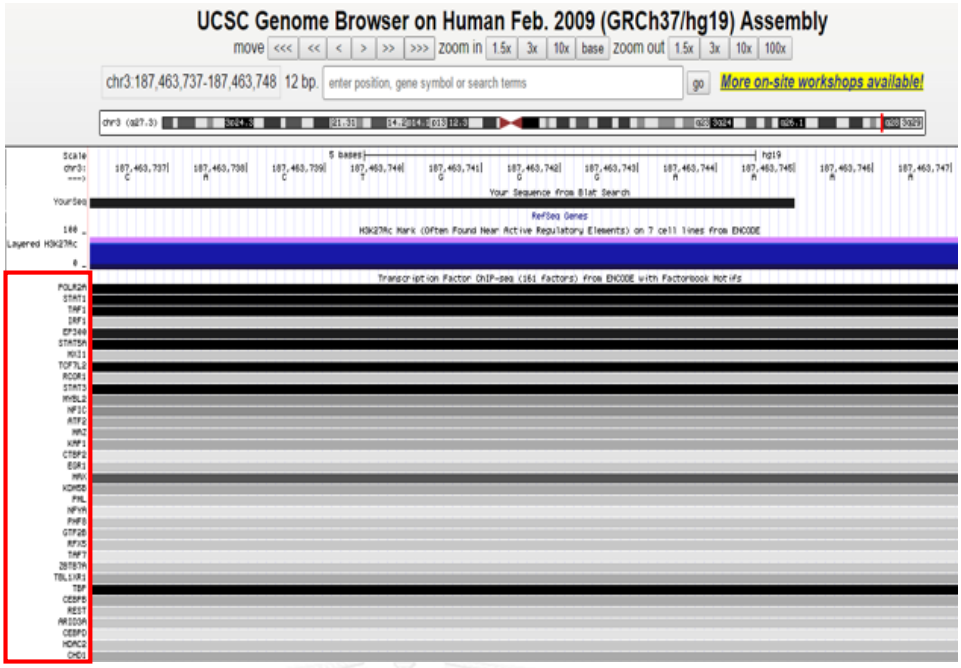


Figure 17 The transcription factors that bind nearly the CSL binding sites on human BCL6 promoter

The association of transcription factor on the human *BCL6* promoter analyzed by ChIP-seg were repoted in UCSC database. The transcription factors such as STAT1, IRF1, STAT3 and RCOR1 that bind near the CSL binding sites are shown in red box.

4.6.2 Investigation of the binding of Notch/CSL complexes on *BCL6* promoter by ChIP assay

Notch signaling have been well-known that involved in regulation of many genes such as *MYC, HES1*, *HEY1* and *HEYL*. Notch have been study that regulates HES1 by binding on *HES1* promoter by ChIP/PCR assay. In this study, binding of Notch1 on *HES1* promoter in Jurkat which are human T lymph ocyte cell line was used as control of systemic ChIP assay. The primers used in this experiment are reference from Alexi et al., 2013 (61). According to prior study, Nocth1 binds directly on *HES1* promoter (Figure 18). This result indicates that ChIP assay system were completed successfully.

ChIP method were selected to investigate the binding of Notch/CSL complex on *BCL6* promoter of TFH. Besides TFH and nonTFH, Jurkat was used as control. Genomic DNA from freshly isolated TFH ,nonTFH and Jurkat were sonicated to smear DNA to an average size between 200 and 1000 bps as shown in Figure 19. After the sonication step, optimal sheared DNA samples were incubated with anti-Notch1 antibody or normal rabbit IgG. Input was served as control for starting material of immunoprecipitation. DNA-Notch1 protein binding complexes were pulled down by protein G agarose beads. The complexes were revered crosslinking and purification of DNA were performed. The *BCL6* promoter region were determined by PCR using primers indicated in Figure16. The results of ChIP were shown in Figure 20. This PCR result showed that sheared DNA of TFH which was immunoprecipitated by anti Notch1 antibody can be amplified using primer set spanning the region of CSL binding site in the *BCL6* promoter whereas sheared DNA of nonTFH did not show any

detectable bands. These results can imply that Notch1 directly associated with BCL6 promoter in TFH and may partially regulates the promoter activity of *BCL6* in TFH.

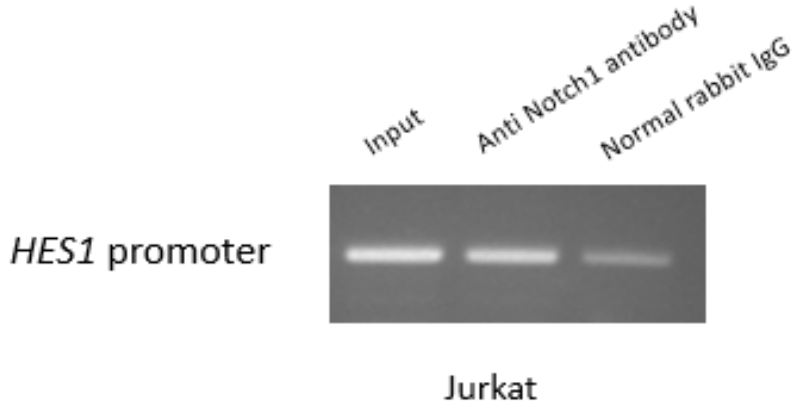


Figure 18 ChIP analysis of Notch1 binding to HES1 promoter in Jurkat T cell line

Chromatin immunoprecipitation of Notch on *HES1* promoter was performed as positive control for ChIP assay. Antibody used in immunoprecipitation were anti Notch1 antibody and control normal rabbit IgG. The binding region of DNA-protein complexes were determined by PCR.

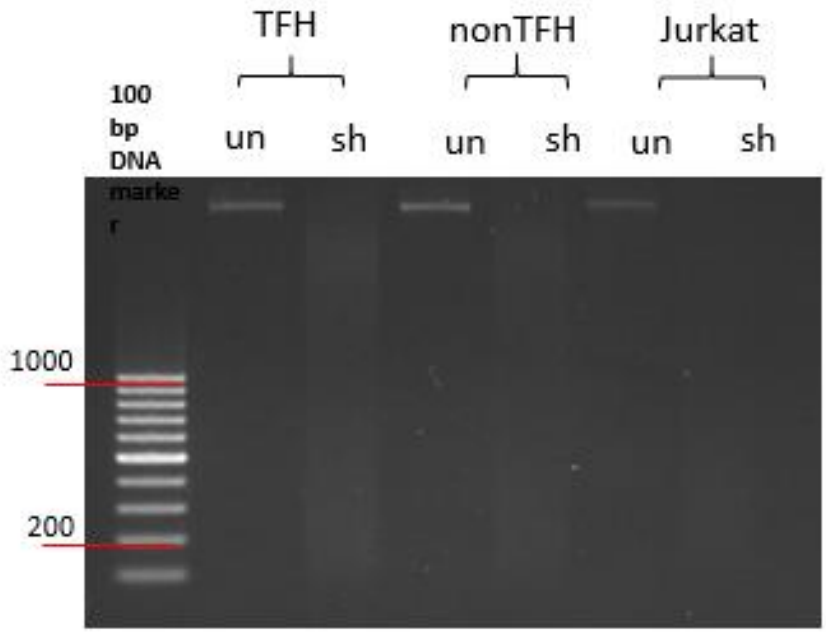


Figure 19 Genomic DNA pattern after sonication

Genomic DNA from TFH, nonTFH and Jurkat were subjected to fragment by exposure to brief periods of sonicator. Unsheared and sheared DNA were performed agarose gel electrophoresis. The majority of the DNA were sheared to a length between 200 and 1000 bp.

(un: unsheared DNA, sh: sheard DNA)

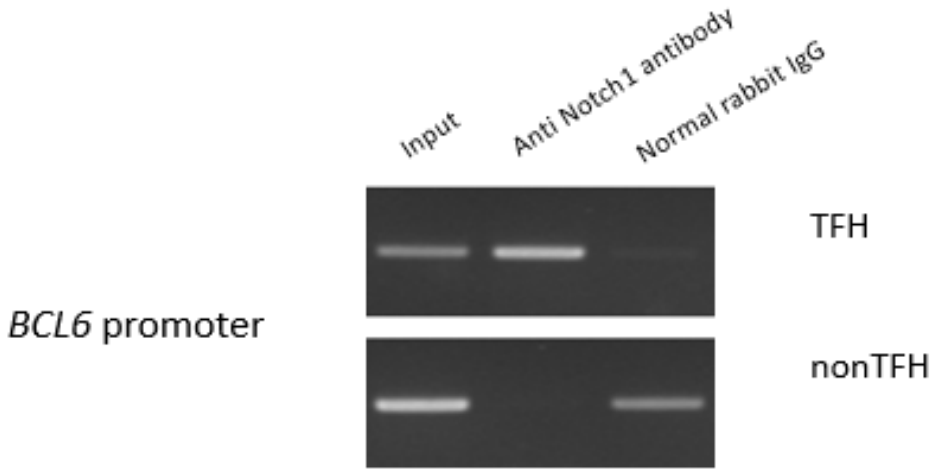


Figure 20 ChIP analysis of association between Notch1/BCL6 promoter in TFH and nonTFH

Chromatin immunoprecipitation was performed using sheared DNA of freshly sorted TFH, nonTFH and either anti Notch1 antibody or control normal rabbit IgG as the immunoprecipitating antibody. The binding region of DNA-protein complexes were determined by PCR.

CHAPTER V
DISCUSSION

Human tonsillar helper T cells can be divided into 3 subsets based on expression of ICOS and CXCR5. The gating strategy of human tonsillar helper T cells used in this study was similar to a previous study (7) but different from Inrun's study (1). Therefore, TFH from freshly dissected tonsils were sorted using $CD4^{+}CXCR5^{hi}ICOS^{hi}$ as marker. The expression of *BCL6* and *IL21* were higher in sorted TFH, compared with nonTFH. These results confirmed characteristic of the TFH in sorted cell population. Interestingly, cleaved Notch1 and Notch1 protein were found in freshly isolated TFH and nonTFH, which implies that Notch1 is activated in both cell types.

For T cell isolation from tonsils, the attempt has been made to isolate cells from tonsils by enzymatic method using collagenase (62, 63) but we could not detect the surface markers of CXCR5 and ICOS on $CD4^{+}$ T cells. This may due to the presence of collagenase which disrupts the CXCR5 and ICOS protein on cell surface or interferes with the fluorescence tagged with CXCR5 and ICOS antibodies (64). Alternatively, in this study, we used the mechanical method to isolate cells from tonsils.

The expression profiles of mRNA of Notch receptors and their ligands were found higher in freshly isolated TFH, compared with nonTFH. But, the levels of expression of Notch receptors and theirs ligands varied widely among patients. It may be the result of differences in genetic backgrounds, age, gender, including disease or pathogen exposure history of each patient. To demonstrate, the patients E and F have remarkably higher mRNA level of *BCL6* and *IL21* expression than the patient D. It may be explained by the accumulation of activated lymphocyte in the germinal center as it is reported in pathological studies of patients E and F. The demographic of all patients is shown in Table 4. However, a larger number of samples are required in analysis to reach the conclusion.

The expression of *Notch1* and *Jagged2* is higher, compared to other Notch receptors and ligands, in freshly isolated TFH. However, these genes were down-regulated after *in vitro* stimulation using anti-CD3/CD28 antibodies. In addition, the expression of *BCL6* and *IL21* were also decreased. These downregulations were possibly due to the upregulation of *IL2* expression via TCR signaling. IL-2 is able to suppress *BCL6* expression as reported by Ballesteros et al. and Oestreich et al. (32, 33). Previous study reported that *IL21* expression was decreased in TFH after

stimulation with anti-CD3 and CD28 beads (63), which is consistent with our finding. The likely explantion is that TFH are terminally differentiated cells and stimulation via CD3/CD28 may lead to negative feedback and inhibit it's effector function. Hence, the neutralizing antibody against IL-2 should be added in the culture system for TFH stimulation in the future in order to facilitate TFH growth.

In contrast to Inrun's resuts, the mRNA expression of *Notch1* and *Notch3* were hihger in freshly soreted TFH than nonTFH. In addition, the mRNA expression of *BCL6* and *IL21* were not different between freshly sorted TFH and nonTFH, but were higher in *in vitro* stimulated TFH via CD3/CD28 antibodies (1). According to gating stretegy of Inrun's study, the total population of TFH were combined with $CXCR5^{int}ICOS^{int}$ cells, which were not used in this study. Population of $CXCR5^{int}ICOS^{int}$ cells may be the precursor of TFH that cause misleading results which are different form our study.

Previous study reported the involvement of Notch in *BCL6* expression. The mRNA expression of *BCL6* were lower in $N1/N2^{-/-}$ mice immunized with *S. mansoni* eggs, compared to wild type mice (60). Furthermore, *IL21* mRNA expression were also decreased. Downregulation of *BCL6* expression results in impairment of TFH development and defective antibody production. Therefore, this research support that Notch regulate the *BCL6* expression and play roles in TFH deferentiation and function.

The potential CSL-binding site on human *BCL6* gene identified at approximately 849 bp upstream of transcription starting site. ChIP analysis revealed specific association of Notch1 with *BCL6* promoter in TFH but not in nonTFH. According to the Western blot results, we found that Notch 1 is activated in both TFH and nonTFH where as the binding of NICD on the *BCL6* promoter cannot be found in nonTFH. These results suggest that Notch is activated and might not regulate BCL6 in nonTFH but other Notch target genes. Therefore, this is the first study to propose that Notch signaling is directly involved in TFH function and/or differentiation by controlling the expression of *BCL6,* a master regulator of TFH, by directly control its promoter.

There are two limitations in this study. First is sample size of the patients. A small number of sample size in this study may obscure some certain effects. For example, TFH in each patient have statistically significant higher expression of *BCL6* and *IL21* than those of nonTFH. On the contrary, when we calculated the results by accumulating relative mRNA expression from each patient, the expression of *BCL6* and *IL21* was not different between TFH and nonTFH group. Therefore, a larger samples

size are required in further research for more reliable results. The second limitation is generalizability. Due to ethical problem, we cannot get the sample from healthy people. The candidates for tonsillectomy are patients who have tonsillar hypertrophy because of recurrent infections, airway obstruction and others that described in material and method. So, these sample might not be a good representatives of healthy people. However, there are few exclusion criteria which purpose is to rule out inappropriate samples. The patients with chronic infections which may have defects in function of TFH such as HBV and HIV are excluded (65-67). The patients with genetic disorders are also excluded because genetic abnormality may distort the result and conclusion. Tonsillar abscess or neoplasia are ruled out because of cytopathic effects of cells.

TFH is one of the promising targets of vaccine development. There is evidence that TFH is important for effective vaccination by enhancing humoral immune response (68, 69). A previous study shown that malaria antigen with nanoparticle vaccines can stimulate the expansion of antigen-specific TFH. Nanoparticles can induce TFH to promote more robust germinal center formation and high affinity antibody production than soluble protein or alum (70). In addition, patients with HIV infection are well-known to have suboptimal humoral immunity. A recent study found that the frequency of TFH in HIV-infected patients are correlated with HIV broadly neutralizing antibody (BNAb) titers (71, 72). However, to date, there was no HIV vaccine or other vaccine that targets to enhance the effectiveness of TFH. The basic knowledge on the involvement of Notch signaling in controlling *BCL6* expression in TFH may help better design of therapy targeting humoral immune response. In the future, Notch ligands may be a supplement in vaccine in order to enhance TFH function and elicit robust antibody response.

Table 4 The demographic of patients

Samples used for Western blot

Patient	Gender	Age (years)	Diagnosis	Underlying diseases
A	Male	9	ATH	• Attention Deficit Hyperactive Disorder • Snoring • Submucous resection of nasal septum
B	Female	3	ATH + OSA	• Snoring • Adhesion of tongue
C	Female	3	ATH	• Snoring • Allergic rhinitis • Acute and chronic inflammation (Pathology)

***Diagnosis**
ATH: Adenotonsillar Hypertrophy
OSA: Obstructive Sleep Apnea

Samples used for qPCR

Patient	Gender	Age (years)	Diagnosis	Underlying diseases
D	Male	3	ATH	• Morbid obesity • Snoring
E	Female	5	ATH + OSA	• Snoring • ATH with fail medication
F	Female	2	ATH+ OSA	• Sleep apnoea • Snoring • Obesity due to excess calories • Vasomotor rhinitis

***Diagnosis**
ATH: Adenotonsillar Hypertrophy
OSA: Obstructive Sleep Apnea

Samples used for ChIP

Patient	Gender	Age (years)	Diagnosis	Underlying diseases
G	Male	4	ATH + OSA	• Thalassemia • Occult bacteriamia • Reactive G6PD deficiency • Airway disease with allergic rhinitis, asthma

***Diagnosis**
ATH: Adenotonsillar Hypertrophy
OSA: Obstructive Sleep Apnea

CHAPTER VI

CONCLUSION

1. Human tonsillar helper T cells can be divided into 3 subsets, based on the expression of CXCR5 and ICOS.

2. The expression level of *BCL6* and *IL21* mRNA were higher in sorted TFH ($CD4^{+}CXCR^{hi}ICOS^{hi}$), compared with nonTFH ($CD4^{+}CXCR5^{lo}ICOS^{lo}$).

3. Cleaved Notch1 and Notch1 protein were found in freshly isolated TFH and nonTFH.

4. The expression profiles of mRNA of all Notch receptors and their ligands were higher in freshly isolated TFH than nonTFH, especially *Notch1* and *Jagged2.*

5. Expression of mRNA of *BCL6, IL21*, Notch receptors and ligands were downregulated in *in vitro* stimulated TFH.

6. The potential CSL-binding site on human *BCL6* gene identified at approximately 849 bp upstream of the transcription starting site.

7. ChIP analysis revealed specific association of Notch1 with *BCL6* promoter in TFH but not in nonTFH.

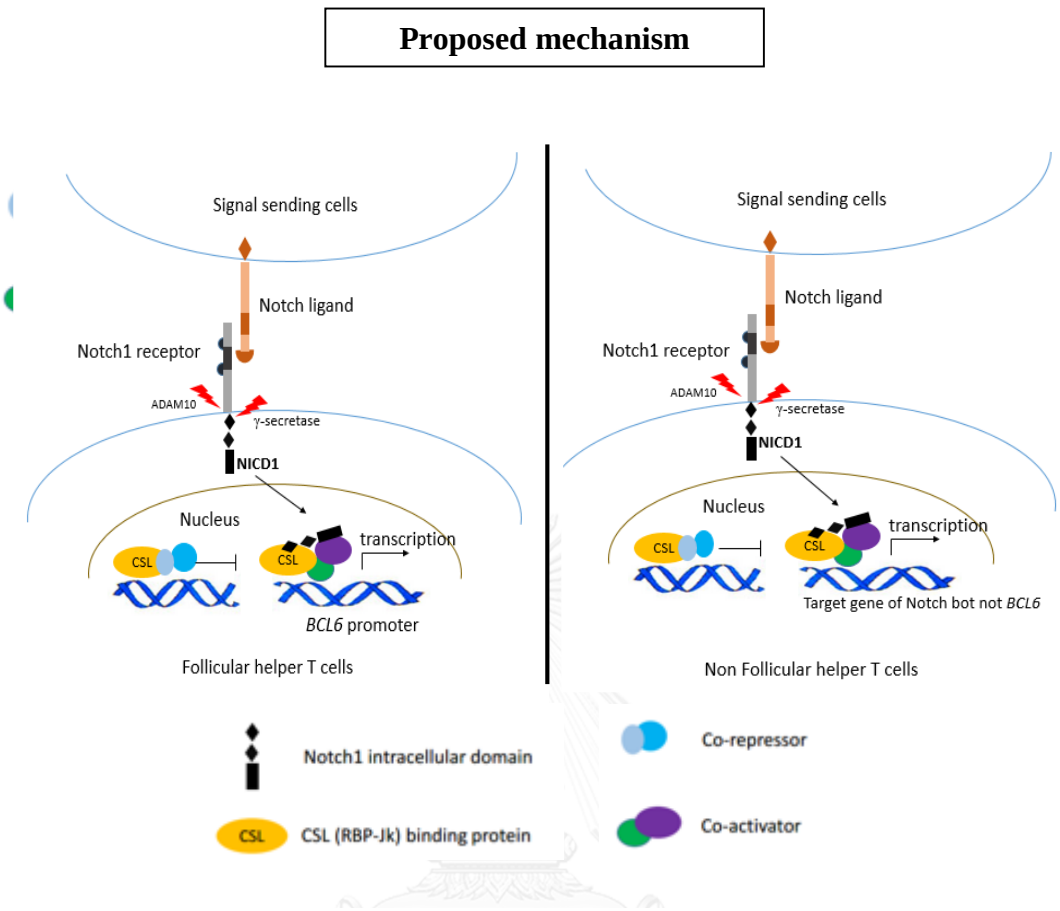


Figure 21 Regulation of *BCL6* expression by Notch signaling in TFH and nonTFH

Interaction of Notch receptors and ligands initiates Notch signaling. The cleavage of Notch receptor by enzyme ADAM10 and γ-secretase releases Notch intracellular domain (NCID). NICD then translocates into nucleus and binds to CSL binding protein. NICD/CSL complexes recruit co-activators and drive gene expression. In TFH, cleaved Notch1 translocates into nucleus and binds to CSL binding protein on the *BCL6* promoter to regulate *BCL6* expression. In spite of Notch1 activation, nonTFH do n

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APPENDIX

APPENDIX

1. Completed RPMI1640

RPMI 1640	90%
FBS	10%
Penicillin	100 U/ml
Streptomycin	100 µg/mL
Sodium pyruvate	1%
HEPES	1%

2. FBS inactivation

   Commercial FBS was thaw at 4°C for a night and inactivated at 56°C for 30 minutes in water baht. Inactivated FBS was stored at -20°C.

3. Protein extraction reagent

   2.1 Buffer A

1. 10 mM EGTA	1 ml
2. 10 mM DTT	1 ml
3. 500 mM Tris HCL (pH 7.2)	1 ml
4. 1.4 M KCL	1 ml
5. 25 mM $Mgcl_2$	1 ml
6. Sterile ddH_2O	5 ml
7. Protease Inhibitor cocktail Tablets	1 tablet

   2.2 Buffer B

1. Buffer A	990 µl
2. Nonidet P-40	10 µl

4. Blocking solution for Western blot

Non-fat dry milk	3 g
PBST	100 ml

5. Washing solution PBST for western blot

1xPBS	500 ml
Tween20	0.05%

6. Gel preparation

6.1 10% separating gel

1. H_2O	3.836 ml
2. 40% Acrylamide/Bis	2 ml
3. 1.5 M Tris-HCL pH 8.8	2 ml
4. 10% SDS	0.08 ml
5. 10% AP	0.08 ml
6. TEMMED	0.004 ml

6.2 5% stacking gel

1. H_2O	1.204 ml
2. 40% Acrylamide/Bis	0.250 ml
7. 1.5 M Tris-HCL pH 8.8	0.504 ml
8. 10% SDS	0.02 ml
9. 10% AP	0.02 ml
10. TEMMED	0.002 ml

7. 5xrunning buffer for western blot (1000 ml)

Trisma base	15.1 g
Glycine	94 g
SDS	5 g

Deionized water was add to adjust volume into 1000 ml

8. 2xLaemmli buffer (SDS-dye) 10 ml

1M Tris-HCL pH 8.8	1 ml
10%SDS	4 ml
99.5% glycerol	2.01 ml
Bromphenol blue	0.001 g

HPLC water was add to adjust volume into 10 ml

9.Transfer buffer for Western blot

Trisma base	5.08 g
Glycine	2.9 g
SDS	0.37 g
Absolute methanol	200 ml

Transfer buffer was added with deionized water to adjust volume into 1000 ml

10. Protein detection

10.1 Reagent A

1. 100 mM Tris pH 8.5	2.5 ml
2. 90 mM Coumaric acid	11 µl
3. 250 mM Luminol	25 µl

10.2 Reagent B

1. 100 mM Tris pH8.8	2.5 ml
2. 30% H_2O_2	1.5 µl

11. ECL substrate of HRP

90 mM of Coumaric acid was prepared in 10 ml of DMSO, 11 µl of Coumaric acid was aliquoted per tube and keep at -20°c

250 mM of Luminol was dissolved in DMSO in total volume 10 ml, 25 µl of Luminol was aliquoted per tube and keep at -20°c

12. Film developer and fixer

Each 10 ml of Film developer and fixer were mixed in 40 ml of tap water

13. 10x glycine

1.25M glycine in sterile deionized water

14. SDS lysis buffer

Protease inhibitor were added to SDS lysis buffer before using. Working SDS comprises of 1% SDS, 10mM EDTA, 50mM Tris pH8 and protease inhibitor.

15. 10 mg/ml RNaseA

Rnase A	1000 µg
Sterile deionized water	100 µl

16. 0.5M EDTA

0.5M EDTA in sterile deionized water, pH 8.0

17. 1M Tris-HCL

1 M Tris-HCL in sterile deionized water, pH 6.5

18. 10 mg/ml proteinase k

1000 µg of proteinase k in 100 µl of 50mM Tris-HCL, pH8.0 and 10 mM CaCl

19. ChIP Dilution buffer

ChIP Dilution buffer comprises of 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris HCL pH8.1 and 167 mM NaCl.

20. Low salt immune complex wash buffer

Low salt immune complex wash buffer comprises of 0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCL pH8.1 and 150 mM NaCl.

21. High salt immune complex wash buffer

High salt immune complex wash buffer comprises of 0.01% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCL pH8.1 and 500 mM NaCl.

22. Licl immune complex wash buffer

Licl immune complex wash buffer comprises of 0.25 M Licl, 1% Nonidet P-40, 1% deoxycholic (sodium salt), 1 mM EDTA and 10 mM Tris HCL pH8.1.

23. TE buffer

TE buffer comprises of 10 mM Tris HCL and 1 mM EDTA pH8.0

24. Elution buffer

Elution buffer comprises of 20%SDS, 20 µl 1M $NaHCO_3$ and 170 µl sterile deionized water

(1 reaction; total volume 200 µl)

25. 50X TAE buffer for agarose gel electrophoresis

Trisma base	96.8 g
Glacial acetic acid	22.84 ml
0.5 M EDTA	40 ml

50X TAE buffer was adjusted pH 8.0 in volume to 400 µl of sterile deionized water.

26. 0.5X TAE Running buffer for agarose gel electrophoresis

50X TAE buffer was diluted in sterile deionized water at final concentration 0.5X TAE

27. Agarose gel preparing for electrophoresis

a. 1.5% agarose gel

Agarose gel	0.6 g

1X TAE	40 ml

b. 2% agarose gel

Agarose gel	0.8 g
1X TAE	40 ml

28. 30% Glycerol

15 ml of Glycerol in 35 ml of distilled water was sterilized by an autoclave

VITA

I got a Bachelor's degree from the Department of Medical Technology, Faculty of Allied Health Science, Chulalongkorn University in 2009. I was certified a medical technologist and worked as a medical technologist in Lupus Research Unit, Faculty of Medicine, Chulalongkorn university and Klang hospital. In 2011, I enrolled in Master degree of Medical microbiology, Graduate School, Chulalongkorn University.

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