ความสัมพันธ์ระหว่างการแสดงออกของ Notch1 receptor ของเซลล์เม็ดเลือดขาวชนิดที กับการดำเนินโรคของผู้ป่วยเอส แอล อี

นางสาวพิมพเยาว์ สดใส

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2549
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

CORRELATION OF NOTCH1 RECEPTOR EXPRESSION IN T LYMPHOCYTES FROM SLE PATIENTS WITH DISEASE PROGRESSION

Miss Pimpayao Sodsai

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Microbiology
(Interdisciplinary Program)
Graduate school
Chulalongkorn University
Academic Year 2006

Copyright of Chulalongkorn University

Thesis Title	CORRELATION OF NOTCH1 RECEPTOR EXPRESSION IN T LYMPHOCYTES FROM SLE PATIENTS WITH DISEASE PROGRESSION
By	Miss Pimpayao Sodsai
Field of study	Medical Microbiology
Thesis Advisor	Associate Professor Nattiya Hirankarn, M.D. Ph.D.
Thesis Co-advisor	Assistant Professor Tanapat Palaga, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Graduate School
(Assistant Professor M.R. Kalaya Tingsabadh, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Anan Chongthaleong, M.D.)

.....Thesis Advisor
(Associate Professor Nattiya Hirankarn, M.D. Ph.D.)

.....Thesis Co-advisor
(Assistant Professor Tanapat Palaga, Ph.D.)

.....Member
(Assistant Professor Yingyos Avihingsanon, M.D.)

.....Member
(Assistant Professor Surapon Piboonpocanun, Ph.D.)

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

พิมพเยาว์ สดใส : ความสัมพันธ์ระหว่างการแสดงออกของ Notch1 receptor ของเซลล์เม็ดเลือดขาวชนิดทีกับการดำเนินโรคของผู้ป่วยเอส แอล อี (CORRELATION OF NOTCH1 RECEPTOR EXPRESSION IN T LYMPHOCYTES FROM SLE PATIENTS WITH DISEASE PROGRESSION) อ.ที่ปรึกษา : รศ.พญ.ดร. ณัฏฐิยา หิรัญกาญจน์, อ. ที่ปรึกษาร่วม : ผศ.ดร. ธนาภัทร ปาลกะ, 77 หน้า.

โรคเอส แอล อี เป็นต้นแบบของโรคภูมิต้านทานเนื้อเยื่อตนเองโดยจะพบการสะสมของ immune complex ได้ตามเนื้อเยื่อต่างๆของร่างกาย ในปัจจุบันยังไม่ทราบสาเหตุที่แน่ชัดของโรคนี้ ความผิดปกติของ T lymphocyte เป็นปัจจัยหนึ่งที่ส่งผลให้การควบคุมเพื่อไม่ให้เกิดภูมิคุ้มกันต่อแอนติเจนของตนเองสูญเสียไป (loss of self-tolerance) อันจะนำไปสู่การสร้าง autoantibody ที่มีบทบาทสำคัญต่อการเกิดพยาธิสภาพของโรคเอส แอล อี สัญญาณ Notch เป็นวิถีสัญญาณที่มีบทบาทสำคัญในการควบคุมพัฒนาการของ T lymphocyte, ขบวนการกระตุ้นเซลล์ (activation), การเพิ่มจำนวนของเซลล์ (proliferation), การแปรรูปของเซลล์ (differentiation) รวมถึงการทำงานของ T lymphocyte ด้วย โดยมีการศึกษามากมายที่แสดงให้เห็นถึงความสัมพันธ์ของความผิดปกติของวิถีสัญญาณ Notch กับโรคภูมิต้านทานเนื้อเยื่อตนเองหลายชนิด งานวิจัยนี้ทำการศึกษาถึงความสัมพันธ์ระหว่างการแสดงออกของโปรตีน Notch1 ใน T lymphocyte กับการดำเนินโรคเอส แอล อี โดยทำการศึกษาในผู้ป่วยเอส แอล อีจำนวน 22 คนและคนปกติ 11 คน จากผลการศึกษาระดับการแสดงออกของยีน *Notch1* ด้วยวิธี real-time RT-PCR ใน T lymphocyte ที่ผ่านการกระตุ้นด้วย PHA พบว่า ในผู้ป่วยเอส แอล อีที่มีอาการรุนแรง (active SLE) มีการแสดงออกของ *Notch1* ลดลงอย่างมีนัยสำคัญทางสถิติ (p=0.025) ในขณะที่การแสดงออกของ *Notch1* ในผู้ป่วยเอส แอล อี ที่มีอาการสงบ (inactive SLE) ไม่มีความแตกต่างเมื่อเปรียบเทียบกับคนปกติ ซึ่งก็ให้ผลสอดคล้องกับการทดสอบด้วยวิธี conventional RT-PCR โดยพบว่าในผู้ป่วยเอส แอล อี มีการแสดงออกของ *Notch1* ลดลงอย่างมีนัยสำคัญทางสถิติ (p=0.015) เมื่อศึกษาระดับการแสดงออกของโปรตีน Notch1 พบว่ามีการลดลงในผู้ป่วยเอส แอล อี ทีมีอาการรุนแรงโดยเปรียบเทียบกับคนปกติ (p=0.001) และผู้ป่วยเอส แอล อีที่มีอาการสงบ (p=0.037) สำหรับการแสดงออกของยีน *Hes-1* ซึ่งเป็นยีนเป้าหมายของวิถีสัญญาณ Notch ไม่พบมีความแตกต่างระหว่างผู้ป่วยเอส แอล อีกับคนปกติ นอกจากนี้การเพิ่มจำนวนของเซลล์ของผู้ป่วยเอส แอล อี หลังการกระตุ้นแบบ in vitro ก็ไม่พบมีความปกติผิดเช่นกัน จากผลข้างต้นแสดงให้เห็นถึงการแสดงออกของยีน *Notch1* กับความรุนแรงของโรคเอส แอล อี มีทิศทางที่สวนกัน ดังนั้นความผิดปกติในการแสดงออกของโปรตีน Notch1 ใน T lymphocyte หลังถูกกระตุ้น อาจจะเป็นอีกปัจจัยหนึ่งที่สำคัญต่อการเกิดพยาธิสภาพของโรคเอส แอล อี

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา.....2549..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4789119620 : MAJOR MEDICAL MICROBIOLOGY

KEYWORD : SLE / NOTCH RECEPTOR / T LYMPHOCYTES

PIMPAYAO SODSAI : CORRELATION OF NOTCH1 RECEPTOR EXPRESSION IN T LYMPHOCYTES FROM SLE PATIENTS WITH DISEASE PROGRESSION. THESIS ADVISOR : ASSOC.PROF. NATTIYA HIRANKARN, MD. Ph.D., THESIS CO-ADVISOR : ASST.PROF. TANAPAT PALAGA, Ph.D., 77 pp.

Systemic lupus erthematosus (SLE) is a prototype of autoimmune disease characterized by tissue deposition of autoantibody immune complex formation. However, etiology of disease remains unclarified. Defects of T lymphocytes lead to loss of immunological tolerance and support autoantibody production suggested that they may consistently have a central role in pathogenesis of SLE. Notch signaling is an evolutionarily conserved pathway responsible for thymocyte development, activation, proliferation, differentiation and T cell functions. Several evidences suggest Notch signaling involvement in autoimmune disorders. The aim of this study was to investigate the correlation of Notch1 receptor expression in T lymphocytes with disease progression. Twenty-two Thai SLE patients and eleven healthy controls were recruited for the study. *Notch1* expression in PHA-stimulated T lymphocytes of SLE patients that indicated significantly defective regulation of *Notch1* in activated T lymphocytes of SLE patients with active stage ($p=0.025$) while stimulated T lymphocytes of SLE patients with inactive stage were indifferent expression of *Notch1* compared with healthy controls that quantified by real-time RT-PCR. It was confirmed by conventional RT-PCR that showed deceleration of Notch1 expression in SLE ($p=0.015$). As well as Notch1 protein expression, it was downregulated in active SLE compared to controls and inactive SLE ($p=0.001$ and 0.037, respectively). However, *Hes1* that was target of Notch signaling did not reduce expression in SLE T lymphocytes. Moreover, proliferation capacity in SLE patients did not defect. These results showed converse correlation of Notch1 expression with severity of SLE. The data reveal the defective Notch1 in T cells that is possibly uncovered new factor of pathogenesis in SLE.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Field of study Medical Microbiology Student's signature.....Pimpayao Sodsai.....

Academic year..... 2006..... Advisor's signature.....Nattiya Hirankarn.....

Co-advisor's signature.....Tanapat Palaga.....

ACKNOWLEDGEMENTS

I wished to express my thankfulness to all those who participated in the success of this thesis. I would like to thank my advisor, Associate Professor Dr.Nattiya Hirankarn, Department of Microbiology, Faculty of Medicine, Chulalongkorn University, for her stimulating guidance, valuable advice and supporting. Her expertise in immunology improved my research skills and prepared me for future challenges. I also would like to thank my co-advisor, Assistant Professor Dr. Tanapat Palaga, Department of Microbiology, Faculty of Science, Chulalongkorn University, for his kindness, supporting and invaluable advice in my research. In addition, I wished to thank Assistant Professor Yingyos Avihingsanon, Department of Medicine, Faculty of Medicine, Chulalongkorn University, for his kindness and help to recruit the SLE patients

I wished to thank Miss Chayanit Singkhaselit for collecting blood samples and database as well as Mrs. Ratchada Inwattana for her kindness in guidance throughout the laboratories and providing the beneficial instruments for this study. I am grateful to Mr. Jeerawat Nakkuntod and Miss Thitima Benjachat for helping in molecular techniques. This work would not be accomplished without their help and support.

Furthermore, I would like to thank the committee of Inter-department of Medical Microbiology Program for giving me opportunity to commence this thesis in the first instance, to do the necessary research work. This research is supported in part by research grant from Lupus Research Unit, the 90th Years Anniversary of Chulalongkorn University (Ratchadphiseksomphot) Endowment Fund and the Thai Government Research Fund.

Finally, I would like to express my deepest gratitude to my parents for their love, supporting, encouragement and understanding.

CONTENTS

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

Table Page

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF FIGURES

Figure Page

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

ABBREVIATIONS

ACR	American College of Rheumatology
ANA	anti-nuclear antibody
APC	antigen presenting cell
APS	ammonium persulfate
ARA	American Rheumatism Association
BAFF	B-cell activating factor
BILAG	British Isles Lupus Assessment Group
BSA	bovine serum albumin
°C	degree Celsius
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
Cox-2	cyclooxygenase-2
CpG	cytosine-guanine dinucleotide
cpm	count per minute
CSL	CSF-1/RBP-Jκ for mammals, Suppressor of Hairless for *Drosophila*, and Lag-1 for *C.elegans*
CTLA-4	cytotoxic T lymphocyte antigen-4
DC	dendritic cell
DILE	drug-induced lupus erythematosus
DNA	deoxyribonucleic acid
dNTP	deoxynucletide triphosphates
dsDNA	double-stranded deoxyribonucleic acid
EBV	Epstein-Barr virus
ECLAM	European Consensus Lupus Activity Measurement
EDTA	ethylenedinitrilotetraacetic acid
FBS	fetal bovine serum
FCGR	Fc gamma receptor

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

g	gram
GAPDH	glyceraldehyde-3-phosphatedehydrogenase
GN	glomerulonephritis
3H-TdR	^{3}H-methyl-thymidine
Hes	Hairy enhancer of split
HRT	hormone-replacement therapy
HSC	hematopoietic stem cell
ICN	intracellular domain of Notch
ICs	immune complexes
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	Interleukin
kDa	kilodalton
L	liter
LAI	Lupus Activity Index
μg	microgram
μl	microliter
M	molar
MHC	major histocompatibility complex
mg	milligram
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
ng	nanogram
PARP	poly-ADP-ribosyl transferase
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PBST	phosphate buffered saline tween
PDCD1	programmed cell death 1

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

PTH	parathyroid hormone
qRT-PCR	quantitative reverse transcription polymerase chain reaction
rpm	round per minute
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
sFas	soluble Fas
SI	stimulation index
SLAM	Systemic Lupus Activity Measure
SLE	Systemic Lupus Erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
TAE	Tris-acetate buffer
TCR	T cell receptor
TGF-β1	transforming growth factor-β1
T_H	T helper
TLR	Toll-like receptor
Treg	regulatory T cell
U	unit

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER I

INTRODUCTION

Systemic Lupus Erythematosus (SLE), a chronic systemic inflammatory disease, is characterized by uncontrolled lymphocyte autoreactivity and dysregulated production of autoantibodies from autoreactive B cells leading to formation of immune complexes that can deposit in various organs, causing tissue damages. Individuals with SLE exhibit various clinical manifestations, including glomerulonephritis, rashes, serositis, hemolytic anemia, thrombocytopenia and central nervous system involvement. Women of childbearing age (15-45 years old) are primarily affected (female:male = 9:1). SLE is a worldwide disease and more common in African-Americans, African-Caribbean and Asians than Caucasians(Petri 2002). Although precise cause of SLE is not well understood, a number of genetic and environmental factors may contribute to SLE susceptibility. Genetic evidences from study of twins found that monozygotic twins have a concordance rate of about 24-58%, compared with 5% in dizygotic pairs, suggesting that strong genetic involvement contributes to SLE pathogenesis(Tsao 2003).

Autoimmune disorders are mainly arose from dysregulation of immune responses or breakdown of self-tolerance. The pathogenesis of SLE appears to be the outcome of numerous abnormalities of immune system, including a generalized hyperactivity of T and B cells, leading to autoantibody production. Lupus T cells have intrinsic defects that render them more susceptible to activation through their T cell receptor complex after contact with self-peptides/MHC complex. This hypothesis stems in part from several observations—increased numbers of activated T cells, a lower threshold for T cell activation, a higher threshold for apoptotic death during T cell activation, abnormalities in TCR signaling and apoptosis, and abnormal expression of effector molecules(Kong, Odegard et al. 2003). Moreover, defects in clearance of apoptosis cells play a crucial role in driving dysfunction of immunological tolerance in autoreactive T and B cells. The studies have investigated genetic mutations and

abnormal expression of apoptotic and anti-apoptotic molecules and found that this defective apoptosis machinery results in escaping of pathogenic T and B cells with continuous autoantibody production(Salmon and Gordon 1999). Deficient clearance of apoptotic materials by phagocytes in SLE results in accumulation of apoptotic blebs that promotes prolongation of self-antigen exposure to autoreactive T and B cells and sustains deposition of immune complexes in several tissues(Munoz, Gaipl et al. 2005). However, the major factors responsible for initiation of SLE are not known and they deserve further investigation.

Notch signaling is an evolutionarily conserved pathway regulating many aspects of cell fate decisions in multicellular organisms. Mammals have four Notch receptors (Notch1-Notch4) and five Notch ligands (Jagged1, Jagged2, Delta-like 1, Delta-like 3 and Delta-like 4), most of which are expressed on multiple lineages of cells. Notch signaling is initiated through ligand-receptor interactions leading to proteolytic cleavage and releasing of intracellular domain of Notch (ICN) into nucleus. In the nucleus, ICN forms a complex with transcriptional factors, CSL (CSF-1/RBP-Jκ for mammals, Suppressor of Hairless for *Drosophila*, and Lag-1 for *C.elegans*), and interact with promoters of target genes(Radtke, Wilson et al. 2004). Notch signaling plays a key role in T cell development, activation, proliferation, differentiation, function and apoptosis. The essential function of Notch1 in T cell lineage development is well characterized. Lymphocyte progenitors adopting B cell fate must turn off Notch signaling. Moreover, each step of T cell development in thymus is described to involve Notch signaling as well as differentiation of helper T cells into T_H1/ T_H2 effector T cells(Maillard, Fang et al. 2005). For its involvement in T cell functions, there are several studies describing the role of Notch signaling in helper T cells and regulatory T cells(Maillard, Fang et al. 2005).

Several studies reported evidences of autoimmune diseases in association with anomalies in Notch signaling. For example, in type I diabetes caused by impaired generation of naturally occurring $CD4^+CD25^+$ Treg cells, overexpression of Notch3 enhanced generation of Treg cells and ameliorated progression of experimental

autoimmune diabetes in mice(Anastasi, Campese et al. 2003). Blocking of Notch signaling inhibited T_H1-mediated autoimmune disease progression in mouse experimental autoimmune encephalomyelitis, a model of multiple sclerosis(Minter, Turley et al. 2005). Interestingly, there are some evidences describing the role of Notch signaling in SLE. T lymphocytes in human SLE differentially express *presenilin2* that is a component of γ-secretase, an important enzyme for Notch activation(Xu, Zhang et al. 2004). In mouse model, partial loss of presenilins causes development of several autoimmune phenotypes, particularly SLE-like autoimmune disease(Tournoy, Bossuyt et al. 2004).

With the current knowledge on the involvement of Notch pathway in T cell functions, several investigators indicated the involvement of Notch signaling in autoimmune disorders, including SLE. The aim of this study is to investigate and compare the expression level of *Notch1* and *Hes1*, target gene of Notch signaling, in stimulated T lymphocytes from patients with SLE and healthy controls. In addition, this study also examined population profiles of T lymphocytes and proliferative capability of T lymphocytes from SLE patients, compared with those from healthy controls. We hypothesized that Notch signaling may be defective in T cells from SLE patients, with abnormal presenilins observed in SLE. The knowledge from this research might lead to the better understanding of mechanisms of SLE and the development of new treatment and prevention.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

OBJECTIVE

The objectives of this study are:

To compare levels of expression of *Notch1* and *Hes1*, one of the target genes of Notch signaling, in stimulated T lymphocytes from SLE and healthy controls. In addition, the population profiles and proliferative capacity of T lymphocytes from SLE patients compare to those from healthy donors will be studied.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER III

LITERATURE REVIEW

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a prototype of a multisystem autoimmune disease characterized by the production of pathogenic autoantibodies secreting from autoreactive B cells. Circulating IgG autoantibodies are often exhibit specific for self-antigen such as dsDNA, nuclear proteins and cytoplasmic components. The immune complexes of antigen-antibody formation directly deposit on various tissues resulting in inflammation and malfunctions of multiple organs. Clinical manifestations could be diversed, most commonly including arthralgia, arthritis, rash, alopecia, oral ulcers, serositis, leukopenia, central nervous system and renal involvement(Manson and Isenberg 2003).

Epidemiology

The incidence of SLE has more than tripled over the past four decades. Using the 1982 American College of Rheumatology criteria, the adjusted incidence of SLE was 5.56 per 100,000 during 1980–92, compared with 1.51 per 100,000 during 1950–79. A review of 19 papers published between 1965 and 1995 gave a pooled incidence of SLE of 7.3 per 100 000(Ruiz-Irastorza, Khamashta et al. 2001). SLE is a worldwide disease with a prevalent range from 5.8-1,000 per 100,000 individuals showing increase in recent series(Petri 2002). Ethnicity also influences the incidence of SLE. African-Americans and African-Caribbeans are approximately two to four times more likely to contract the disease than Caucasians. SLE is much more common in women than in men with a female to male ratio of ~ 9:1 between 24-64 years old(Nguyen, Limaye et al. 2002; Petri 2002). Overall, increasing the 10-year survival rate has been 75–85%, with more than 90% of patients surviving longer than 5 years(Ruiz-Irastorza, Khamashta et al. 2001; Petri 2002).

Classification of SLE

The 1971 preliminary criteria of the American Rheumatism Association (ARA) for SLE were revised to incorporate newly acquired immunologic knowledge and improve disease classification in 1982. Recently, the American College of Rheumatology (ACR) classification criteria for SLE were updated in 1997 as shown in Table1. Patients are classified as having SLE when they have at least four of eleven of classification criteria, serially or simultaneously(Hochberg 1997). In addition, it is crucial to distinguish disease activity of SLE from infection, chronic damages and co-morbid disease. Over the past 20 years, many indices have been developed to objectively measure lupus disease activity and several of these have been validated. The widely used indices are the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (Table2), the British Isles Lupus Assessment Group (BILAG) index, the European Consensus Lupus Activity Measurement (ECLAM), the Systemic Lupus Activity Measure (SLAM), and the Lupus Activity Index (LAI). All these indices have been validated and have excellent reliability, validity and responsiveness to change(Griffiths, Mosca et al. 2005).

Etiology

Although the exact etiology of SLE is not entirely understood, an extremely complicated and multifactorial interaction among various genetic and environmental factors is probably involved(Mok and Lau 2003). The findings provide strong evidence of genetic factors, but they also suggest that environmental causes are important as well.

Genetic Factors

Genetic factors in the pathogenesis of SLE are strongly supported by studies in humans and animal models in which SLE spontaneously develops. The predisposition to SLE has been studied in twins who were shared the same environment. Dizygotic twins have a disease concordance rate approximately 5% that is similar to that for other family members but is still six to eight times greater than that for unrelated persons. On the other hand, the concordance rate in monozygotic twins is between 30 and 50 percent.

Table 1 Revised American Rheumatism Association criteria for classification of systemic lupus erythematosus(Hochberg 1997)

1. Malar rash
2. Discoid rash
3. Photosensitivity
4. Oral ulcers
5. Arthritis
6. Serositis
   a) pleuritis, or
   b) pericarditis
7. Renal disorder
   a) proteinuria>0.5g/24 hrs or 3+, persistently, or
   b) cellular casts
8. Neurological disorder
   a) seizures or
   b) psychosis (having excluded other causes)
9. Hematological disorder
   a) hemolytic anemia or
   b) leucopenia or<4.0×10^9/1 on two or more occasions
   c) lymphopenia or<1.5×10^9/1 on two or more occasions
   d) thrombocytopenia or<100×10^9/1
10. Immunological disorder
   a) raised anti-native DNA antibody binding or
   b) anti-Sm antibody or
   c) positive finding of antiphospholipid antibodies based on
      i. An abnormal serum level of IgG or IgM anticardiolipin antibodies
      ii. A positive test result for lupus anticoagulant using a standard method
      iii. A false-positive serological test for syphilis, present for at least 6 months
11. Anti-nuclear antibody in raised titer

Table 2 Systemic Lupus Erythematosus Disease Activity Index(SLEDAI) (Griffiths, Mosca et al. 2005)

Wt	Present	Descriptor	Definition
8	□	Seizure	Recent onset. Exclude metabolic, infectious or drug cause
8	□	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, impoverished thought content, marked illogical thinking, bizarre, disorganized, or catatonic behavior. Excluded uremia and drug causes.
8	□	Organic Brain Syndrome	Altered mental function with impaired orientation, memory or other intelligent function, with rapid onset fluctuating clinical features. Include clouding of consciousness with reduced capacity to focus, and inability to sustain attention to environment, plus at least two of the following: perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious or drug causes.
8	□	Visual Disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serious exodate or hemorrhages in the choroids, or optic neuritis. Exclude hypertension, infection, or drug causes.
8	□	Cranial Nerve Disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	□	Lupus Headache	Severe persistent headache: may be migrainous, but must be non-responsive to narcotic analgesia.
8	□	CVA	New onset of cerebrovascular accident(s). Exclude arteriosclerosis
8	□	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual, infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis
4	□	Arthritis	More than 2 joints with pain and signs of inflammation (i.e. tenderness, swelling, or effusion).
4	□	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/adolase or electromyogram changes or a biopsy showing myositis.
4	□	Urinary Casts	Heme-granular or red blood cell casts
4	□	Hematuria	>5 red blood cells/high power field. Exclude stone, infection or other cause.
4	□	Proteinuria	>0.5 gm/24 hours. New onset or recent increase of more than 0.5 gm/24 hours.
4	□	Pyuria	>5 white blood cells/high power field. Exclude infection.
2	□	New Rash	New onset or recurrence of inflammatory type rash.
2	□	Alopecia	New onset or recurrence of abnormal, patchy or diffuse loss of hair.
2	□	Mucosal Ulcers	New onset or recurrence of oral or nasal ulcerations
2	□	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	□	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram confirmation.
2	□	Low Complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory.
2	□	Increased DNA binding	>25% binding by Farr assay or above normal range for testing laboratory.
1	□	Fever	>38°C. Exclude infectious cause
1	□	Thrombocytopenia	<100,000 platelets/mm3
1	□	Leukopenia	<3,000 White blood cell/mm3. Exclude drug causes.

This 10-fold difference in the disease concordance rate between identical twins and fraternal twins suggests that multiple genes shared between each pair of twins greatly influence the susceptibility to SLE(Mills 1994; Mok and Lau 2003; Tsao 2003).

More than 50 loci on chromosomes 1, 4, 7 and 17 have been found to affect susceptibility to lupus. Eight of the best-supported SLE susceptibility loci are 1q23, 1q25-31, 1q41-42, 2q35-37, 4p16-15.2, 6p11-21, 12p24 and 16q12. Case-control association studies have identified several genes exhibiting convincing evidence for allelic association with SLE including many major histocompatibility complex (MHC) and non-MHC genes interacting with each other(Nguyen, Limaye et al. 2002; Tsao 2004). In Table 3, it is shown that gene disruptions of candidate disease genes that are involved in a variety of lymphocytic interactions, apoptosis, or antigen clearance are silenced or overexpressed(Nguyen, Limaye et al. 2002; Tsao 2003; Tsao 2004). Anomalies in one single gene is insufficient to cause the disease. The different combinations of genes may be associated with disease development in different families.

Environmental Factors

Although genetic factors may create a predisposition towards SLE, the initiation of the disease probably results from several environmental triggers and exogenous factors. Environmental exposures may lead to the production of autoreactive lymphocytes and autoantibodies, the stimulation of pro- and anti-inflammatory cytokines, and target end-organ damage, but there are no convincing evidence pointing to any particular agents. Viral and bacterial infections are common in SLE. Some Epstein-Barr virus (EBV) proteins share sequence homologies with SLE autoantigens that may cross-react with autoantigens and promote the proliferation and antibody production of B cells. It is also known that parvovirus B19 (B19), retrovirus and cytomegalovirus (CMV) infections induce a number of autoimmune abnormalities resembling those found in SLE (Sarzi-Puttini, Atzeni et al. 2005). The list of medications implicated as etiological agents in drug-induced lupus erythematosus (DILE) continues to grow and now includes more than 80 drugs such as hydralazine, procainamide, isoniazide, methyldopa, chlorpromazine, quinidine and minocycline(Sarzi-Puttini, Atzeni et al. 2005). In addition,

Table 3 Candidate susceptibility genes in SLE

Genes	functions	characteristics	references
complement genes *C1q, C2, C4*	- a mediator of inframmation - clearance of apoptotic cells	- develop GN with immune deposits - high levels of ANAs - a number of apoptotic bodies - impaired immune complex clearance	(Botto, Dell'Agnola et al. 1998; Chen, Koralov et al. 2000; Walport 2002)
Fc-receptor genes *FCGR2A, FCGR3A, FCGR2B, FCGR3B*	- clearance IgG ICs	- delay clearance of IgG containing ICs - deposit of IgG containing ICs - increase releasing pro-inflammatory cytokines - develop autoantibodies and GN	(Bolland and Ravetch 2000; Ravetch and Lanier 2000)
PDCD1	- activation-induced cell death	- develop GN	(Nishimura, Nose et al. 1999)
CTLA-4	- inhibit B7-CD28 interaction	- hyperproliferative T cells - increase titers of serum antibodies	(Waterhouse, Penninger et al. 1995)
Fas, FasL	- regulate T cell tolerance	- produce T cell-dependent autoantibodies	(Watanabe-Fukunaga, Brannan et al. 1992; Takahashi, Tanaka et al. 1994)
Bcl-2	- anti-apoptosis	- increase proliferation and expansion - produce ANAs - develop lupus-like GN	(Strasser, Whittingham et al. 1991; Mehrian, Quismorio et al. 1998)
PARP	- apoptosis	- accumulate autoimmune cells	(Tsao, Cantor et al. 1999)
Type I Interferon genes	- enhance antigen presentation - promote T_H1 response - enhance antibody response	- attenuation of autoantibody production	(Ivashkiv 2003)

GN = glomerulonephritis, ICs = immune complexes, ANAs = anti-nuclear antibodies

synthetic exogenous female sex hormones are widely used in clinical practice for various therapeutic applications, mainly oral contraception and post-menopausal hormone replacement therapy (HRT) that may induce or unmask SLE(Mok and Lau 2003; Sarzi-Puttini, Atzeni et al. 2005). Photosensitivity is a common presenting symptom of SLE. UV light exposure causes the release of pro-inflammatory cytokines and increases the rate of keratinocyte apoptosis (Manson and Isenberg 2003). Autoimmune-

like syndromes have been associated with dietary exposure for example, alfalfa sprouts and saturated fats diet affect the production of inflammatory mediators(Mok and Lau 2003). Furthermore, SLE is a predominant female disease. Sex hormones seem to play an important role as modulators of the disease onset/perpetuation. Thus, excessive estrogenic but inadequate androgenic hormonal activity in both men and women with SLE might be responsible for the alteration of the immune responses. It has suggested a role for endogenous sex hormones in disease predisposition(Mok and Lau 2003; Cutolo, Sulli et al. 2004). The impingement of these environmental triggers upon predisposed individuals is probably highly variable and could be a further explanation for disease heterogeneity.

SLE and Immune system

It is well recognized that B cells are hyperactive and produce excessive amounts of immunoglobulins and a variety of autoantibodies, resulting in formation of immune complexes, which is central to the effector phase of the disease. On the other hand, reported evidences also suggest that SLE T cells participate in the attack on target cells or tissues through overproduction of pro-inflammatory cytokines or an increase in cell-to-cell adhesion, ultimately leading to the apoptosis and inflammation of target tissues. Thus, both SLE B cells and T cells play a central role in the pathogenesis of SLE(Takeuchi, Tsuzaka et al. 2005).

B cell abnormalities in SLE

Several lines of evidence strongly suggest a primary role for B cells in systemic lupus erythematosus. The importance of primary B cell abnormalities is also strongly supported by genetic studies. B cell hyper-responsiveness is genetically determined in lupus mice by several recessive loci which mediates the loss of tolerance to nuclear antigens and decreases the activation threshold of B cells. Moreover, manipulating the expression of molecules that modulate immunoglobulin mediated signaling and influence B cell tolerance can produce SLE-like phenotypes(Looney, Anolik et al. 2004).

Escaping from deletion by autoreactive B cells may be mediated by one signal from the B-cell activating factor (BAFF). This survival factor inhibits negative selection by interaction with BAFF receptor. Mice overexpressing BAFF exhibit an autoantibody mediated, lupus like disease(Mackay, Woodcock et al. 1999). Moreover, soluble-active BAFF has been found to be elevated in the serum of patients with active SLE(Zhang, Roschke et al. 2001). CD154–CD40 interactions are essential for formation of germinal centers and the differentiation of memory and plasma cell effector populations. Treatment with blocking anti-CD154 antibody improves serologic activity and decreases hematuria in patients with active SLE(Boumpas, Furie et al. 2003). Altered signals of B cells such as CD80/CD86, CD19 and CD22 also might be involved in autoimmunity (Grammer and Lipsky 2003; Renaudineau, Pers et al. 2004). Additionally, somatic hypermutation of immunoglobulin variable regions, abnormality of receptor editing, antigen-drived autoantibody production and defect of idiotypic networks could be associated with SLE development(Kalden, Winkler et al. 1991; Williams, Malone et al. 1995; Spatz, Iliev et al. 1997; Suzuki, Mihara et al. 1997).

T cell abnormalities in SLE

- *Antigen processing and presentation*

It is widely believed that T cells provide help to autoantibody-producing B cells in SLE. There are possible mechanisms by which T cells may escape immunological tolerance leading to clinical manifestations of SLE. The hypothetical points of abnormalities of T cell–antigen-presenting cell interaction or abnormal signalings might lead to loss of immunological tolerance in SLE as shown in Table4. In addition, chromatin-containing CpG motif-rich DNA or ribonucleoprotein antigens containing double-stranded RNA (dsRNA) could potentially trigger adaptive immune responses in the pathogenesis of SLE by providing accessory signals through Toll-like receptor 9 (TLR9) on human dendritic cells, macrophages, or B cells, or through TLR 3 on human dendritic cells(Hoffman 2004).

Table 4 Breaking immune tolerance in SLE(Hoffman 2004)

Potential mechanisms
- Exposure of cryptic or neoepitopes during apoptosis
- Abnormal antigen processing
- Inappropriate help by cytokines
- Excess or defective co-stimulation via accessory molecules
- Abnormal activation threshold for cell signaling
- Insufficient Treg cells

- *Co-stimulatory and accessory molecules*

T cells from SLE patients exhibiting defects in regulatory functions are key to prolong overexpression of CD40L leading to prolong co-stimulation that sustains autoantibody producing B cells(Yi, McNerney et al. 2000). CD154 has been found to be overexpressed on lymphocytes in secondary lymphoid tissues from active SLE patients (Devi, Van Noordin et al. 1998). In addition, B7-CD28 co-stimulatory signaling has defects resulting in incomplete T cell activation. Furthermore, overexpression of adhesion molecules such as lymphocyte function associated antigen 1 (LFA-1), sVAM-1 and sICAM-1 has been reported to occur in SLE(Hoffman 2004).

- *Apoptosis and apoptotic clearance*

Normally, autoreactive cells are anergic or undergo activation-induced cell death upon stimulation and are therefore functionally eliminated. Studies have identified defects of activation-induced cell death in T cells from SLE patients. T cells of SLE resist to anergy and apoptosis by upregulating *Cox-2* and *Bcl-2* expression, both of which encoding anti-apoptotic proteins(Aringer, Wintersberger et al. 1994; Budagyan, Bulanova et al. 1998; Xu, Zhang et al. 2004). Fas-FasL pathway is one of apoptotic mechanisms in lymphocytes undergoing activation-induced cell death. Mutations in Fas or FasL as well as elevated soluble Fas levels are revealed in T cells of SLE patients(Drappa, Vaishnaw et al. 1996; Wu, Wilson et al. 1996; Vaishnaw, Toubi et al. 1999). These evidences have indicated the breakdown of tolerance in SLE. Furthermore, impaired clearance of apoptotic materials has been implicated in the pathogenesis of

SLE. Macrophages from SLE patients are defective in the phagocytosis of autologous apoptotic materials(Herrmann, Voll et al. 1998). Therefore, the impaired clearance might affect the accumulation of apoptotic cells in tissues of some SLE patients. It has been found that the number of functional macrophages, which usually ingest apoptotic materials in the germinal centers of lymph nodes, was strongly reduced in some SLE patients(Baumann, Kolowos et al. 2002).

- *Regulatory T cells*

The development and maintenance of Treg cells constitute one of essential mechanisms of peripheral tolerance. Defects in either ontogeny of Treg cell population or suppressive functions of Treg cells are linked to various autoimmunity. In SLE, it is reported that suppressive activity of $CD4^{+}CD25^{+}$ Treg cells decreased(Monk, Spachidou et al. 2005), while some investigators reported that Treg cells is depleted in active SLE but have normal phenotype and function(Crispin, Alcocer-Varela et al. 2003; Miyara, Amoura et al. 2005; Lee, Wang et al. 2006). Therefore, the likely involvement of Treg cells in SLE is still controversial and need further investigation.

Notch signaling

Notch signaling is an evolutionarily conserved pathway that was first described as a neurogenic gene locus in *Drosophila melanogaster* and has functions in regulation of cell fate decisions during neuronal development(Wharton, Johansen et al. 1985; Kidd, Kelley et al. 1986; Artavanis-Tsakonas, Rand et al. 1999). *Drosophila* genome encodes one Notch receptor and two ligands, Serrate and Delta, whereas the mammalian genome encodes four Notch receptors (Notch1, 2, 3 and 4). Mammalian Notch receptors have five ligands; Jagged1 and Jagged2 (homologs of Serrate) and Delta-like1, Delta-like3 and Delta-like4 (homologs of Delta). Notch receptors are single-pass transmembrane receptors that are cleaved within the trans-Golgi network during biosynthesis to yield a heterodimeric complex before transported to cell surface. Notch signaling was initiated by receptor-ligand interaction between adjacent cells. Ligand binding causes a cascade of proteolytic cleavages resulting in the release of

intracellular domain of Notch (ICN). The first cleavage occurs just external to the transmembrane domain and is mediated by an ADAM metalloprotease. The second cleavage, mediated by a multiprotein complex with γ-secretase activity containing presenilin and nicastrin proteins, occurs within the transmembrane domain. The ICN translocates to the nucleus and heterodimerizes with the transcription factor CBF1, a member of CSL family protein, converting it from a repressor into an activator by displacing corepressors and recruiting coactivator complex(Maillard, Adler et al. 2003; Pear and Radtke 2003; Radtke, Wilson et al. 2004). The direct effect of Notch activation is transcription of Notch target genes Hairy enhancer of split (Hes) family, such as *Hes1* and *Hes5* and the related *Herp* (Hes-related repressor protein) transcription factor family that encodes transcriptional repressors (Iso, Kedes et al. 2003). Hes is basic helix-loop-helix type of transcription factors and suppresses expression of downstream target genes such as tissue-specific transcriptional activators. Thus, Hes directly effects cell fate decisions as a primary Notch effector(Iso, Kedes et al. 2003). Moreover, Notch signaling is modulated at extracellular, cytoplasmic and nuclear level.

Notch and Immune System

Notch signaling regulates developmental cell fate decisions, cell growth and differentiation of multiple organs during embryogenesis and adult(Hoyne 2003). Several Notch receptors and ligands are temporally and spatially expressed during hematopoiesis.

Notch and hematopoietic stem cells

Mutations in Notch receptors and ligands lead to abnormalities in development of several tissues, including vessels, thymus, central nervous system as well as hematopoietic cells(Iso, Kedes et al. 2003). Notch receptors and ligands are expressed in the hematopoietic system, implying the important roles its play during hematopoiesis. During embryonic development, Notch1 and Notch4 are expressed in hemangioblasts, bipotential developing to both endothelial and hematopoietic cells, while hematopoietic progenitors express Notch1 and Notch2. Signaling through Notch1 but not Notch2 was

strongly demonstrated as an essential for generation of hematopoietic stem cells (HSC) derived from endothelial cells(Kumano, Chiba et al. 2003). The osteoblasts, a critical cell type in HSC niches, upregulate Jagged1 when actvated by parathyroid hormone (PTH) or PTH-related protein. It results in regulation of HSC homeostasis through Notch activation(Calvi, Adams et al. 2003). The ligand-stimulated Notch activation, or expression of constitutively active form of Notch1 can promote self-renewal of adult HSCs(Varnum-Finney, Xu et al. 2000; Stier, Cheng et al. 2002; Varnum-Finney, Brashem-Stein et al. 2003).

Notch and T cell development

The essential function of Notch in T cell fate decisions is one of the most well established roles during lymphoid development. In Notch1 loss of function mutation or CSL deficiency, bone marrow progenitors enter thymus and adopt B lineage fate instead of T cells(Radtke, Wilson et al. 1999; Wilson, MacDonald et al. 2001; Han, Tanigaki et al. 2002). Conversely, gain of function by Notch1-IC expression in bone marrow progenitors leads to T cell lineage development as well as Delta-like1 expression in stromal cells(Pui, Allman et al. 1999; Jaleco, Neves et al. 2001). These reports suggested that Notch1 signaling mediated by CSL is necessary and sufficient for T cell lineage commitment.

After the initial T lineage commitment, developing thymocytes undergo binary cell fate decision to become either αβ or γδ TCR lineage T cells. In study using mixed bone marrow chimeras with Notch1$^{+/-}$ and Notch1$^{+/+}$ progenitors, Notch1$^{+/-}$ progenitors are more likely to develop as γδ T cells than as αβ T cells(Washburn, Schweighoffer et al. 1997). It is possible that Notch1 signaling may preferentially influence αβ T cell lineage. Furthermore, conditional inactivation of Notch1 driven by expression of *Cre* under a proximal *Lck* promoter (before the pre-TCR checkpoint) defected αβ T cell differentiation whereas it has no apparent effect on γδ T cell number or phenotype (Wolfer, Wilson et al. 2002). These results indicate that Notch1 signaling might not influence γδ lineage choice. However, the possibility that Notch1 inactivation may occur after αβ-γδ lineage commitment in this experiment system that also does not conclude

role of Notch1 in γδ T cell lineage. For pre-TCR checkpoint, Notch1 deficiency leads to a partial block of αβ T cell development at pre-TCR checkpoint during VDJβ rearrangement(Wolfer, Wilson et al. 2002).

For further differentiation along the αβ T cell lineage, $CD4^+CD8^+$ T cells develop to either $CD4^+$ or $CD8^+$ T cells. Although still controversial, results from gain-of-function experiments suggested that constitutively active Notch influences CD4 and CD8 single positive thymocytes development while loss-of-function studies show that Notch does not effect the CD4 versus CD8 decision(Robey, Chang et al. 1996; Deftos, Huang et al. 2000; Wolfer, Bakker et al. 2001).These results indicated that other Notch family members may influence this lineage decision. Some reports show normality of CD4:CD8 ratio or rate of generation in the conditional CSL knockout mice(Han, Tanigaki et al. 2002). Thus it is now apparent that Notch signaling is not involved in CD4-CD8 lineage decision.

Notch and B cell development

Bone marrow progenitors expressing active form of Notch1 fail to develop into B cells but instead develop into T cells(Pui, Allman et al. 1999). Notch signaling must be shut down to allow B cell development in bone marrow. In spite of the expression of Notch receptors and their ligands in bone marrow progenitors and stromal cells, the development of B cells can continue because of expression of Pax5. It is proposed that Pax5 promotes B cell lineage due to interfering with T lineage commitment by repressing Notch1 transcription(Souabni, Cobaleda et al. 2002). In periphery, Notch signaling is necessary for marginal zone B cell development. Notch2 expression predominates in splenic B cell and increases during B cell maturation. Moreover, CSL-mediated Notch2/delta-like1 interaction promotes marginal zone B cell development (Saito, Chiba et al. 2003; Hozumi, Negishi et al. 2004).

Notch and peripheral T cells

$CD4^+$ and $CD8^+$ T cells differentiate to become effector cells in the periphery. Antigen presenting cells (APC) have an important role for initiating immune responses

by interaction with and signaling through TCR and co-stimulatory molecules on naïve T cells. Cascade of other signalings that involve in activation is not well understood.

Notch ligands, including Jagged1, Jagged2, Delta-like1 and Delta-like4 are expressed on APCs, macrophages and dendritic cells (DC)(Yamaguchi, Chiba et al. 2002). Naïve $CD4^+$ T cells express both Notch1 and Notch2 while naïve $CD8^+$ T cells express only Notch1(Hoyne, Le Roux et al. 2000; Amsen, Blander et al. 2004). After stimulation, peripheral $CD4^+$ T cells increase Notch1-4 expression. Moreover, cleaved form of Notch1 is detected as well as Hes-1, one of target genes of Notch, and CD25. Conversely, inhibiting Notch signaling results in decreased production of IL-2, CD25 expression and diminished proliferation, whereas the opposite results are obtained in constitutively active Notch1 condition(Adler, Chiffoleau et al. 2003; Palaga, Miele et al. 2003). These results suggest that Notch signaling enhances responsiveness to TCR-mediated stimulation. On the other hand, Notch1 signaling is reported as regulator of peripheral T cell activation in vitro and in vivo. Coligation of Notch1 together with TCR inhibits T cell activation, proliferation and cytokine production(Eagar, Tang et al. 2004). Signal using high concentrations of an activating ligand, Delta-like1-Fc, suppresses T cell proliferation(Maekawa, Tsukumo et al. 2003). While another study reports that proliferation is comparable between activated T cells from $Notch^{+/+}$ and $Notch^{-/-}$ mice (Radtke, Wilson et al. 2002). Thus, Notch signaling may either promote or inhibit T cell activation depending on the microenvironment, ligand expression, redundant Notch receptors and different stages of activation, maturation, or differentiation.

After encountering antigen, naïve T cells differentiate to be either one of two functional classes depending on several factors. T helper type 1 cells (T_H1) is promoted by interleukin12 (IL-12)/IFNγ and secrete effector cytokines such as interferon-γ (IFN-γ). The other effector lineage is T helper type 2 cells (T_H2) which is promoted by IL-4 and secrete effector cytokines such as IL-4, IL-5 and IL-13. Furthermore, specific transcription factors, T-bet and GATA-3, are considered master regulators in differentiation of T_H1 and T_H2 cells, respectively(Murphy and Reiner 2002). Notch ligands and receptors have been implicated in helper T cell differentiation. Notch1 signaling

directly controls expression of T-bet, which is encoded by *Tbx21*, through complex formation on *Tbx21* promoter under T_H1 polarization condition. After inhibition of Notch signaling by γ-secretase inhibitor, preventing activation of all Notch receptors, expression of Notch, *Tbx21* and IFN-γ are impeded in T_H1 polarized $CD4^+$ cells and can be restored by active form of Notch1(Minter, Turley et al. 2005). $CD4^+$ T cell differentiation is partly controlled by APCs, which translate information of microbial threat to T cells and promote T_H1 or T_H2 differentiation. Expression of the Delta-like family on DCs induces T_H1 differentiation while expression of the Jagged family induces T_H2 fate, independent of IL-4/STAT6. This polarization is determined by cytokine profile. It is suggested that APCs use the Notch pathway to regulate $CD4^+$ differentiation based on the types of Notch ligands(Amsen, Blander et al. 2004). Additionally, Notch receptors is shown to be involved in the differentiation of helper T cells. Expression of activated form of Notch1 in $CD4^+$ T cells promotes T_H2 differentiation and IL-4 production whereas an active form of Notch3, but not activated form of Notch1, generated by interaction with Delta-like1, promotes T_H1 phenotype, T-bet expression and IFN-γ production(Maekawa, Tsukumo et al. 2003; Amsen, Blander et al. 2004). The role of Notch signaling in promoting T_H2 immune response has demonstrated. $CD4^+$ T cells lacking Notch signaling by overexpressing a dominant negative MAML protein, impairs T_H2 differentiation but have normal T_H1 differentiation. These genetically manipulated mice failed to develop a protective T_H2 cell response against infection with *Trichuris muris* ,but can normally control T_H1 cell response(Tu, Fang et al. 2005). In contrast, γ-secretase inhibitor does not impede T_H2 differentiation ,but inhibit T_H1 differentiation (Minter, Turley et al. 2005). However, whether Notch signals are directly involved in differentiation of either T_H1 or T_H2 cells, or both. It remains poorly understood and controversial.

Several mechanisms control recognition and distingushing between self and non-self such as deletion of autoreactive cells in central lymphoid organs and induction of anergy in peripheral lymphoid organs. The existence of subpopulation of T cells that specialize in this suppressive activity in immune responses is called regulatory T cell

(Treg). However, the cellular and molecular mechanisms for these suppressive activities are currently unknown. There are many evidences suggesting that Notch signaling induces differentiation of naïve T cells towards Treg cells. Constitutive expression of Jagged1 on DCs contributes to differentiation of naïve peripheral $CD4^+$ T cells to Treg cells which inhibit primary and secondary immune responses and decrease cytokine production, IL-2 and IFN-γ. This antigen-specific tolerance can be transferred from donor to recipient mice(Hoyne, Le Roux et al. 2000). In further investigation, stimulation of naïve human $CD4^+$ T cells with APCs overexpressing Jagged1 reduce proliferation, production of IL-2, IL-5 and IFN-γ, whereas transforming growth factor-β1 (TGF-β1) is upregulated. Furthermore, stimulated T cells show hyporesponsiveness to stimulation and inhibitory activities against cytotoxic activity to alloantigen stimuli which indicates a T_H3-type Treg cell phenotype(Yvon, Vigouroux et al. 2003). In another report with similar condition, it was demonstrated that the same results were obtained except cytokine production such as IL-10 upregulation, which indicates Tr1-type Treg cell phenotype(Vigouroux, Yvon et al. 2003). A striking feature of transgenic mice with activated form of Notch3 is CD25 upregulation in thymocytes. Therefore, dysregulated CD25 expression may underlie Notch3 pathway that lead to Treg cell generation in thymus(Bellavia, Campese et al. 2000). Furthermore, suppressive function of Treg cells expressing $Foxp3^+$ TGF-β^{m+} depends on activation of TGF-β^{m+} and Notch1 pathways in target cells. Blocking Notch1 activation inhibits suppressive activity of TGF-β^{m+} Treg cells to allergic airways disease *in vivo* (Ostroukhova, Qi et al. 2006). The role Notch signaling plays in controlling peripheral T cells are currently emerging, however, the molecular mechanisms leading to such functions are largely unknown.

Notch Signaling and Autoimmune Diseases

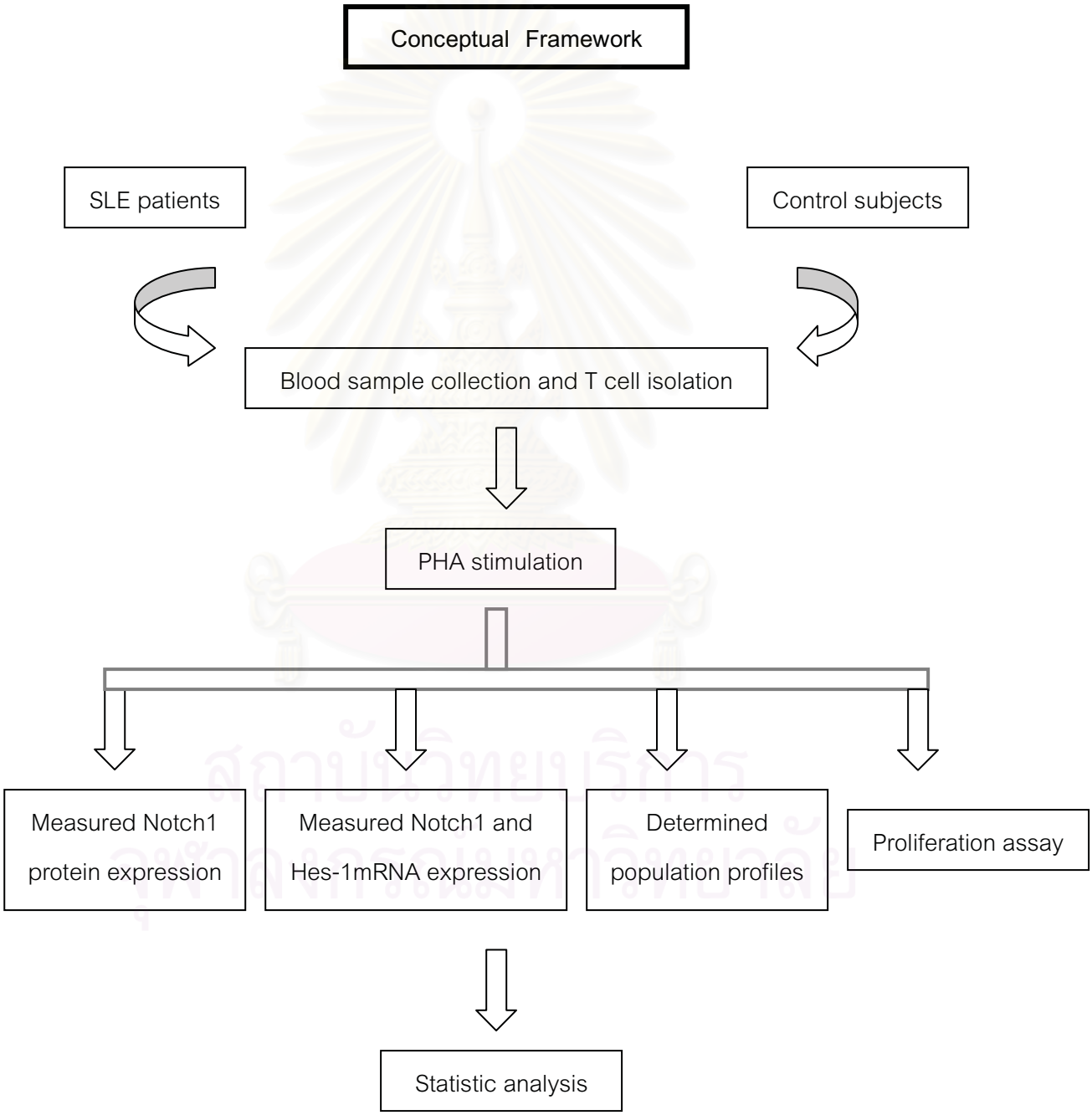
Diseases caused by failure of self-tolerance and subsequent immune responses against self-antigens are called autoimmune diseases. There are number of evidences of autoimmune diseases associated with the Notch signaling. Thymic-derived dysregulated tolerance due to impaired generation of naturally occurring $CD4^+CD25^+$

Treg cells leads to an imbalance between β cell Ag self-reactive T cells and regulating factors, which is a key event in autoimmune diabetes. Thus, overexpression of active form of Notch in developing thymocytes enhances generation of naturally occurring Treg cells, IL-4 and IL-10 expression, and especially protection from experimental autoimmune diabetes in mice(Anastasi, Campese et al. 2003). Multiple sclerosis is an autoimmune disease of the central nervous system which $CD4^+$ T cells of the T_H1 subset reacts against self myelin antigens. *In vivo*, γ-secretase inhibitor, blocking all Notch signals, inhibits T_H1-mediated autoimmune disease progression in mouse model of experimental autoimmune encephalomyelitis model of multiple sclerosis(Minter, Turley et al. 2005). There are some reports demonstrating that Notch signaling pathway is involved in SLE. T lymphocytes of human SLE differentially express *presenilin2* that is a component of γ-secretase which is an important enzyme for Notch activation(Xu, Zhang et al. 2004). Interestingly, partial loss of presenilins in adult mice causes development of several autoimmune phenotypes, including SLE-like autoimmune disease. The disease manifestations include kidney immunoglobulin deposition in glomerula, leukocyte invasion, high titers of anti-nuclear autoantibody, proteinuria, hematuria and increased levels of anti-ssDNA antibody(Tournoy, Bossuyt et al. 2004).

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER IV

MATERIALS AND METHODS



Patients and healthy controls

Twenty-two Thai SLE patients, 21 females and 1 male, from King Chulalongkorn Memorial hospital diagnosed with SLE according to the American College of Rheumatology criteria (Table1) were included in this study. SLE patients were classified into two groups according to SLE disease activity index (SLEDAI). The first group included 10 inactive SLE patients (mean age 32.80 ±6.21 years old) receiving low doses of prednisolone at less than 10 milligrams per day within at least one month before blood collection (SLEDAI < 3). The other group included 12 active SLE patients (mean age 26.33 ± 6.01 years old) receiving low to intermediate doses of corticosteroids (2.5-25 milligrams per day of prednisolone with or without mycophenolate mofetil or azathioprine or myfortic) (SLEDAI ≥ 3) as shown in Table 5. Eleven age- and sex-matched healthy controls were included (mean age 24.55 ± 1.04 years old, 10 females and 1 male). The study was approved by the ethics committee of the King Chulalongkorn University and obtained informed consents from all subjects.

Cells and Cell Cultures

Twenty milliliters (ml) heparinized blood samples were collected and processed within 2 hours of collection. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque reagent (Robbins Scientific Corporation, Sunny vale, CA) at ratio 3:4 (v/v; Ficoll-Hypaque reagent : whole blood). This solution was centrifuged at 2,000 rpm for 25 minutes at room temperature. For density-gradient centrifugation technique, separated PBMCs in interface by specific density were collected and washed twice in 10 ml of phosphate buffered saline (PBS) by centrifugation at 1,500 rpm for 10 minutes at 4^{o}C. Some experiments used purified T lymphocytes which were isolated by positive selection by CD3 magnetic beads (Miltenyi Biotec). PBMCs were suspended in 80 microliters (µl) of buffer (see appendix A) per 10^7 total cells and added 20 µl of CD3 microbeads per 10^7 cells followed by a through mixing. After incubation for 15 minutes at 4-8^{o}C, stained cells were washed with 1 ml of buffer per 10^7 cells and centrifuged at 300 g for 10 minutes. Pellets were resuspended with cells upto 10^8 cells in 500 µl of

buffer. Separating column were prepared by rinsing with 500 µl of buffer and placed in magnetic field. Cell suspension was loaded onto the column and unlabelled cells were allowed to flow through. The column was washed three times with 500 µl of buffer and removed from the magnetic field separator and placed on top of collection tube. After Pipetting 500 µl of buffer onto the column, the magnetically labeled cells which were CD3+ T lymphocytes were immediately flushed out by firmly applying the plunger on columns. Cells adjusted to 2×10^6 cells/ml by hemacytometer chamber were cultured at density of 1×10^6 cells per well in 24-well plate containing of RPMI1640 medium supplemented with 10% fetal bovine serum (GIBCO BRL), penicillin, streptomycin and non-essential amino acids (GIBCO BRL). For stimulation, cells were stimulated with phytohemagglutinin (PHA) (10 µg/ml) at 37°C in 5% CO_2 for 72 hr or as indicated.

RNA extraction and complementary DNA synthesis

Stimulated cells were harvested and 1 ml of Trizol (Molecular Research Center) was added, followed by thorough pipetting. The homogenate was stored for 10 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Next, 200 µl of chloroform were added and following by shaking vigorously. After centrifugation at 12,000 g for 15 minutes at 4°C, RNA which remained exclusively in the aqueous phase was collected into fresh tube. RNA was precipitated by mixing with 500 µl of isopropanol and incubated for 10 minutes at room temperature and then centrifuged at 12,000 g for 10 minutes at 4°C. Precipitated RNA was washed with 1 ml of 75% absolute ethanol at 12,000 g for 10 minutes at 4°C. Finally, RNA pellet was air-dried for 5 minutes and dissolved subsequently with RNase-free water stored at -80°C until use. RNA concentration was determined using absorbance value from a spectrophotometer for complementary DNA (cDNA) generation. mRNA was reverse-transcribed to cDNA using a commercial kit (Applied Biosystems, Branchburg, New Jersey). Two hundred and fifty nanograms (ng) of each RNA sample were added with 10x RT buffer, 25 mM $MgCl_2$, 10 mM dNTP, 50 µM random hexamer primer, 20 U/µl RNase inhibitor, 50 U/µl superscript reverse transcriptase and RNase-free water

according to the manufacturer's protocol. Subsequently, mRNA was reverse-transcribed at 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes using Perkin Elmer/GeneAmp PCR system, and cDNA was kept at -20°C.

Conventional reverse transcription polymerase chain reaction (RT-PCR)

Expression of genes of interest and housekeeping genes were confirmed by conventional RT-PCR. The reaction volume of the amplification reaction was 10 μl, containing 2 μl of 8.3 ng/μl cDNA, 0.2 μl of 5.0 U/μl Taq polymerase (Promega), 1 μl of 10x PCR buffer (20mM Tris-HCl pH 8.0, 100 mM KCl), 0.8 μl of 25 mM $MgCl_2$, 0.64 μl of 10 mM dNTP, 0.2 μl (10 pmol) of each primer and 4.96 μl of sterile water. Each primer sequence was shown in Table 6. The PCR step consisted of an initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation (94°C, 30 seconds), annealing (55°C for *Notch1* gene or 60°C for *GAPDH* gene, 30 seconds), extension (72°C, 30 seconds) and final extension at 72°C for 10 minutes. Amplification was performed in Perkin Elmer/ GeneAmp PCR system 2400. The PCR products were loaded in 1.5% Tris-acetate agarose gel and analyzed using electrophoresis containing Tris-acetate buffer at 100 voltages for 40 minutes followed by staining 50 μg/ml ethidium bromide and visualizing under UV light by Gel Doc™ MZL (BIO-RAD). Negative controls without DNA template were included in each experiment. DNA molecular ladder of 100 bp (Promega) was used to estimate the size of the PCR fragments.

Quantitative Real-time RT-PCR

A real time RT-PCR assay was developed for detection and quantification of Notch1 and Hes-1 transcripts using β-actin housekeeping transcripts as endogenous control. Primers were indicated in Table 6. Each polymerase chain reaction was set up for 20 ml reaction volume. PCR amplification was performed with 2x QuantiTect SYBR Green PCR Master Mix with 0.5 μM primers, 16 ng cDNA and nuclease-free water according to the manufacturer's protocol (Qiagen). Polymerase chain reaction amplification included an initial activation at 95°C for 15 minutes, denaturation at 95°C

for 15 seconds, annealing at 55°C (*Notch1*) or 57°C (*Hes-1*) for 30 seconds and extension at 60°C for 30 seconds followed by repeating for 40 cycles. The mRNA levels were measured by a Light Cycler machine (Roche Molecular Biochemicals, Indianapolis, IN). Levels of mRNA were expressed as threshold cycle (C_T) and used comparative C_T method for analysis. For relative quantification, the expression of *Notch1* and *Hes-1* as target genes were normalized by expression of *β-actin* (housekeeping gene) as endogenous reference and relative to a calibrator (a sample used as the basis for comparative results). Finally, the amount of target was given by $2^{-\Delta\Delta CT}$.

$$\Delta C_T = C_T(\text{target}) - \Delta C_T(\text{reference})$$

$$\Delta\Delta C_T = \Delta C_T(\text{normalized target}) - \Delta C_T(\text{normalized calibrator})$$

Proliferation assay

Purified T lymphocytes (2×10^6 cells/ml) were plated at 100 µl (2×10^5 cells/well) in 96-well plate and were stimulated with 10 µg/ml of PHA for 72 hr at 37°C in 5% CO_2. Cells were pulsed with 1 µCi/ml of ^{3}H-methyl-thymidine (3H-TdR) (Amersham Biosciences) for the final 6 hr of culture. After incubation, cells were harvested and 3H-TdR incorporation were measured with a scintillation counter (Packard Instruments, Downers Grove, IL) as mean counts per minute (cpm) and the results were shown with SD of triplicate measurements. The following formulas were used to calculate Δcpm.

$$\Delta\text{cpm} = \text{cpm (PHA-stimulated cells) - cpm (unstimulated cells)}$$

$$\text{SI} = \frac{cpm(PHA-stimulated\ \ cells)}{cpm(unstimulated\ \ cells)}$$

normal value : Δcpm > 2,000

: SI > 5

Western Blot

PBMCs stimulated with or without PHA as described above were harvested on day 3. Cells were washed with PBS and lysed by 200 µl of buffer A (see appendix C), followed by centrifugation at 3,000 rpm for 5 minutes at 4°C. Twenty-five microliters of buffer B (see appendix C) and 1 µl of protease inhibitor cocktails were added with gentle mixing and centrifuged at 5,000 rpm for 5 minutes at 4°C. Cell lysates in aqueous phase was collected as protein samples and stored at -80°C. Amounts of proteins were measured using BCA protein assay kit (Pierce; Rockford, IL). Two hundred of mixed solution (A:B = 50:1) for assay were added to 1 µl of cell lysates plus 1 µl of sterile water within 96-well plate. After incubation 37°C for 30 minutes, the cell lysates were measured the density using microplate reader at $OD_{540\ nm}$ and concentration of total proteins were calculated from protein standard curve of six-fold diluted bovine serum albumin (BSA). 30 µg of total proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were added with β-mercaptoethanol (5%) and laemmli loading buffer, followed by boiling for 5 minutes for denaturing proteins to primary structure and changing to negative charge. Proteins were loaded and separated onto SDS-PAGE containing running buffer (see appendix D) in Protein III system (Bio-Rad) at 100 voltages for 90 minutes. Proteins were electrotransferred onto PVDF membrane (Amersham Biosciences) and blocked twice in PBS containing 3% nonfat dry milk and 0.05%(v/v) Tween 20. For primary antibody, blots were probed with rabbit IgG anti-Notch1 antibody at 1:1000 dilution for Notch1 detection (Santa Cruz Biotech), while mouse anti-β-actin monoclonal antibody was used at 1:5000 dilution for β-actin detection (Chemicon). Blots were washed with phosphate buffered saline tween (PBST) and then probed with HRP-conjugated donkey anti-rabbit IgG antibody or HRP-conjugated sheep anti-mouse IgG antibody at 1:4000 dilution for Notch1 and β-actin protein assay, respectively. After washing, signals were detected using ECL Western blotting analysis system (Amersham Biosciences) which is a light emitting non-radioactive method according to the manufacturer's protocol. The density of protein band was quantitated using software of Gel Doc analysis.

Flow Cytometry

Purified T lymphocytes stimulated with or without PHA as describe above were harvested at 72 hr and washed with PBS. Cells were stained with cocktail of anti-CD3-FITC (clone S4.1), anti-CD4-PE (clone S3.5), anti-CD8-PE-Cy5.5 (clone 3B5) and anti-CD25-APC (clone CD25-3G10) (Caltag, Burlingame, CA) for 20 minutes at room temperature. After washing off excess antibody, cells were fixed in 1% paraformaldehyde in PBS followed by analyzing on a FACS Calibur flow cytometer using Cellquest Software (Becton Dickinson).

Statistical analysis

Mean ± SD of independent experiments were analyzed. Intergroup comparisons in expression of Notch1 protein was analyzed using Chi-square Test (χ^2) and the other experiments were analyzed using independent *t* test of SPSS software (version 11.5). A *P* value of <0.05 was considered statistical significance.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 5 Characteristics of SLE patients included in this study

No. patients	stage	Age (years)	Sex	Treatment	Steroid dose (mg/d)	SLEDAI score	Clinical and biological features
1	active	21	F	Pred, MMF	10, 1,500	4	HP
2	active	24	F	Pred	15	4	HP
3	active	29	F	Pred, MMF	25, 1,000	13	AR, RI, HP, AN, PI
4	active	24	M	Pred, MFT	12.5, 540	5	RI, HP, AN
5	active	25	F	Pred, AZT	10, 75	5	ED, RI
6	active	38	F	Pred, AZT	10, 100	13	ED, RI, PI, HE, HP
7	active	20	F	Pred, AZT	12.5, 50	10	RA, RI, HE, HP, CY
8	active	27	F	Pred, AZT	12.5, 50	8	HP, HE
9	active	16	F	Pred, AZT	5, 75	4	HE
10	active	30	F	Pred	5	10	AR, RA, FE, ED, RI, LP, TC
11	active	29	F	Pred, MMF	2.5, 1,000	3	AL, RI, PI
12	active	33	F	Pred	2.5	18	RA, AL, RI, HP, PI, HE, LC
13	inactive	35	F	Pred	5	0	
14	inactive	25	F	Pred	2.5	0	
15	inactive	32	F	Pred	5	1	ED
16	inactive	38	F	Pred	5	0	
17	inactive	26	F	Pred	2.5	0	
18	inactive	41	F	Pred	2.5	1	AN
19	inactive	34	F	Pred	5	0	
20	inactive	39	F	Pred	7.5	0	
21	inactive	23	F	Pred	2.5	0	
22	inactive	35	F	none	-	1	ED

Pred, prednisolone; AZT, azathioprine; MMF, mycophenolate mofetil; MFT, myfortic; AL, alopecia; AR, arthritis; RI, renal involvement; HP, high proteinuria; AN, anemia; PI, pyuria; HE, hematuria; ED, edema; RA, rash; CY, cytopenia; FE, fever; LP, leucopenia; TC, thrombocytopenia; LC, low complement

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

Table 6 Primers used for analysis of the *Notch1*, *Hes-1*, *β-actin* and *GAPDH* gene

primers	Sequence (5'→3')	Size
Notch1		298 bp
- Forward	CAGCCTGCACAA CCAGACAGA	
- Reverse	TGAGTTGATGAGGTCCTCCAG	
Hes-1		259 bp
- Forward	ACCAACTGGGACGACATGGAGAA	
- Reverse	GTGGTGGTGAAGCTGTAGCC	
β-actin		380 bp
- Forward	ACCAACTGGGACGACATGGAGAA	
- Reverse	GTGGTGGTGAAGCTGTAGCC	
GAPDH		380 bp
- Forward	ACCACAGTCCATGCCAT	
- Reverse	ACCACCCTGTTGCTGTA	

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER V

RESULTS

1. Notch1 protein expression in lymphocytes of SLE patients

Twenty SLE patients (14 active SLE patients and 6 inactive SLE patients) receiving drug regimens as described in methods and twenty healthy controls were recruited for this study. Representations of expression of Notch1 protein as detected (ICN) by western blot is shown in Figure1A. In PHA-stimulated lymphocytes, Notch1 protein expression was detected in 2/14 (ratio=0.14) of active SLE patients, 5/6 (ratio=0.83) of inactive SLE patients and 17/20(ratio=0.85) of normal controls. Expression of Notch1 protein in active SLE is significantly downregulated by comparing to healthy controls and inactive SLE ($p=0.0002$, $\chi^2=13.96$ and $p=0.01$, $\chi^2=6.03$ respectively) (Figure 1B). Comparable upregulation of Notch1 is observed between controls and SLE patients in an inactive stage. Similarly, unstimulated lymphocytes showed significant Notch1 downregulation in active SLE compared to healthy controls ($p=0.001$, $\chi^2=10.77$).

Additionally, the level of Notch1 protein was measured by semi-quantitative method as showed in Figure 2. The results showed that the level of Notch1 proteins was significant decreased in stimulated lymphocytes of active SLE patients, as compared to healthy controls and inactive SLE ($p=0.001$ and 0.037, respectively). For unstimulated lymphocytes from active SLE, the level of Notch1 protein was also significantly reduced compared to healthy controls ($p=0.007$). After combining active and inactive SLE, the level of Notch1 protein was significant lower in stimulated and unstimulated lymphocytes of active SLE patients than healthy controls ($p=0.017$ and 0.029, respectively).

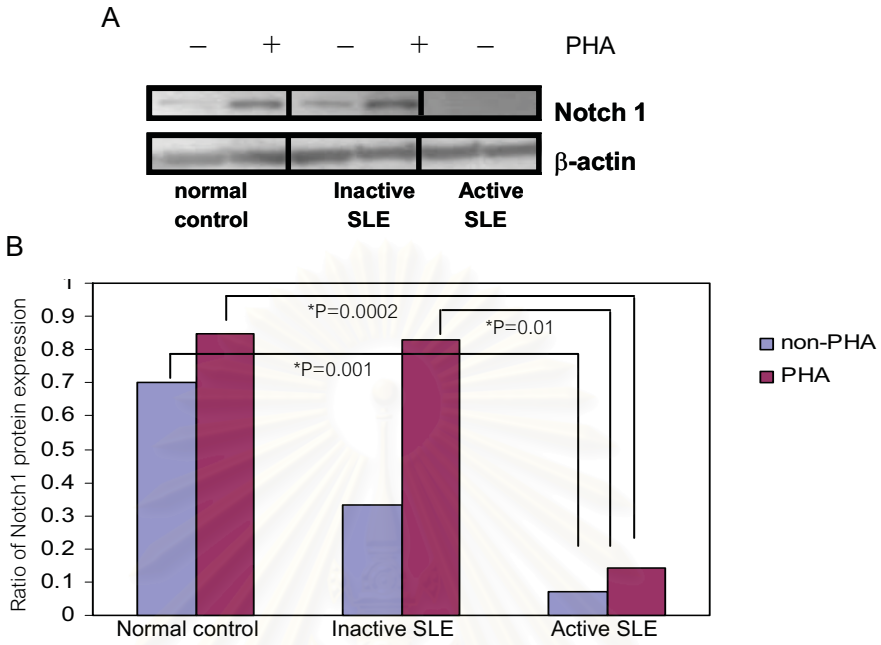


Figure 1. A, a representative of western blot of Notch1 expression in lymphocytes with or without PHA activation. ICN of Notch1 protein with molecular weight of approximately 110 kDa were detected. β-actin was used as a loading control. B, Western blot density quantitation was shown as ratio of the level of Notch1 protein normalized to the level of β-actin, number of samples with positive Notch1 protein expression per all samples in each group.

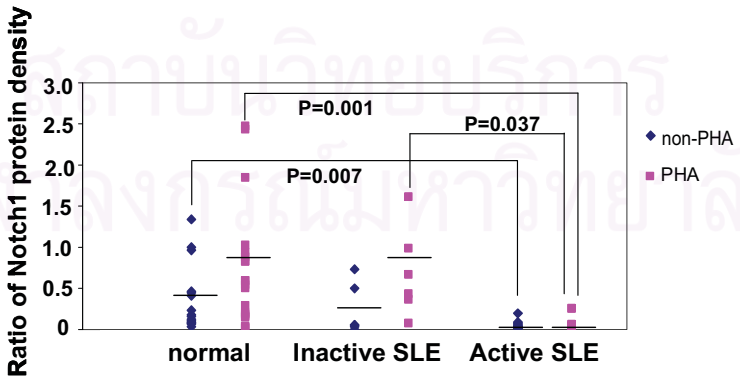


Figure 2. Graph was showed quantification of Notch1 protein in lymphocytes with or without PHA stimulation. The level of Notch1 protein was normalized by the level of β-actin and measured as density of Notch1 protein band.

2. Conventional Notch1 RT-PCR analysis in T lymphocytes

To confirm the result of quantitative Notch1 RT-PCR, (Figure 3A). Four patients with SLE and three healthy controls were recruited for this experiment. The level of *Notch1* mRNA expression was measured by semi-quantitative RT-PCR. *Notch1* mRNA was found to be decreased in stimulated T lymphocytes from SLE patients, as compared to those from healthy controls (p=0.015) as shown in Figure 3B.

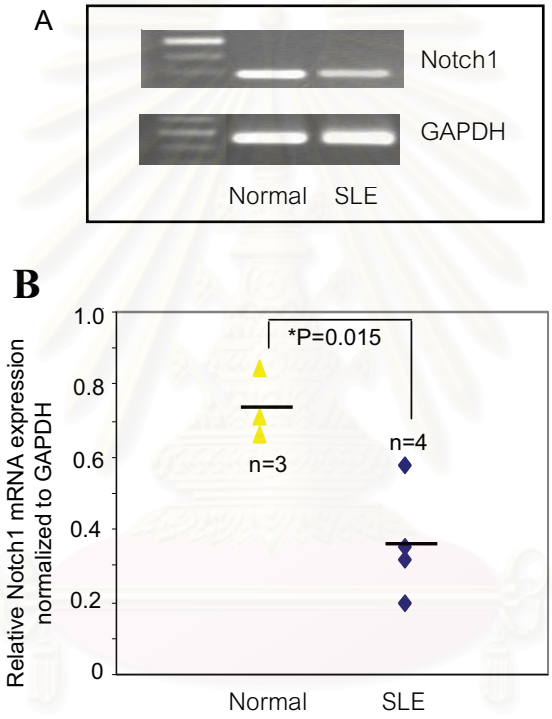


Figure 3. A, a representative of *Notch1* mRNA expression in T lymphocytes with PHA stimulation is shown. Notch1 expression in SLE patients was lower than normal control, and GAPDH was used as internal control. B, Semi-quantitative assay was shown as ratio of *Notch1* expression normalized to GAPDH. Dots were indicated *Notch1* mRNA levels and means in PHA-stimulated T lymphocytes of SLE patients and healthy controls.

3. Quantitative Real-time Notch1 RT-PCR Analysis in T lymphocytes

The results of Notch1 protein expression shown previously indicated that Notch1 was downregulated in activated lymphocytes of SLE. Types of lymphocytes with this defect is of interest, especially T lymphocyte. Therefore, expression of *Notch1* mRNA in PHA-activated T lymphocytes was investigated by quantitative RT-PCR. Twenty-two SLE patients (12 active SLE and 10 inactive SLE patients) and eleven healthy controls were recruited. The level of *Notch1* mRNA expression was quantified by real-time RT-PCR using SYBR and found similarly significant decrease in stimulated T lymphocytes from total cases of SLE patients compared with healthy controls ($p=0.05$) as shown in Figure 4A. Interestingly, when active SLE patients alone were compared with healthy controls, the level of Notch1 expression was remarkably lower ($p=0.025$). In contrary, T lymphocytes of inactive SLE patients did not show significant downregulation of mRNA Notch1 expression, as compared with controls and active SLE patients (Figure 4A). Surprisingly, stable inactive SLE (n=3) that was inactive stage at least 2 years before collecting blood samples and at least 6 months following up was showed mRNA Notch1 expression similar to healthy controls (Figure 4B). But mRNA Notch1 expression of unstable inactive SLE (n=7) was significantly decelerated compare to healthy controls and stable inactive SLE ($p=0.008$ and $p<0.0001$, respectively) (Figure 4B). To rule out any effects from immunosuppressive medications taken by patients, T lymphocytes from active SLE patients receiving low doses of steroid (n=3) and intermediate doses of steroid (n=9) were subjected to qRT-PCR and showed indifferent mRNA Notch1 expression (Figure 4C). Taken together, this result confirmed the western blot study that expression of Notch1 is downregulated in stimulated T lymphocytes from active SLE patients.

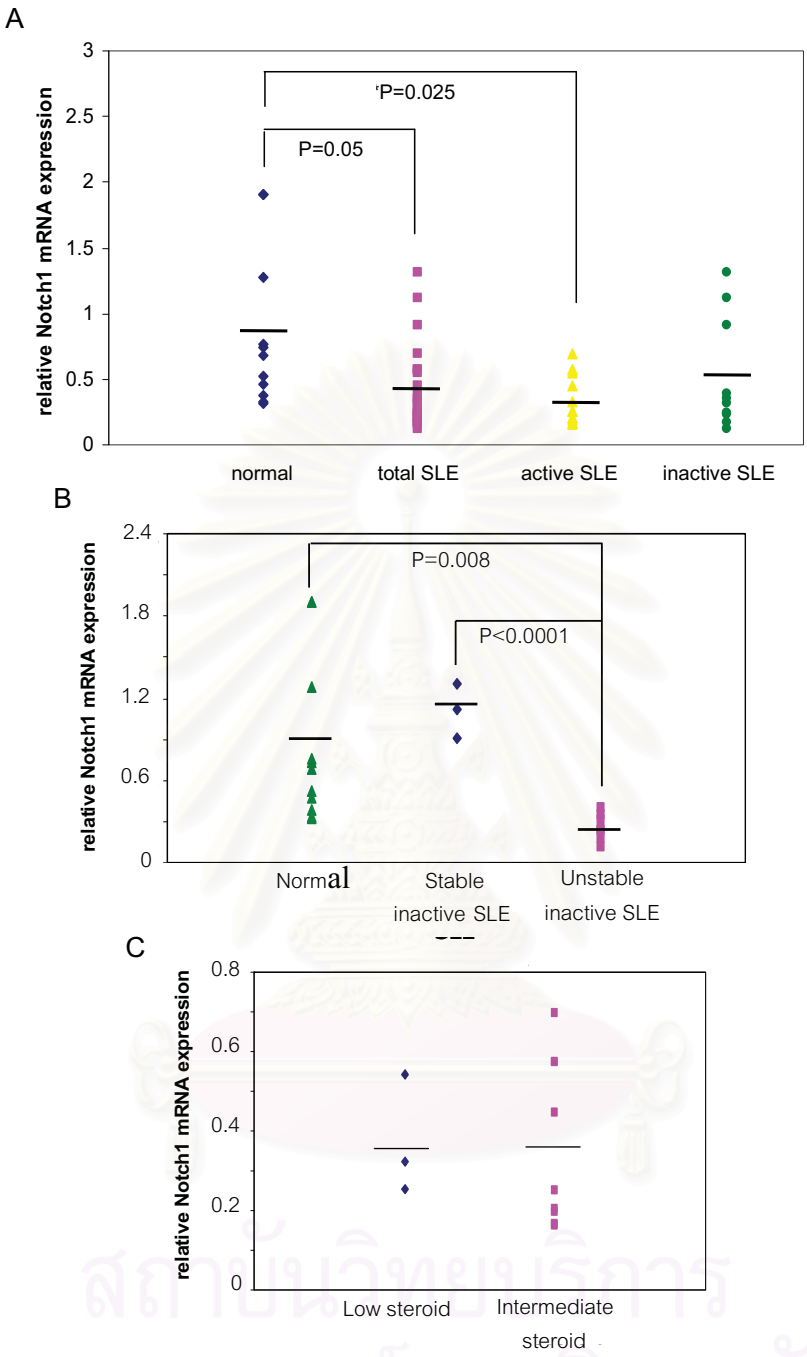


Figure 4. Summary of qRT-PCR on *Notch1* expression in PHA-stimulated T lymphocytes. A, dots were indicated *Notch1* mRNA levels and means in PHA-stimulated T lymphocytes of SLE patients and healthy controls. B, dots were indicated *Notch1* mRNA levels and means in PHA-stimulated T lymphocytes of inactive SLE patients with stable and unstable stage and healthy controls. C, to determine any effects from immunosuppressive medications taken by patients, dots were indicated *Notch1* mRNA levels and meaned in PHA-stimulated T lymphocytes of active SLE patients.

4. Quantitative Real-time Hes-1 RT-PCR analysis in T lymphocytes

Since *Hes-1* is one of the target genes of Notch signaling, activation of Notch1 induces expression of *Hes-1*. To investigate *Hes-1* expression in SLE, all subjects and method resembled to those used in Notch1 verification were employed. In PHA-activated T lymphocytes, it was found that the level of Hes-1 mRNA expression in active and inactive SLE was at a comparable level with those from healthy controls (Figure 5).

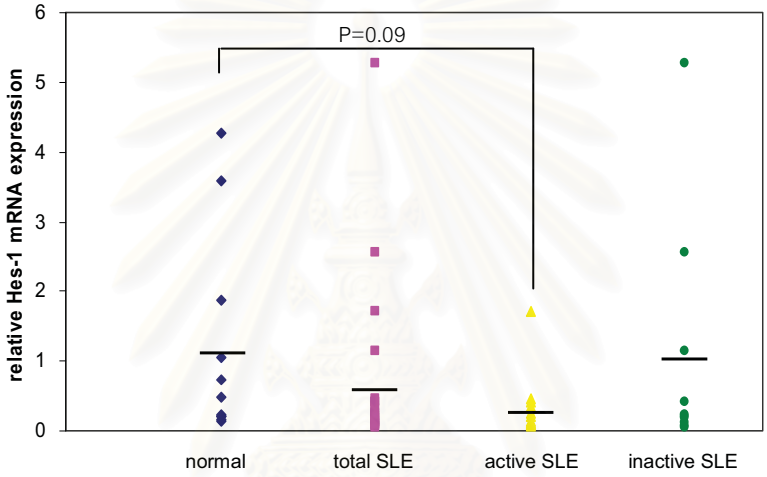


Figure 5. Summary of Hes1 mRNA expression by qRT-PCR. Dots and bars were indicated *Hes-1* mRNA levels and means in PHA-stimulated T lymphocytes of SLE patients and healthy controls.

5. Proliferation assay

In order to study the consequence of Notch1 downregulation from T lymphocytes of SLE patients, proliferation upon stimulation were measured ^{3}H-methyl-thymidine (3H-TdR) incorporation. Proliferation in both active SLE and inactive SLE group were similar as those of normal controls (Figure 6). This result suggests that Notch1 downregulation did not affect proliferative response in T lymphocytes upon i*n vitro* stimulation.

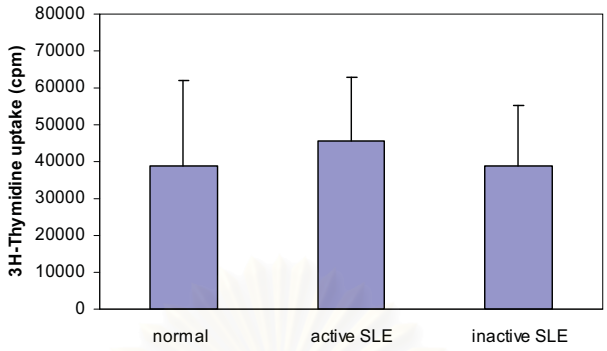


Figure 6. Proliferation of T lymphocytes from SLE patients and normal controls was assayed by 3H-Thymidine uptake. The results are shown as triplicate ± SD.

6. Profiles of Lymphocyte Populations in SLE Patients

To determine cell populations of PBMC used in this study, cell surface markers including CD4, CD8 and CD25 were used to follow T lymphocytes of SLE patients with or without PHA stimulation for 72 hr (Figure 7A and 8A). In unstimulated condition, the frequency of $CD4^+$ T cells was decreased in active and inactive SLE compared with healthy controls ($p=0.0004$ and <0.0001, respectively) whereas this of $CD8^+$T cells was increased in active and inactive SLE compared with healthy controls ($p=0.0004$ and <0.0001, respectively) as shown in Figure 7B. Similarly, the frequency of $CD4^+$ T cells and $CD8^+$ T cells in stimulated condition showed similar population patterns as seen with unstimulated condition ($p=0.002$ and <0.01, respectively) (Figure 8B). Moreover, level of CD25 expression in activated $CD4^+$ T cells in healthy controls and SLE patients by the flow cytometric analysis was shown in Figure 9. Level of CD25 expression in activated $CD4^+$ T cells of SLE patients was lower than healthy controls in three times repeated experiments.

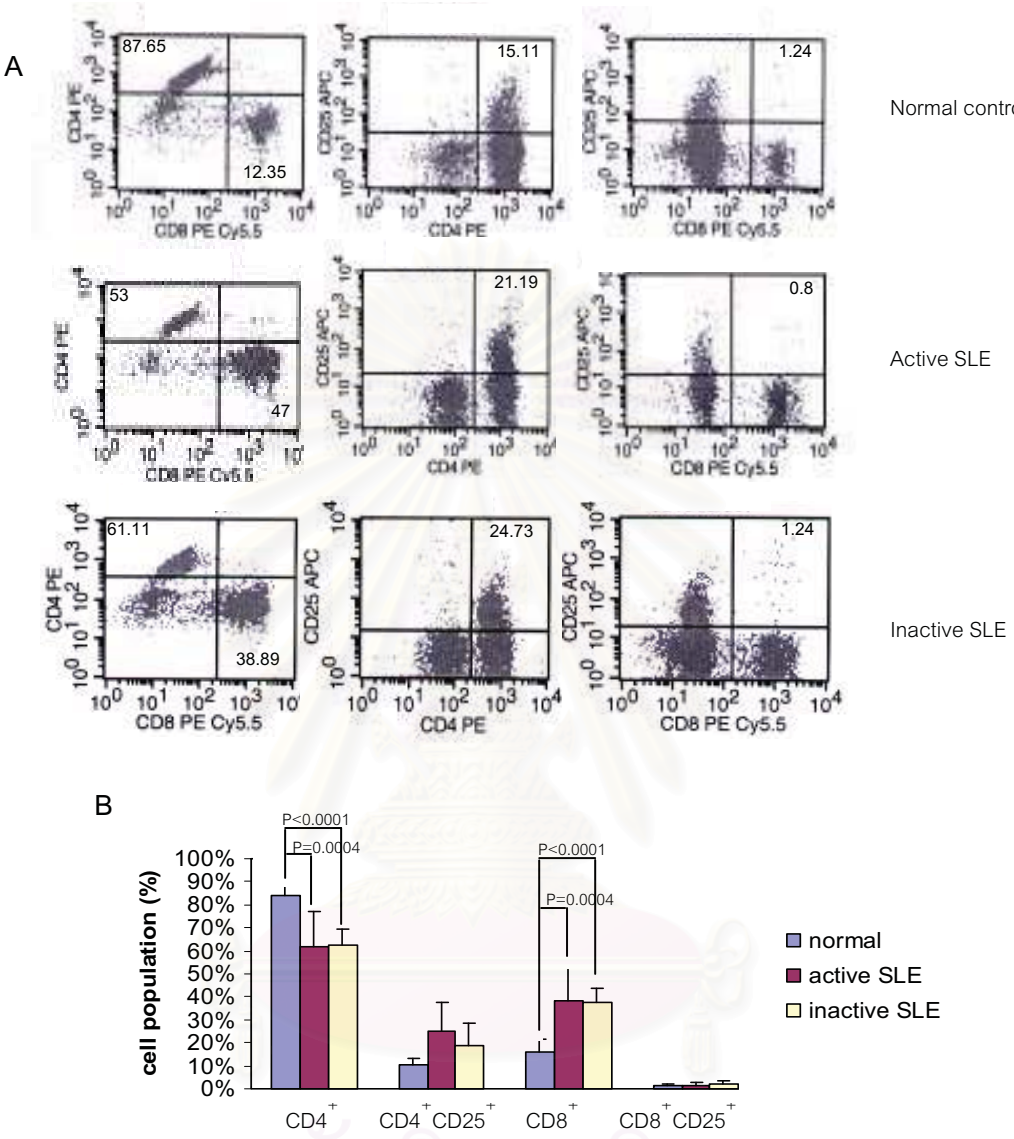


Figure 7. In unstimulated condition, representative dot plots of population profiles including $CD4^+$, $CD8^+$, $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells in healthy controls, active and inactive SLE patients by the flow cytometric analysis was shown in A. B were indicated means±SD of percentage of T cell populations.

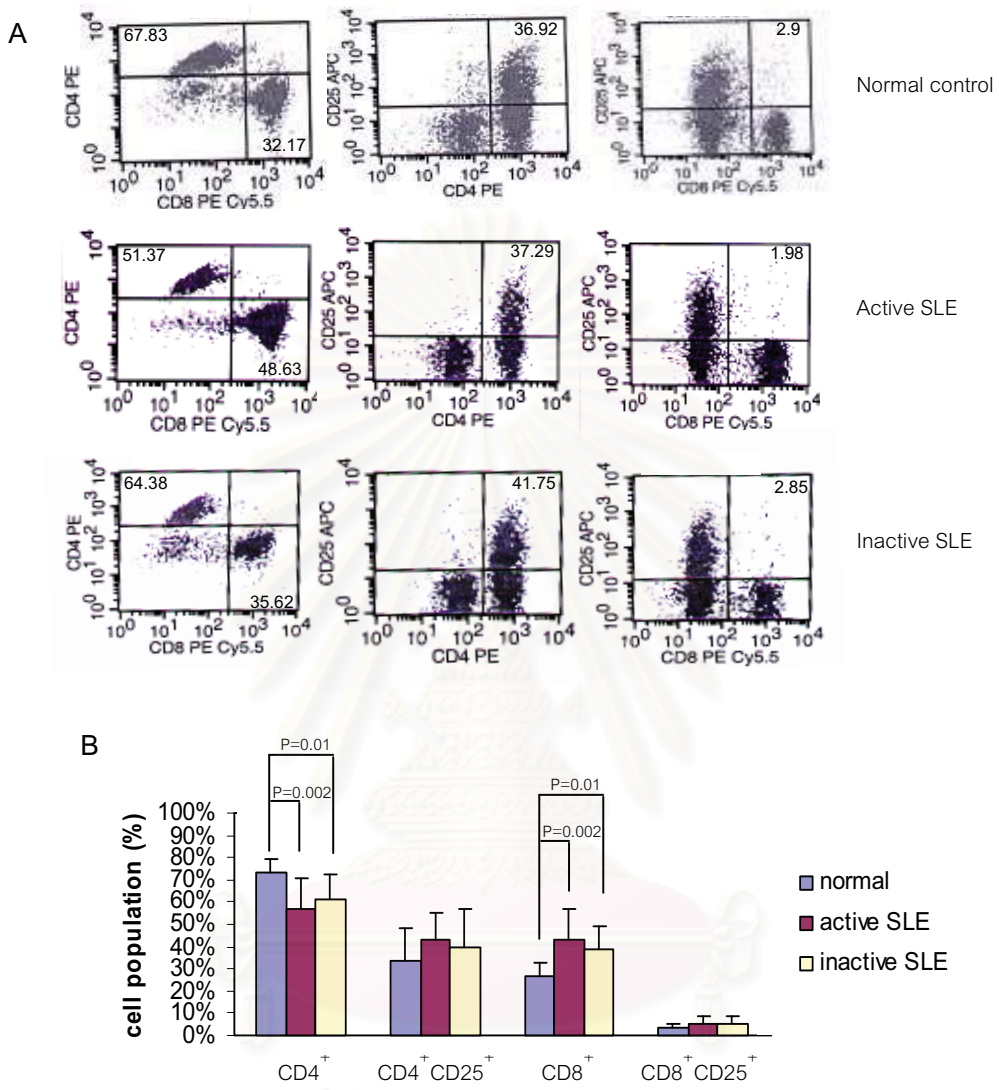


Figure 8. In stimulated condition, representative dot plots of population profiles including $CD4^+$, $CD8^+$, $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells in healthy controls, active and inactive SLE patients by the flow cytometric analysis was shown in A. B were indicated means±SD of percentage of T cell populations.

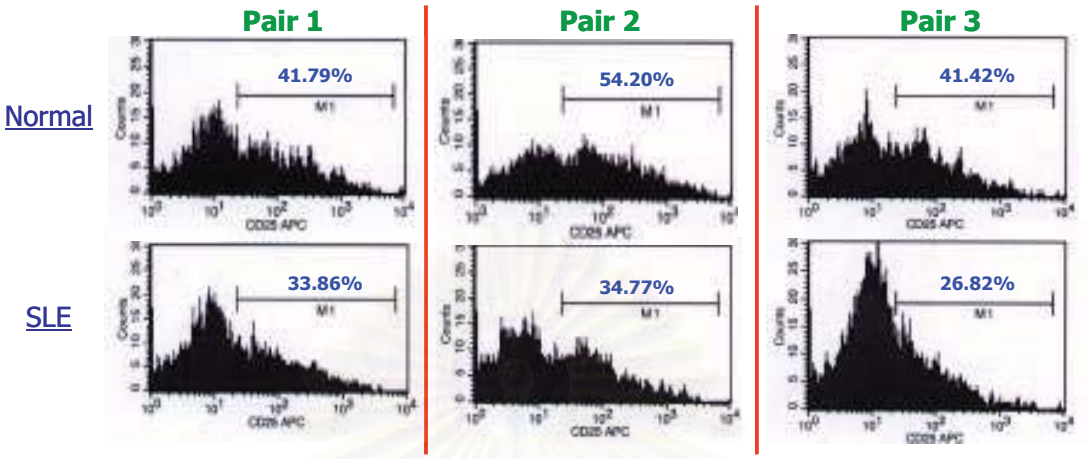


Figure 9. In stimulated condition, histograms of level of CD25 expression in activated $CD4^+$ T cells in healthy controls and SLE patients by the flow cytometric analysis was shown. This experiment was tripicated independently.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER VI

DISCUSSION

Notch signaling plays an important role in regulating T cell development, activation, differentiation, apoptosis and effector function(Radtke, Wilson et al. 2004). This study found for the first time that mRNA *Notch1* expression is reduced in T lymphocytes from SLE patients upon PHA-activation, especially in active SLE, while inactive SLE showed only modest downregulation of *Notch1*. The result demonstrates that there was a converse correlation of Notch1 expression with severity of SLE. Interestingly, stable inactive SLE was showed mRNA Notch1 expression similar to healthy controls while unstable inactive SLE was significantly decelerated compare to healthy controls and stable inactive SLE. Notch1 expression might be also a new marker to predict stage of SLE and the disease progression. Due to the treatment, patients with SLE who were treated with low to intermediate doses of steroid drugs, immunosuppressive and anti-inflammatory drugs(Goldblatt and Isenberg 2005), the phenomena observed in this study might be due to the effects of medications. In order to rule out this possibility, activated T lymphocytes from active SLE patients receiving low and intermediate doses of steroid drugs were studied and found that they were similar to mRNA Notch1 expression. This result suggested that downregulation of *Notch1* in SLE T cells was not the result from interference by medications. However, using T lymphocytes of untreated SLE patients would be more ideal for this study. Additionally, despite defects in Notch1 expression, T cell proliferation in SLE patients was normal. These data implied that SLE T cells did not have defects in proliferative response thus decreasing in Notch1 expression was not caused by defect in proliferation *per se*. Moreover, the defect in Notch1 did not influence T cell proliferation that might be redundant effect of the other Notch members (Radtke, Wilson et al. 2002; Adler, Chiffoleau et al. 2003; Palaga, Miele et al. 2003). On the other hand, defective proliferation in SLE T cells was reported resulting from co-stimulatory signal defect of B7

on antigen presenting cells(Garcia-Cozar, Molina et al. 1996). It conflicted to our study because of using purified T cells but not PBMCs in our proliferation assay that also not showed defective proliferation in SLE T cells. Furthermore, T cells were activated with high concentration that resulted in unsightedly defective proliferation as shown in our study(Adler, Chiffoleau et al. 2003). Then it should repeat stimulation in this experiment with low PHA concentration. However, the role of Notch in T cell proliferation is still controversial. Moreover, heterodimeric complex is not an active form of Notch receptors. For further study, if we also determine ICN of Notch1 that would be showed believable result and confirming our previous results. Ligand expression is one of interesting points which should be studied in ligand-Notch1 interaction.

Notch1 is involved in T cell development in thymus and peripheral T cell responses in periphery including T cell activation, proliferation, differentiation and effector cytokine production(Maillard, Adler et al. 2003). There are numerous evidences indicated that facilitation of T_H1 differentiation by Notch1 signaling is linked with T_H1-mediated autoimmune disease(Minter, Turley et al. 2005). Notch1 signaling is required for TCR-mediated activation that interferes with NF-κB activation and regulation of IFN-γ production(Palaga, Miele et al. 2003; Shin, Minter et al. 2006). In this study, it is shown that downregulation of *Notch1* in stimulated T lymphocytes of SLE patients is one of the newly discovered features of SLE T lymphocytes. Because of imbalance in T_H1/ T_H2 cells affected by Notch signaling, decreased Notch signaling may prefer T_H2 differentiation and promote autoimmune disorders involving in T_H2 cell response. On the other hand, some studies reported that Notch1 signaling promote T_H2 lineage choice while Notch3 signaling promote T_H1 fate. Additionally, expression of the Delta-like family on DCs induces T_H1 differentiation but Jagged family sustains T_H2 differentiation (Maekawa, Tsukumo et al. 2003; Amsen, Blander et al. 2004). Therefore, the effect of the Notch pathway on Th1 versus Th2 differentiation may depend on the balance between Jagged and Delta-like ligands on APCs, as well as on the balance between different Notch family members. Thus, the overall effect is difficult to predict and the different experimental models lead to different conclusions. However, pathologies of SLE cannot

be decidedly characterized whether it is T_H1 or T_H2-type autoimmune disease. Interestingly, mouse model with abnormal presenilins, the essential enzymes for Notch processing, developed SLE-like phenotype like human SLE. In light of our finding in this study, abnormal Notch signaling might be linked with SLE-like phenotypes(Tournoy, Bossuyt et al. 2004; Xu, Zhang et al. 2004).

Self-tolerance occurs during thymic development by clonal deletion and induction of anergy in periphery. Breakdown of self-tolerance is a crucial step of developing autoimmune disorders. There are various reports suggesting that abnormal Treg cells correlates with autoimmunity. Deficiency in suppressive function of $CD4^+CD25^+$ Treg cells has been observed in several autoimmune diseases such as type I diabetes, multiple sclerosis, rheumatoid arthritis, autoimmune polyglandular syndrome type II and psoriasis(Ehrenstein, Evans et al. 2004; Kriegel, Lohmann et al. 2004; Viglietta, Baecher-Allan et al. 2004; Lindley, Dayan et al. 2005; Sugiyama, Gyulai et al. 2005). In SLE, it is indicated that T lymphocytes have decrease in suppressive sensitivity against $CD4^+CD25^+$ regulatory T cells(Monk, Spachidou et al. 2005), while some investigators have reported that regulatory T cells are depleted in active SLE ,but have normal phenotype and function(Crispin, Alcocer-Varela et al. 2003; Miyara, Amoura et al. 2005; Lee, Wang et al. 2006). Generation and function of Treg cells may be regulated by Notch signaling. A striking feature of mice expressing active form of Notch3 is CD25 upregulation in thymocytes and increase generation of naturally occurring Treg (Bellavia, Campese et al. 2000). Therefore, dysregulated CD25 expression may underlie Notch3-specific events that lead to Treg cell generation. Overexpression of the Jagged-1 induces alloantigen-specific human Treg cells which increases TGF-β and IL-10 production(Vigouroux, Yvon et al. 2003; Yvon, Vigouroux et al. 2003). Furthermore, the correlation between Notch pathway and function of Treg cells is demonstrated by the study showing that suppressive function of regulatory T cells expressing $Foxp3^+$ TGF-β^{m+} depends on Notch1 signaling and TGF-β^{m+} pathway in target cells(Ostroukhova, Qi et al. 2006). Therefore, SLE T lymphocytes that fail to upregulate *Notch1* may tolerate the

suppressive function of Treg cells. Further study into the relationship of Notch signaling and Treg functions will clarify this fascinating link.

Previous reports demonstrated that Notch signaling can protect activated T lymphocytes from TCR-induced cell death in apoptotic mechanism. Notch1 signaling can upregulate the expression of inhibitor of apoptotic proteins(Jehn, Bielke et al. 1999; Sade, Krishna et al. 2004). *Cox-2* is one of anti-apoptotic proteins that is upregulated in apoptotic-resistant T cells of human SLE(Xu, Zhang et al. 2004). Study of apoptosis in SLE can be divided into two T cell populations. 1) apoptotic resistance in autoreactive SLE T lymphocytes, these autoreactive SLE T cells can escape programmed cell death and induce T-dependent pathogenic B cells to secrete autoantibody. 2) apoptosis in general lymphocytes from SLE patients has been accelerated that was hypothesized to be the source of nuclear antigen(Emlen, Niebur et al. 1994). In our study, we observed all T cell populations from PBMC not only autoreactive T cell clone that showed the defective Notch1 expression in SLE T cells. It might be promote apoptotic rate in general T cells. While autoreactive T cells might be upregulate Notch1 expression supporting escape apoptotic mechanism that need to more further studies. Moreover, there are evidences that reported IL-2 regulation of apoptosis through CD25 depending on Fas/FasL signaling (Choi, Simon-Stoos et al. 2002; Du, Guan et al. 2005). In our study, level of CD25 expression in activated $CD4^+$ T cells of SLE patients was lower than healthy controls. Thus Notch1 signaling might be regulated CD25 expression that also resulted in decreasing of apoptotic rate in SLE T cells and promoting survival of autoreactive T cells in SLE patients.

Hes1 is a known target gene of activated Notch. To determine whether Notch1 expression has functional consequence, we observed expression of *Hes-1* that had a trend of low expression of *Hes-1* in SLE T cells. This may be due to functional redundancy among other Notch members or Hes1 may not be the direct target of Notch1. Several reports suggest that Hes5, Nrarp and Deltex1 are also physiological target of Notch signaling(Radtke, Wilson et al. 2004).

Taken together, intact etiology of SLE is currently not concluded, but this finding might be a clue of role in Notch1 signaling for T_H1/T_H2 development and suppressive function of Treg cells in SLE that need to further observation.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER VII

CONCLUSION

The aim of this study was to determine *Notch1* expression in stimulated T lymphocytes of SLE patients that indicated significantly defective regulation of *Notch1* in activated T lymphocytes of SLE patients with active stage while stimulated T lymphocytes of SLE patients with inactive stage were indifferent expression of *Notch1* compared with healthy controls. Interestingly, stable inactive SLE was showed mRNA Notch1 expression similar to healthy controls while unstable inactive SLE was significantly decelerated compare to healthy controls and stable inactive SLE. That not only showed conversely correlation of Notch1 expression with severity of SLE but also Notch1 expression might be a new marker to predict stage of SLE and the disease progression. This finding might be a clue of role in Notch1 signaling for T_H1/T_H2 development and suppressive function of Treg cells in SLE that need to further discovery.

Although at the present several reports indicated that Notch signaling plays an important role in periphery, most of these functions are still inconclusive and controversial. Further investigating the potential roles of Notch pathway in lymphocytes are necessary. These insights may yield novel diagnostic and therapeutic application for SLE.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

REFERENCES

Adler, S. H., E. Chiffoleau, et al. (2003). "Notch signaling augments T cell responsiveness by enhancing CD25 expression." J Immunol **171**(6): 2896-903.

Amsen, D., J. M. Blander, et al. (2004). "Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells." Cell **117**(4): 515-26.

Anastasi, E., A. F. Campese, et al. (2003). "Expression of activated Notch3 in transgenic mice enhances generation of T regulatory cells and protects against experimental autoimmune diabetes." J Immunol **171**(9): 4504-11.

Aringer, M., W. Wintersberger, et al. (1994). "High levels of bcl-2 protein in circulating T lymphocytes, but not B lymphocytes, of patients with systemic lupus erythematosus." Arthritis Rheum **37**(10): 1423-30.

Artavanis-Tsakonas, S., M. D. Rand, et al. (1999). "Notch signaling: cell fate control and signal integration in development." Science **284**(5415): 770-6.

Baumann, I., W. Kolowos, et al. (2002). "Impaired uptake of apoptotic cells into tingible body macrophages in germinal centers of patients with systemic lupus erythematosus." Arthritis Rheum **46**(1): 191-201.

Bellavia, D., A. F. Campese, et al. (2000). "Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice." Embo J **19**(13): 3337-48.

Bolland, S. and J. V. Ravetch (2000). "Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis." Immunity **13**(2): 277-85.

Botto, M., C. Dell'Agnola, et al. (1998). "Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies." Nat Genet **19**(1): 56-9.

Boumpas, D. T., R. Furie, et al. (2003). "A short course of BG9588 (anti-CD40 ligand antibody) improves serologic activity and decreases hematuria in patients with proliferative lupus glomerulonephritis." Arthritis Rheum **48**(3): 719-27.

Budagyan, V. M., E. G. Bulanova, et al. (1998). "The resistance of activated T-cells from SLE patients to apoptosis induced by human thymic stromal cells." Immunol Lett **60**(1): 1-5.

Calvi, L. M., G. B. Adams, et al. (2003). "Osteoblastic cells regulate the haematopoietic stem cell niche." Nature **425**(6960): 841-6.

Chen, Z., S. B. Koralov, et al. (2000). "Complement C4 inhibits systemic autoimmunity through a mechanism independent of complement receptors CR1 and CR2." J Exp Med **192**(9): 1339-52.

Choi, Y., K. Simon-Stoos, et al. (2002). "Hypo-active variant of IL-2 and associated decreased T cell activation contribute to impaired apoptosis in autoimmune prone MRL mice." Eur J Immunol **32**(3): 677-85.

Crispin, J. C., J. Alcocer-Varela, et al. (2003). "Immunoregulatory defects in patients with systemic lupus erythematosus in clinical remission." Lupus **12**(5): 386-93.

Cutolo, M., A. Sulli, et al. (2004). "Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity." Lupus **13**(9): 635-8.

Deftos, M. L., E. Huang, et al. (2000). "Notch1 signaling promotes the maturation of CD4 and CD8 SP thymocytes." Immunity **13**(1): 73-84.

Devi, B. S., S. Van Noordin, et al. (1998). "Peripheral blood lymphocytes in SLE--hyperexpression of CD154 on T and B lymphocytes and increased number of double negative T cells." J Autoimmun **11**(5): 471-5.

Drappa, J., A. K. Vaishnaw, et al. (1996). "Fas gene mutations in the Canale-Smith syndrome, an inherited lymphoproliferative disorder associated with autoimmunity." N Engl J Med **335**(22): 1643-9.

Du, C., Q. Guan, et al. (2005). "IL-2-mediated apoptosis of kidney tubular epithelial cells is regulated by the caspase-8 inhibitor c-FLIP." Kidney Int **67**(4): 1397-409.

Eagar, T. N., Q. Tang, et al. (2004). "Notch 1 signaling regulates peripheral T cell activation." Immunity **20**(4): 407-15.

Ehrenstein, M. R., J. G. Evans, et al. (2004). "Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy." J Exp Med **200**(3): 277-85.

Emlen, W., J. Niebur, et al. (1994). "Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus." J Immunol **152**(7): 3685-92.

Garcia-Cozar, F. J., I. J. Molina, et al. (1996). "Defective B7 expression on antigen-presenting cells underlying T cell activation abnormalities in systemic lupus erythematosus (SLE) patients." Clin Exp Immunol **104**(1): 72-9.

Goldblatt, F. and D. A. Isenberg (2005). "New therapies for systemic lupus erythematosus." Clin Exp Immunol **140**(2): 205-12.

Grammer, A. C. and P. E. Lipsky (2003). "B cell abnormalities in systemic lupus erythematosus." Arthritis Res Ther **5 Suppl 4**: S22-7.

Griffiths, B., M. Mosca, et al. (2005). "Assessment of patients with systemic lupus erythematosus and the use of lupus disease activity indices." Best Pract Res Clin Rheumatol **19**(5): 685-708.

Han, H., K. Tanigaki, et al. (2002). "Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision." Int Immunol **14**(6): 637-45.

Herrmann, M., R. E. Voll, et al. (1998). "Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus." Arthritis Rheum **41**(7): 1241-50.

Hochberg, M. C. (1997). "Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus." Arthritis Rheum **40**(9): 1725.

Hoffman, R. W. (2004). "T cells in the pathogenesis of systemic lupus erythematosus." Clin Immunol **113**(1): 4-13.

Hoyne, G. F. (2003). "Notch signaling in the immune system." J Leukoc Biol **74**(6): 971-81.

Hoyne, G. F., I. Le Roux, et al. (2000). "Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral CD4(+) T cells." Int Immunol **12**(2): 177-85.

Hozumi, K., N. Negishi, et al. (2004). "Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo." Nat Immunol **5**(6): 638-44.

Iso, T., L. Kedes, et al. (2003). "HES and HERP families: multiple effectors of the Notch signaling pathway." J Cell Physiol **194**(3): 237-55.

Ivashkiv, L. B. (2003). "Type I interferon modulation of cellular responses to cytokines and infectious pathogens: potential role in SLE pathogenesis." Autoimmunity **36**(8): 473-9.

Jaleco, A. C., H. Neves, et al. (2001). "Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation." J Exp Med **194**(7): 991-1002.

Jehn, B. M., W. Bielke, et al. (1999). "Cutting edge: protective effects of notch-1 on TCR-induced apoptosis." J Immunol **162**(2): 635-8.

Kalden, J. R., T. H. Winkler, et al. (1991). "Pathogenesis of SLE: immunopathology in man." Rheumatol Int **11**(3): 95-100.

Kidd, S., M. R. Kelley, et al. (1986). "Sequence of the notch locus of Drosophila melanogaster: relationship of the encoded protein to mammalian clotting and growth factors." Mol Cell Biol **6**(9): 3094-108.

Kong, P. L., J. M. Odegard, et al. (2003). "Intrinsic T cell defects in systemic autoimmunity." Ann N Y Acad Sci **987**: 60-7.

Kriegel, M. A., T. Lohmann, et al. (2004). "Defective suppressor function of human CD4+ CD25+ regulatory T cells in autoimmune polyglandular syndrome type II." J Exp Med **199**(9): 1285-91.

Kumano, K., S. Chiba, et al. (2003). "Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells." Immunity **18**(5): 699-711.

Lee, J. H., L. C. Wang, et al. (2006). "Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus." Immunology **117**(2): 280-6.

Lindley, S., C. M. Dayan, et al. (2005). "Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes." Diabetes **54**(1): 92-9.

Looney, R. J., J. Anolik, et al. (2004). "B lymphocytes in systemic lupus erythematosus: lessons from therapy targeting B cells." Lupus **13**(5): 381-90.

Mackay, F., S. A. Woodcock, et al. (1999). "Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations." J Exp Med **190**(11): 1697-710.

Maekawa, Y., S. Tsukumo, et al. (2003). "Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells." Immunity **19**(4): 549-59.

Maillard, I., S. H. Adler, et al. (2003). "Notch and the immune system." Immunity **19**(6): 781-91.

Maillard, I., T. Fang, et al. (2005). "Regulation of lymphoid development, differentiation, and function by the Notch pathway." Annu Rev Immunol **23**: 945-74.

Manson, J. J. and D. A. Isenberg (2003). "The pathogenesis of systemic lupus erythematosus." Neth J Med **61**(11): 343-6.

Mehrian, R., F. P. Quismorio, Jr., et al. (1998). "Synergistic effect between IL-10 and bcl-2 genotypes in determining susceptibility to systemic lupus erythematosus." Arthritis Rheum **41**(4): 596-602.

Mills, J. A. (1994). "Systemic lupus erythematosus." N Engl J Med **330**(26): 1871-9.

Minter, L. M., D. M. Turley, et al. (2005). "Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21." Nat Immunol **6**(7): 680-8.

Miyara, M., Z. Amoura, et al. (2005). "Global natural regulatory T cell depletion in active systemic lupus erythematosus." J Immunol **175**(12): 8392-400.

Mok, C. C. and C. S. Lau (2003). "Pathogenesis of systemic lupus erythematosus." J Clin Pathol **56**(7): 481-90.

Monk, C. R., M. Spachidou, et al. (2005). "MRL/Mp CD4+,CD25- T cells show reduced sensitivity to suppression by CD4+,CD25+ regulatory T cells in vitro: a novel

defect of T cell regulation in systemic lupus erythematosus." Arthritis Rheum **52**(4): 1180-4.

Munoz, L. E., U. S. Gaipl, et al. (2005). "SLE--a disease of clearance deficiency?" Rheumatology (Oxford) **44**(9): 1101-7.

Murphy, K. M. and S. L. Reiner (2002). "The lineage decisions of helper T cells." Nat Rev Immunol **2**(12): 933-44.

Nguyen, C., N. Limaye, et al. (2002). "Susceptibility genes in the pathogenesis of murine lupus." Arthritis Res **4 Suppl 3**: S255-63.

Nishimura, H., M. Nose, et al. (1999). "Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor." Immunity **11**(2): 141-51.

Ostroukhova, M., Z. Qi, et al. (2006). "Treg-mediated immunosuppression involves activation of the Notch-HES1 axis by membrane-bound TGF-beta." J Clin Invest **116**(4): 996-1004.

Palaga, T., L. Miele, et al. (2003). "TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells." J Immunol **171**(6): 3019-24.

Pear, W. S. and F. Radtke (2003). "Notch signaling in lymphopoiesis." Semin Immunol **15**(2): 69-79.

Petri, M. (2002). "Epidemiology of systemic lupus erythematosus." Best Pract Res Clin Rheumatol **16**(5): 847-58.

Pui, J. C., D. Allman, et al. (1999). "Notch1 expression in early lymphopoiesis influences B versus T lineage determination." Immunity **11**(3): 299-308.

Radtke, F., A. Wilson, et al. (2002). "The role of Notch signaling during hematopoietic lineage commitment." Immunol Rev **187**: 65-74.

Radtke, F., A. Wilson, et al. (2004). "Notch regulation of lymphocyte development and function." Nat Immunol **5**(3): 247-53.

Radtke, F., A. Wilson, et al. (1999). "Deficient T cell fate specification in mice with an induced inactivation of Notch1." Immunity **10**(5): 547-58.

Ravetch, J. V. and L. L. Lanier (2000). "Immune inhibitory receptors." Science **290**(5489): 84-9.

Renaudineau, Y., J. O. Pers, et al. (2004). "Dysfunctional B cells in systemic lupus erythematosus." Autoimmun Rev **3**(7-8): 516-23.

Robey, E., D. Chang, et al. (1996). "An activated form of Notch influences the choice between CD4 and CD8 T cell lineages." Cell **87**(3): 483-92.

Ruiz-Irastorza, G., M. A. Khamashta, et al. (2001). "Systemic lupus erythematosus." Lancet **357**(9261): 1027-32.

Sade, H., S. Krishna, et al. (2004). "The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells." J Biol Chem **279**(4): 2937-44.

Saito, T., S. Chiba, et al. (2003). "Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development." Immunity **18**(5): 675-85.

Salmon, M. and C. Gordon (1999). "The role of apoptosis in systemic lupus erythematosus." Rheumatology (Oxford) **38**(12): 1177-83.

Sarzi-Puttini, P., F. Atzeni, et al. (2005). "Environment and systemic lupus erythematosus: an overview." Autoimmunity **38**(7): 465-72.

Shin, H. M., L. M. Minter, et al. (2006). "Notch1 augments NF-kappaB activity by facilitating its nuclear retention." Embo J **25**(1): 129-38.

Souabni, A., C. Cobaleda, et al. (2002). "Pax5 promotes B lymphopoiesis and blocks T cell development by repressing Notch1." Immunity **17**(6): 781-93.

Spatz, L., A. Iliev, et al. (1997). "Studies on the structure, regulation, and pathogenic potential of anti-dsDNA antibodies." Methods **11**(1): 70-8.

Stier, S., T. Cheng, et al. (2002). "Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome." Blood **99**(7): 2369-78.

Strasser, A., S. Whittingham, et al. (1991). "Enforced BCL2 expression in B-lymphoid cells prolongs antibody responses and elicits autoimmune disease." Proc Natl Acad Sci U S A **88**(19): 8661-5.

Sugiyama, H., R. Gyulai, et al. (2005). "Dysfunctional blood and target tissue CD4+CD25high regulatory T cells in psoriasis: mechanism underlying unrestrained pathogenic effector T cell proliferation." J Immunol **174**(1): 164-73.

Suzuki, N., S. Mihara, et al. (1997). "Development of pathogenic anti-DNA antibodies in patients with systemic lupus erythematosus." Faseb J **11**(12): 1033-8.

Takahashi, T., M. Tanaka, et al. (1994). "Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand." Cell **76**(6): 969-76.

Takeuchi, T., K. Tsuzaka, et al. (2005). "T cell abnormalities in systemic lupus erythematosus." Autoimmunity **38**(5): 339-46.

Tournoy, J., X. Bossuyt, et al. (2004). "Partial loss of presenilins causes seborrheic keratosis and autoimmune disease in mice." Hum Mol Genet **13**(13): 1321-31.

Tsao, B. P. (2003). "The genetics of human systemic lupus erythematosus." Trends Immunol **24**(11): 595-602.

Tsao, B. P. (2004). "Update on human systemic lupus erythematosus genetics." Curr Opin Rheumatol **16**(5): 513-21.

Tsao, B. P., R. M. Cantor, et al. (1999). "PARP alleles within the linked chromosomal region are associated with systemic lupus erythematosus." J Clin Invest **103**(8): 1135-40.

Tu, L., T. C. Fang, et al. (2005). "Notch signaling is an important regulator of type 2 immunity." J Exp Med **202**(8): 1037-42.

Vaishnaw, A. K., E. Toubi, et al. (1999). "The spectrum of apoptotic defects and clinical manifestations, including systemic lupus erythematosus, in humans with CD95 (Fas/APO-1) mutations." Arthritis Rheum **42**(9): 1833-42.

Varnum-Finney, B., C. Brashem-Stein, et al. (2003). "Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability." Blood **101**(5): 1784-9.

Varnum-Finney, B., L. Xu, et al. (2000). "Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling." Nat Med **6**(11): 1278-81.

Viglietta, V., C. Baecher-Allan, et al. (2004). "Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis." J Exp Med **199**(7): 971-9.

Vigouroux, S., E. Yvon, et al. (2003). "Induction of antigen-specific regulatory T cells following overexpression of a Notch ligand by human B lymphocytes." J Virol **77**(20): 10872-80.

Walport, M. J. (2002). "Complement and systemic lupus erythematosus." Arthritis Res **4 Suppl 3**: S279-93.

Washburn, T., E. Schweighoffer, et al. (1997). "Notch activity influences the alphabeta versus gammadelta T cell lineage decision." Cell **88**(6): 833-43.

Watanabe-Fukunaga, R., C. I. Brannan, et al. (1992). "Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis." Nature **356**(6367): 314-7.

Waterhouse, P., J. M. Penninger, et al. (1995). "Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4." Science **270**(5238): 985-8.

Wharton, K. A., K. M. Johansen, et al. (1985). "Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats." Cell **43**(3 Pt 2): 567-81.

Williams, R. C., Jr., C. C. Malone, et al. (1995). "Active systemic lupus erythematosus is associated with depletion of the natural generic anti-idiotype (anti-F(ab')2) system." J Rheumatol **22**(6): 1075-85.

Wilson, A., H. R. MacDonald, et al. (2001). "Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus." J Exp Med **194**(7): 1003-12.

Wolfer, A., T. Bakker, et al. (2001). "Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8T cell development." Nat Immunol **2**(3): 235-41.

Wolfer, A., A. Wilson, et al. (2002). "Inactivation of Notch1 impairs VDJbeta rearrangement and allows pre-TCR-independent survival of early alpha beta Lineage Thymocytes." Immunity **16**(6): 869-79.

Wu, J., J. Wilson, et al. (1996). "Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease." J Clin Invest **98**(5): 1107-13.

Xu, L., L. Zhang, et al. (2004). "Human lupus T cells resist inactivation and escape death by upregulating COX-2." Nat Med **10**(4): 411-5.

Yamaguchi, E., S. Chiba, et al. (2002). "Expression of Notch ligands, Jagged1, 2 and Delta1 in antigen presenting cells in mice." Immunol Lett **81**(1): 59-64.

Yi, Y., M. McNerney, et al. (2000). "Regulatory defects in Cbl and mitogen-activated protein kinase (extracellular signal-related kinase) pathways cause persistent hyperexpression of CD40 ligand in human lupus T cells." J Immunol **165**(11): 6627-34.

Yvon, E. S., S. Vigouroux, et al. (2003). "Overexpression of the Notch ligand, Jagged-1, induces alloantigen-specific human regulatory T cells." Blood **102**(10): 3815-21.

Zhang, J., V. Roschke, et al. (2001). "Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus." J Immunol **166**(1): 6-10.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX A

Reagent for cell culture

1. culture media

RPMI1640	1	pack
Sodium bicarbonate solution	10	ml
Non-essential amino acid solution	10	ml
Penicillin-Streptomycin	10	ml
1M Hepes solution	10	ml

Adjust volume up to 1 liter (L) with distilled water and sterile with filter 0.2 μm and store at 4°C. Completed culture media is supplemented with FBS (RPMI1640 with 10%FBS)

2. 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 4°C.

3. Fetal bovine serum (FBS)

Heat-inactivate 56°C 30 min and store at -20°C.

4. Ficoll-Hypaque solution

store in the dark at 4°C.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

5. 1x Phosphate buffered saline (PBS)

Na_2HPO_4	4.88	g
$NaH_2PO_4.H_2O$	1.54	g
NaCl	3.04	g

Adjust pH to 7.2-7.4, add distilled water up to 1 liter, sterilize by autoclaving at 121°C for 15 min and store at room temperature.

6. Buffer (for T cell sorting)

Prepare a solution containing PBS pH 7.2, 0.5% bovine serum albumin (BSA) and 2 mM EDTA and degass before using. Store at 4°C.

7. 20 µCi/ml H^3-thymidine

Dilute 1:50 of 1 mCi/ml H^3-thymidine with steriled PBS and store in the dark at 4°C.

8. 1 mg/ml Purified phytohemaggutinin (PHA)

Lyophilized PHA	5	mg
steriled PBS	5	ml

Store at -20°C.

9. 1% Trypan blue

Trypan blue	0.3	g
distilled water	30	ml

10. 1% paraformaldehyde

Paraformaldehyde	1	g
distilled water	100	ml

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX B

Reagent for agarose gel electrophoresis

1. 50x Tris-acetate buffer (TAE)

Tris base	424.0	g
Glacial acetic acid	57.1	g
0.5 M EDTA pH 8.0	100	ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilized by autoclaving at 121°C for 15 min.

2. 10 mg/ml Ethidium bromide

Ethidium bromide	1.0	g
Distilled water	100	ml

Mix the solution and store in the dark at 4°C.

3. 1.5% Agarose gel

Agarose	0.3	g
1x TAE	20	ml

Dissolve by heating in microwave oven and occasional mix unit no granules of agarose are visible.

4. 5x Loading buffer

Tris HCL	0.6	g
EDTA	1.68	g
SDS	0.5	g
Bromphenol Blue	0.1	g
Sucrose	40	g

Adjust volume to 100 ml with distilled water and store at 4°C.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX C

Reagent for protein extraction

1. Buffer A

500 mM Tris-HCl	1	ml
1.4 M KCl	1	ml
25 mM $MgCl_2$	1	ml
10 mM EGTA	1	ml
10 mM DTT	1	ml
Distilled water	5	ml

Store at 4°C.

2. Buffer B

Buffer A	990	µl
1% nonidet P-40	10	µl

Store at 4°C.

3. protease inhibitor

50 µg/ml PMSF	5	mg
1 µg/ml Aprotinin	100	µg
1 µg/ml Leupeptin	100	µg
1 µg/ml Pepstatin	100	µg

Adjust volume up to 1 ml with absolute ethanol and store at -20°C.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX D

Reagent for western blot assay

1. 1.5 M Tris (pH 8.8)

Trizma base	90.855	g
Distilled water	500	ml

2. 1.0 M Tris (pH 6.8)

Trizma base	90.855	g
Distilled water	500	ml

3. 10% w/v Ammonium persulfate (APS)

APS	0.5	g
Distilled water	5	ml

Store at 4°C.

4. 10% w/v SDS (pH 7.2)

SDS	1	g
Distilled water	10	ml

5. Separating gel (8% Acrylamide)

Distilled water	5.3	ml
40% Acrylamide	2.0	ml
1.5 M Tris (pH 8.8)	2.5	ml
10% SDS	0.1	ml
10% APS	0.1	ml
TEMED	6	µl

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

6. Stacking gel

Distilled water	1.4825	ml
40% Acrylamide	0.2475	ml
1 M Tris (pH 6.8)	0.25	ml
10% SDS	0.02	ml
10% APS	0.02	ml
TEMED	2	µl

7. 5x running buffer

Trisma base	15.1	g
Glycine	94	g
SDS	5	g
Distilled water	1	L

8. Transfer buffer

Trisma base	5.08	g
Glycine	2.9	g
SDS	0.37	g
Distilled water	800	ml
Absolute methanol	200	ml

Store at 4°C.

9. 2x Laemmli buffer

10% SDS	4	ml
Glycerol	2	ml
1 M Tris (pH 6.8)	1.2	ml
HPLC	2.8	ml
Bromphenol blue	0.001	g

Store at 4°C.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

10. 10x PBS (pH 7.2)

NaCl	80	g
KCl	2	g
Na_2HPO_4	14.4	g
KH_2PO_4	2.4	g
Distilled water	1	L

11. Blocking solution

Tween20	125	µl
Non-fate dried milk	7.5	g
1x PBS	250	ml

Store at 4°C.

12. Solving solution (PBST)

Tween20	0.5	ml
1x PBS	1	L

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

BIOGRAPHY

Miss Pimpayao Sodsai was born on November 12, 1981 in Chonburi, Thailand. She graduated with Bachelor's of Science in Microbiology (Second Class Honors), from Faculty of Science, Chulalongkorn University in 2004. She enrolled in the Inter-Department of Medical Microbiology, Graduate School, Chulalongkorn University for her Master's degree in 2004.

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย