การแสดงออกของ IL-17 และ IL-23R ในทีลิมโฟซัยท์จากผู้ป่วยเอสแอลอีชาวไทย

นางสาวหทัยภัทร ภูวิภิรมย์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาจุลชีววิทยาทางการแพทย์ (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

EXPRESSION OF IL-17 AND IL-23R IN T LYMPHOCYTES FROM THAI SLE PATIENTS

Miss Hathaipat Phuwipirom

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 2009

Copyright of Chulalongkorn University

Thesis Title	EXPRESSION OF IL-17 AND IL-23R IN T LYMPHOCYTES FROM THAI SLE PATIENTS
By	Miss Hathaipat Phuwipirom
Field of Study	Medical Microbiology
Thesis Advisor	Assistant Professor Tanapat Palaga, Ph.D.
Thesis Co-Advisor	Associate Professor Nattiya Hirankarn, M.D., 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
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Ariya Chindamporn, Ph.D.)

…….. Thesis Advisor
(Assistant Professor Tanapat Palaga, Ph.D.)

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

…….. Examiner
(Associate Professor Yingyos Avihingsanon, M.D.)

…….. External Examiner
(Associate Professor Primchanien Moongkarndi, Ph.D.)

หทัยภัทร ภูวิภิรมย์ : การแสดงออกของ IL-17 และ IL-23R ในทีลิมโฟซัยท์จากผู้ป่วยเอสแอลอีชาวไทย. (EXPRESSION OF IL-17 AND IL-23R IN T LYMPHOCYTES FROM THAI SLE PATIENTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ.ดร.ธนาภัทร ปาลกะ , อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.พญ.ดร.ณัฏฐิยา หิรัญกาญจน์, 118 หน้า.

โรคเอสแอลอี (Systemic Lupus Erythematosus; SLE) คือโรคออโต้อิมมูนชนิดหนึ่ง ส่งผลกระทบต่อร่างกายในหลายระบบ สาเหตุของโรคยังไม่ทราบชัดเจน คาดว่าความผิดปกติของเครือข่ายไซโตไคน์และลิมโฟซัยท์มีบทบาทสำคัญต่อการเกิดโรค โดยพบรายงานการเพิ่ม สูงขึ้นของไซโตไคน์หลายชนิดในผู้ป่วยเอสแอลอี เช่น IL-1β IL-6 และ IL-23 เป็นต้น และจากรายงานเมื่อไม่นานมานี้พบว่าทีลิมโฟซัยท์ชนิด $CD4^+$ ที่หลั่งไซโตไคน์ IL-17 เป็นหลัก หรือที่เรียกว่า Th17 มีบทบาทสำคัญในโรคออโต้อิมมูนหลายโรค พัฒนาการของ Th17 อยู่ภายใต้การควบคุมของ IL-1β/IL-6 และ IL-23 ซึ่ง IL-23 ยังมีบทบาทสำคัญในการรักษาสภาพของ Th17 อีกด้วย นอกจาก Th17 แล้วพบว่าเซลล์อีกหลายชนิดก็สามารถหลั่ง IL-17 ได้เช่นกัน งานวิจัยนี้จึงมีจุดประสงค์เพื่อศึกษาระดับของ IL-17 ในผู้ป่วยเอสแอลอี 29 ราย แยกความรุนแรงของโรคด้วย SLEDAI score เป็นผู้ป่วยระยะ active 16 รายและระยะ inactive 13 ราย และกลุ่มควบคุมปกติอีก 10 ราย จากผลการวิเคราะห์การแสดงออกของ IL-23R ใน PBMC ด้วยโฟลไซโตเมทรี พบว่าผู้ป่วยมีเปอร์เซนต์ของ $CD4^+IL\text{-}23R^+$ และ $CD8^+IL\text{-}23R^+$ ทีลิมโฟซัยท์สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ ($p<0.05$) ทั้งใน PBMC ที่ ไม่มีการกระตุ้น (day 0) และที่ได้รับการกระตุ้นด้วยแอนติบอดีต่อ CD3 และ CD28 (day 3) เมื่อวิเคราะห์ การแสดงออกของ IL-17 ใน PBMC โดยวิธีเดียวกัน พบว่าผู้ป่วยมีเปอร์เซ็นต์ของ $CD4^+IL\text{-}17^+$ ที่มีแนวโน้มสูงกว่าและมี $CD8^+IL\text{-}17^+$ สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญที่ day 3 สำหรับการวัดระดับของ IL-17 ในซีรัมและพลาสมาของผู้ป่วยกับกลุ่มควบคุมโดยวิธี ELISA พบว่า มีผู้ป่วยเพียง 2 รายที่ตรวจพบ IL-17 ได้ในระดับ 5.12, 6.78 และ 5.75, 8.50 pg/ml ตามลำดับ ส่วนการวัดระดับ IL-23 โดย ELISA นั้นตรวจไม่พบในทุกตัวอย่าง เมื่อวิเคราะห์การแสดงออกของ *IL-17A* mRNA โดยวิธี quantitative realtime RT-PCR พบว่า PBMC จากผู้ป่วยมีแนวโน้มเพิ่มขึ้นของการแสดงออกของ *IL-17A* เมื่อเปรียบเทียบกับกลุ่มควบคุมแต่ยังไม่ถึงระดับที่มีนัยสำคัญ ดังนั้น ผลที่ได้จากงานวิจัยนี้บ่งชี้ว่าทีลิมโฟซัยท์ในผู้ป่วยเอสแอลอีมีการแสดงออกของ IL-23R และ IL-17 มากกว่าในกลุ่มควบคุม ซึ่งอาจจะมีบทบาทสำคัญต่อการเกิดพยาธิสภาพของโรคเอสแอลอี

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

4989220420 : MAJOR MEDICAL MICROBIOLOGY

KEYWORDS : SLE / IL-17 / IL-23R

HATHAIPAT PHUWIPIROM: EXPRESSION OF IL-17 AND IL-23R IN T LYMPHOCYTES FROM THAI SLE PATIENTS. THESIS ADVISOR: ASST.PROF. TANAPAT PALAGA, Ph.D., THESIS CO-ADVISOR: ASSOC. PROF. NATTIYA HIRANKARN, M.D., Ph.D., 118 pp.

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder which affects various systems. The exact etiology of SLE is not known but the defects in cytokine network and the functions of T lymphocytes (T cells) may play a vital role. It was reported that SLE patients showed elevated level of various cytokine such as IL-1β, IL-6 and IL-23 and recent reports indicated that $CD4^+$ T cells mainly producing IL-17 called Th17 play a vital role in various autoimmune disorders. Th17 differentiation is under the influence IL-1β/IL-6 and IL-23. Moreover, IL-23 is also vital in maintaining the Th17 phenotype. In addition to Th17 cells, IL-17 is also produced by various cell types. The aim of this study was to investigate the level of cytokine IL-17 in 29 SLE patients that were divided based on the severity of disease by SLEDAI score. Sixteen active, 13 inactive SLE patients and 10 normal subjects were recruited in this study. The frequency of $CD4^+$IL-$23R^+$ and $CD8^+$IL-$23R^+$ T cells in PBMC, as analyzed by flow cytometry, from patients were significantly higher than those of controls (p<0.05), in both freshly isolated PBMC (day 0) and PBMC stimulated by anti-CD3 and CD-28 antibodies (day 3). When IL-17 expression was measured, higher frequency of $CD4^+$IL-17^+ and significantly higher frequency of $CD8^+$IL-17^+ T cells in PBMC on day 3 from patients were observed. The level of IL-17 in serum and plasma were measured by ELISA, but only two SLE were found to show detectable IL-17 at 5.12, 6.78 and 5.75, 8.50 pg/ml, respectively. When IL-23 level measured by ELISA, all samples showed negative results. The expression of *IL-17A* mRNA was analyzed by quantitative realtime RT-PCR, higher but not statistically significant level was observed in PBMC from patients. Taken together, these results suggest that T lymphocytes in SLE patients increase IL-23R and IL-17 expression, which may play an important role in pathology of SLE.

Field of Study : Medical Microbiology ..... Student's Signature .....

Academic Year : 2009 ..... Advisor's Signature .....

Co-Advisor's Signature .....

ACKNOWLEDGEMENTS

I am grateful to all who were always supportive during the experiments and completion of this thesis.

I would like to deeply thank my advisor, Assistant Professor Dr. Tanapat Palaga, Department of Microbiology, Faculty of Science, Chulalongkorn University for his excellent coaching, valuable advices and guidance. His experiences greatly helped me in studying, researching and future working.

I would like to express my sincere gratitude to my co-advisor, Associate Professor Dr. Nattiya Hirankarn, Department of Microbiology, Faculty of Medicine, Chulalongkorn University for excellent advices, her help with data analysis and thesis reviewing and a great encouragement.

I would like to thank Associate Professor Dr. Yingyos Avihingsanon, Department of Medicine, Faculty of Medicine, Chulalongkorn University, for his kindness in helping in recruiting SLE patients.

I would like to thank Dr. Puchaniyada Wicheantham, Bhumibol Adulyadej Hospital, for helping with collecting blood samples and clinical data from the SLE patients.

I would like to thank the committee of Inter-department Program of Medical Microbiology for giving me a permission to commence this dissertation and provided me with the necessary facility to conduct my research.

I am grateful to Miss Supranee Buranapraditkun for her valuable advice, guidance and helping in flow cytometry.

All patients and normal subjects who volunteered in this study are greatly appreciated.

Finally, I would like to express special thanks to my parents and my friends for their love, support, understanding and encouragement.

CONTENTS

PAGE

ABSTRACT (THAI)..... iv

ABSTRACT (ENGLISH)..... v

ACKNOWLEDGEMENTS..... vi

CONTENTS..... vii

LIST OF TABLES..... x

LIST OF FIGURES..... xi

LIST OF ABBREVIATIONS..... xiii

CHAPTER

I INTRODUCTION..... 1

II OBJECTIVE..... 5

III REVIEW OF RELATED LITERATURES..... 6

Systemic Lupus Erythematosus..... 6

SLE Classification Criteria..... 6

Epidemiology..... 10

Etiology..... 10

Genetic Factors..... 11

Environmental Factors..... 11

SLE and Immune System..... 12

B Cells Abnormalities in SLE..... 13

T Cells Abnormalities in SLE..... 13

Antigen Processing and Presentation..... 14

T Cell Effector Surface Molecules and Co-stimulation..... 14

Apoptosis and Apoptotic Clearance..... 15

Regulatory T cells..... 15

T helper-associated cytokines..... 15

PAGE

T Helper 17 Cells..... 16
Discovery of the Th17 Cells..... 19
Definition of Th17 Cells..... 20
Unique Cytokine Environment for Human Th17 Development..... 20
The Signature Cytokine of Th17 Cells..... 24
Interleukin (IL)-17 or IL-17A..... 24
Interleukin (IL)-17F..... 24
Interleukin (IL)-22..... 25
Key Transcription Factors in Th17 Cells..... 27
Surface Receptors on Th17 Cells..... 28
Th17 cells and Infection..... 29
Th17 Cells and Autoimmunity..... 29
Th17 Cells and SLE..... 32
IV MATERIALS AND METHODS..... 35
Patients and Normal Subjects..... 36
PBMCs Isolation and Serum Collections..... 36
PBMCs Cultures..... 37
Antibodies..... 37
PBMCs Stimulation for Intracellular Cytokine Staining..... 38
Surface and Intracellular Staining with Antibodies..... 38
Flow Cytometric Analysis..... 39
RNA Extraction and Complementary DNA Synthesis..... 39
Semi-Quantitative Real-time RT-PCR..... 40
Enzyme Linked Immunosorbent Assay (ELISA) 41
Statistical Analysis..... 42
V RESULTS..... 46
SLE Patients and Normal Subjects..... 46

PAGE

The Percentages of IL-23R$^+$CD4$^+$ T cells, IL-23R$^+$CD8$^+$ T cells, IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$T cells in PBMC from SLE Patients and Normal Subjects..... 51
Detection of IL-17A and IL-23 Levels in Serum and Plasma from SLE Patients and the Normal Subjects..... 59
Semi-Quantitative Real-time PCR Analysis for *IL-17A* in PBMCs from SLE Patients and Normal Subjects..... 63
Semi-Quantitative Real-time RT-PCR Analysis for *RORC* in PBMCs from SLE Patients and Normal Subjects..... 64
Correlations of the Percentages of IL-23R$^+$CD4$^+$ T Cells, IL-23R$^+$CD8$^+$ T Cells, IL-17$^+$CD4$^+$T Cells and IL-17$^+$CD8$^+$T Cells with the Relative mRNA Expression of *IL-17A* and *RORC* in PBMCs from SLE Patients and Normal Subjects..... 66
Correlation with the SLEDAI-2K Scores..... 77
VI DISCUSSION..... 81
VII CONCLUSION..... 87
REFERENCES..... 88
APPENDICES..... 111
Appendix A..... 112
Appendix B..... 114
Appendix C..... 116
BIOGRAPHY..... 118

LIST OF TABLES

TABLE		PAGE
1	The 1997 Revised Criteria for the Classification of Systemic Lupus Erythematosus (SLE).....	8
2	Scoring for SLE Disease Activity Index (SLEDAI) 2000 (SLEDAI-2K)....	9
3	The Factors Involved in the Differentiation of the Murine Th17 Subset..	22
4	Unique Transcriptional Programme of T Helper cells Lineages.....	28
5	Evidence for a Role of Th17 Cells in Immune-Mediated Diseases.....	30
6	Interleukin (IL)-17 in Murine Models and patients with Systemic Lupus Erythematosus.....	33
7	Characteristics of SLE Patients Included in This Study.....	43
8	List of primers used for analysis of the *IL-17A*, *RORC* and β*-actin* gene.....	45
9	Characteristics of Individual Subjects that were Recruited for Flow Cytometric Analysis, Serum Cytokine Detection by ELISA and Semi-Quantitative Real-time RT-PCR Analysis.....	47
10	Characteristics of Individual Subjects that were Recruited for Plasma Cytokine Detection by ELISA.....	50
11	Individual Characteristics and Summary of the Results of Samples that were Recruited for Plasma Cytokine Detection by ELISA.....	61
12	Summary of the Results of the Subjects that were Recruited for Flow Cytometric Analysis, Serum Cytokine Detection by ELISA and Semi-Quantitative Real-time RT-PCR Analysis.....	68

LIST OF FIGURES

FIGURE		PAGE
1	Subsets of Effector T Helper Cells.....	18
2	Molecular Mechanisms of Differentiation and Maturation of Murine and Human Th17 Cells.....	23
3	The Biological Function of IL-17A.....	26
4	Representative FACS Plots and Gating Strategy Used in This Study....	44
5	The Percentages of IL-23R$^+$CD4$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects.....	53
6	The Percentages of IL-23R$^+$CD8$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects.....	54
7	The Percentages of IL-17$^+$CD4$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects.....	55
8	The Percentages of IL-17$^+$CD8$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects.....	56
9	The Fold Increase in the Percentage of IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$ T cells from SLE Patients and Normal Subjects.....	57
10	Correlations between the Percentages of IL-23R$^+$ T Cells and the Percentages of IL-17$^+$ T Cells.....	58
11	Detection of Serum IL-17A Levels in SLE Patients and Normal Subjects by ELISA.....	60
12	Detection of Serum IL-23 levels in SLE Patients and Normal Subjects by ELISA.....	60
13	Detection of Plasma IL-17A Levels in SLE Patients and Normal Subjects by ELISA.....	62
14	Detection of Plasma IL-23 Levels in SLE Patients and Normal Subjects by ELISA.....	62
15	Semi-Quantitative Real-time RT-PCR Analysis for *IL-17A* in PBMCs at Day 3 from SLE Patients and Normal Subjects.....	63

FIGURE		PAGE
16	Semi-Quantitative Real-time RT-PCR Analysis for *RORC* Expression in PBMCs at Day 0 and Day 3 from SLE Patients and Normal Subjects...	65
17	Correlation between Relative *IL-17A* Expression and the Results from Flow Cytometric Analysis.....	71
18	Correlation between Relative *RORC* Expression and the Results from Flow Cytometric Analysis.....	73
19	Correlation Analyses between the Scores of SLEDAI-2K and the Percentages of IL-23R$^+$CD4$^+$ T Cells, IL-23R$^+$CD8$^+$ T Cells, IL-17$^+$CD4$^+$ T Cells and IL-17$^+$CD8$^+$ T Cells in PBMCs from 29 SLE Patients.....	78
20	Correlation Analyses between the Scores of SLEDAI-2K and Relative mRNA Expression in PBMCs from 19 SLE Patients.....	80

LIST OF ABBREVIATIONS

ACD	Acid Citrate Dextrose
ACR	American College of Rheumatology
AhR	aryl hydrocarbon receptor
ARA	American Rheumatism Association
BAFF	B-cell activating factor
BLK	B lymphocyte kinase
CCL20	chemokine (C-C motif) ligand 20
CCR6	C-C chemokine receptor type 6
CIA	Collagen Induced Arthritis
CMV	Cytomegalovirus
COX	Cyclooxygenase
EAE	Experimental Autoimmune Encephalitis
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence Activated Cell Sorting
G-CSF	granulocyte-colony stimulating factor
(GM)–CSF	granulocyte-macrophage
(Gro)-α	growth-related protein
HLA	Human leukocyte antigen
IBD	Inflammatory bowel disease
IFN-γ	Interferon-gamma
IL	Interleukin
IRF	Interferon regulatory factor
LFA	Lymphocyte function antigen

MHC	Major histocompatibility complex
ml	Milliliter
mRNA	Messenger Ribonucleic acid
MS	Multiple sclerosis
ng	Nanogram
NK	Natural killer
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PBMC	Peripheral Blood Mononuclear Cell
PGE_2	prostaglandin E_2
RBC	Red blood cell
RORγt	RAR-related orphan receptor gamma
RT-PCR	Real-time polymerase chain reaction
SLE	Systemic lupus erythematosus
SLEDAI	Systemic Lupus Erythematosus Disease Activity Index
STAT	Signal transducer and activator of transcription
T-bet	T box family protein expressed in T cells
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
Treg	Regulatory T cells
WBC	White blood cell
µg	Microgram
µl	Microliter

CHAPTER I

INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease characterized by the activation of T and polyclonal B lymphocytes, production of autoantibodies, and formation of immune complexes that affect multiple organs [1-3]. SLE has a worldwide distribution, with differences in prevalence related to race, geography and sex [4, 5]. SLE primarily affects women in the child-bearing years, and is estimated to be 9–12 times more common in women than in men [6]. The disease is more frequent in blacks/African Americans, Hispanics and Indians/Native Americans than in Caucasians [4, 5]. The prevalence of SLE in Asia is estimated to be 50 to 100 per 100,000 [7]. In addition, there are also differences in the development of disease by ethnicity. A study by Mok *et al.* indicated that Asian patients with SLE show more severity in the progression of the disease, especially renal involvement [8]. The exact etiology and pathogenesis of SLE remain unclear. An extremely complex and multi-factorial interaction between diverse genetic and environmental factors are possibly involved [9].

Like other autoimmune disorder, SLE is proposed to arose mainly 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 hyperactivation of T and B cells, leading to autoantibody production.

A large body of evidence suggests that T cells are required for the full manifestation of SLE diseases. Presentation of autoantigens could have important effects on breaking T cell tolerance by stimulated T cells from SLE patient and allow them to help B cells leading to the production of autoantibodies [10, 11]. T cells functional abnormalities have been reported in SLE, such as the expression of many different surface molecules

and co-stimulation on T cells that can provide help for autoantibody production [12-17]. Moreover, defects in apoptosis and apoptotic clearance play a crucial role in driving dysfunction of immunological tolerance in autoreactive T and B cells in SLE [18-23].

In addition, an imbalance in T cell subsets and their associated cytokines network have been reported in SLE. Defects in the reduced number and/or suppressive functions of regulatory T (Treg) cells also may have a role in SLE [24-27]. Cytokine expression profiles have been studied extensively in patients with SLE. Some studies have shown that Th2 cytokines IL-6 [28] and IL-10 [29] were elevated while Th1 cytokines IL-2 and IFN-γ [30] decreased in active SLE. Other studies; however, have shown that Th1 cytokines such as IL-12 [31], TNF-α [32] and IFN-γ [33] were significantly higher in SLE patients. These differences imply that the exact T cells subsets and the T helper cytokine response in SLE require further investigation.

More recent findings have shown that another T helper subset that mainly produces the pro-inflammatory cytokine IL-17A, termed 'Th17', exhibits effector functions distinct from Th1 and Th2 cells [34-36]. Th17 cells are considered as a distinct T helper cell subset because: (i) they arise from naïve T cells when primed in the presence of specific factors (TGF-β and IL-6 for mice [37-39], IL-1β and/or IL-23 or undefined factors for human [40-47]); (ii) their differentiation is controlled by exclusive transcription factor (RORγt for mice, RORC for human); (iii) they exhibit a particular cytokine production profile distinct from Th1 and Th2 cells, such as IL-17A; and (iv) their differentiation into Th17 cells excludes the acquisition of other effector phenotypes (i.e. Th1 and Th2). The pattern of chemokine and cytokine receptor expression on human Th17 cells has been investigated extensively [43, 48-50] and it is now generally accepted that they express CCR6 and IL-23R. Th17 mainly produces the IL-17A, a potent pro-inflammatory cytokine, that exerts its effects through activates the production of chemokines and cytokines results in recruitment of monocytes and neutrophils to sites of inflammation as well as the amplification of the immune response by inducing the

production of many cytokines. Additionally, IL-17A synergizes with other cytokines, leading to amplification of inflammatory responses [51]. IL-17 receptor is expressed broadly and mediates its effects through a number of immune and non-immune cells (particularly endothelial and epithelial cells) [52]. It is now known that several cell types are capable of producing IL-17A other than $CD4^+$ T cells. The γδ T cells [53], $CD8^+$ memory T cells [54, 55], NK T cells [56, 57], NK cells [58], eosinophils [59], neutrophils [54] and monocytes [60] can also be a source of IL-17A. Nevertheless, the predominant source of IL-17A remains the $CD4^+$ T cell population [51].

The primary function of Th17 cells appears to be the clearance of pathogens that are not adequately handled by Th1 or Th2 cells [38, 61, 62]. Th17 cells are, however, potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions. A large body of evidence suggests that Th17 and Th17–associated cytokines have a crucial role in the development of a wide range of autoimmune disorders, such as psoriasis [42, 63], rheumatoid arthritis [64-66], multiple sclerosis [67, 68], inflammatory bowel disease [69, 70] and recent study demonstrated that IL-17A is involved in different aspects of SLE pathogenesis [71-73].

Initial studies suggested that the plasma IL-17A level were increased in SLE patients [71]. Another group, however, reported that no different in the serum IL-17A level between SLE patients and normal subjects was seen [72]. In addition, recent studies suggested that IL-17A was the effector cytokine that promote the autoinflammatory responses in SLE [73]. Therefore, the role of IL-17A and Th17, if any, in SLE remained unsettled.

The aim of this study is to investigate and compare the expression of IL-23 receptor (IL-23R) and IL-17A on $CD4^+$ and $CD8^+$ T cells in both recently isolated PBMCs (day 0) and PBMCs cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3) from SLE patients and normal subjects. Moreover, this study

examined the expression level of *IL-17A* and *RORC*, a master regulator of Th17 differentiation, in PBMCs (both on day 0 and day3) from SLE patients, compared with those from healthy controls. This study also investigated and compared the serum and plasma levels of IL-17A and IL-23 from SLE patients and normal subjects. Furthermore, this study determined the correlation between the results that described above and the score of SLDAI-2K. We hypothesized that the expression of the Th17 associated proteins and genes in the SLE patients are different from the normal subjects. The knowledge from this research might show the association of Th17 with SLE.

CHAPER II

OBJECTIVE

Hypothesis

The expressions of the Th17 associated markers in the SLE patients are different from the normal subjects.

Objectives

To investigate and compare the expression of IL-23 receptor (IL-23R) and IL-17A on $CD4^+$ and $CD8^+$ T cells in both recently isolated PBMCs (day 0) and PBMCs cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3) from SLE patients and normal subjects. Moreover, the expression level of *IL-17A* and *RORC*, the Th17-associated genes, in PBMCs (both on day 0 and day3) from SLE patients, were examined and compared with those from healthy controls. In addition, this study also investigated and compared the serum and plasma levels of IL-17A and IL-23 from SLE patients and normal subjects. Furthermore, the correlation between the results that described above and the score of SLEDAI-2K were determined.

CHAPTER III

REVIEW OF RELATED LITERATURES

Systemic Lupus Erythematosus

Systemic Lupus Erythematosus (SLE) is a multisystem disease that is caused mainly by B cell hyperactivity. The production of various autoantibodies and complement fixing immune complex deposition results in tissue damage. Since various autoantibodies can be produced in SLE patients, the different organ-specific targets of these autoantibodies can cause a wide spectrum of clinical manifestations, and are characterized by remission and exacerbations [3]. The most common manifestations including arthralgia, arthritis, rash, alopecia, oral ulcers, serositis, leukopenia, central nervous system, and renal involvement [2]. The pathogenic immune responses probably result from environmental triggers acting in the setting of certain susceptibility genes. Ultraviolet light and certain drugs are the only known environmental triggers identified to date [3].

SLE Classification Criteria

In 1971, the American Rheumatism Association (ARA) (the name was changed to the American College of Rheumatology in 1988) published preliminary criteria for the classification of SLE. These criteria were developed for clinical trials and population studies rather than for diagnostic purposes [74]. The preliminary criteria were revised and updated to include new immunologic knowledge and improve disease classification in 1982 [75]. Most recently, in 1997 the American College of Rheumatology (ACR) updated classification criteria for SLE [76] as shown in Table1. A patient who has four or more of these criteria is classified as having SLE. The classification criteria can guide the initial assessment of the SLE patient in clinic. However, patients also require a

systematic assessment, which can be guided by the components of the SLE disease activity indices. There is no gold standard for measuring disease activity but the most widely used indices are the British Isles Lupus Assessment Group (BILAG) index, the European Consensus Lupus Activity Measurement (ECLAM), the Systemic Lupus Activity Measure (SLAM), the Lupus Activity Index (LAI) and the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (Table2). All these indices have been validated and have excellent reliability, validity and responsiveness to change [77].

Table 1. The 1997 Revised Criteria for the Classification of Systemic Lupus Erythematosus (SLE) [76].

Criterion	Definition
1. Malar Rash	Fixed malar erythema, flat or raised
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5. Arthritis	Nonerosive Arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	a) Pleuritis (convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion), OR b) Pericarditis (documented by electrocardigram or rub or evidence of pericardial effusion)
7. Renal Disorder	a) Persistent proteinuria (> 0.5 g/d or > 3+ if quantitation not performed), OR b) Cellular casts of any type
8. Neurologic Disorder	a) Seizures (in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance), OR b) Psychosis (in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance)
9. Hematologic Disorder	a) Hemolytic anemia (with reticulocytosis), OR b) Leukopenia (< 4,000/mm3 on ≥ 2 occasions), OR c) Lyphopenia (< 1,500/ mm3 on ≥ 2 occasions), OR d) Thrombocytopenia (<100,000/ mm3 in the absence of offending drugs)
10. Immunologic Disorder	a) Anti-DNA (antibody to native DNA in abnormal titer), OR b) Anti-Sm (presence of antibody to Sm nuclear antigen), OR c) Positive finding of antiphospholipid antibodies on: ● an abnormal serum level of IgG or IgM anticardiolipin antibodies, ● a positive test result for lupus anticoagulant using a standard method, or ● a false-positive test result for at least 6 months confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test
11. Antinuclear Antibody	An abnormal titer of antinuclear antibody (ANA) by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs

Table 2. Scoring for SLE Disease Activity Index (SLEDAI) 2000 (SLEDAI-2K) [78].

SLEDAI score	Descriptor	Definition
8	Seizure	Recent onset, exclude metabolic, infectious or drug causes
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, disorganised or catatonic behaviour. Exclude uraemia and drug causes
8	Organic brain syndrome	Altered mental function with impaired orientation, memory, or other intellectual function, with rapid onset and fluctuating clinical features, 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 haemorrhages, serous exudates or haemorrhages in the choroid, 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	Cerebrovascular accident	New onset cerebrovascular accident(s). Exclude arteriosclerosis
8	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter haemorrhages or biopsy or angiogram proof of vasculitis
4	Arthritis	≥ 2 joints with pain and signs of inflammation (i.e. tenderness, swelling or effusion)
4	Myositis	Proximal muscle aching/weakness, associated with elevated creatinine phosphokinase/aldolase, or EMG changes or a biopsy showing myositis
4	Urinary casts	Haem-granular or RBC casts
4	Haematuria	> 5 RBC/high power field. Exclude stone, infection or other cause
4	Proteinuria	> 0.5 g/24 hour
4	Pyuria	> 5 WBC/high power field. Exclude infection
2	Rash	Inflammatory type rash
2	Alopecia	Abnormal, patchy or diffuse loss of hair
2	Mucosal ulcers	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, effusionor ECG or echocardiogram confirmation
2	Low complement	Decrease in CH50, C3 or C4 below lower limit of normal for testing laboratory
2	Increased DNA binding	Increased DNA binding above normal range for testing laboratory
1	Fever	> 38 °C. Exclude infectious cause
1	Thrombocytopenia	< 100×10^9 platelets/l, exclude drug causes
1	Leucopenia	< 3×10^9 WBC/l, exclude drug causes

CH50, total haemolytic compliment (classical pathway functional activity); DNA, deoxyribonucleic acid; ECG, electrocardiogram; EMG, electromyogram; RBC, red blood cell; SLE, systemic lupus erythematosus; WBC, white blood cell.

Epidemiology

SLE has a worldwide distribution, with differences in prevalence related to race, geography and sex [4, 5]. The reported prevalence of SLE in the general population is approximately 20-150 per 100,000 [79-81]. SLE primarily affects women in the child-bearing years, and is estimated to be 9–12 times more common in women than in men [6]. The disease is more frequent in blacks/African Americans, Hispanics and Indians/Native Americans than in Caucasians [4, 5]. The prevalence of SLE in Asia is estimated to be 50 to 100 per 100,000 [7]. In Hong Kong, the annual incidence has been estimated to be as 60 per 100,000. In addition, the outcome of SLE may also differ among racial/ethnic groups. More serious organ manifestations have been reported in Chinese SLE patients than Caucasians, especially renal disease [8]. The frequencies of renal disease were 50% in Chinese patients and 31-39% in Caucasian patients [9, 82, 83]. In Thailand, there was no official report on the incidence or prevalence of SLE. The renal manifestation in Thai SLE patients, however, has been reported with higher frequency than Chinese SLE patients, with the frequency of 78% [84]. Moreover, another manifestation such as neuropsychiatry was also found with higher frequency in Thai patients, compared to Chinese patients [85]. These data showed that Asian SLE patients, especially Thai patients have more severe disease progression.

Etiology

The exact etiology and pathogenesis of SLE remains unclear. An extremely complex and multi-factorial interaction between diverse genetic and environmental factors are possibly involved [9].

Genetic Factors

Several lines of evidence have shown the importance of genetic factors in SLE. For example, the concordance of SLE in identical twins, the increase in frequency of SLE among first degree relatives, and the increased risk of developing the disease in siblings of SLE patients reflect a polygenic inheritance of the disease [9, 86-88]. Many different genes contribute to disease susceptibility. The most important genes are located in the HLA region on chromosome 6 that exhibit strong association with the risk of SLE and production of specific autoantibodies that are commonly present in SLE [89]. Other genes which contain risk variants for SLE are *IRF5* [90-92], *PTPN22* [93], *STAT4*, *BLK* [94], *CDKN1A* [95], *ITGAM* [96], *TNFSF4* and *BANK1* [97]. Some of the susceptibility genes may be population specific.

Environmental Factors

The findings above offer strong evidence of genetic factors contributing to disease susceptibility. The certain environmental factors, however, are also required to activate the disease. There are about 400 medications that can induce a lupus-like syndrome. The highest risk drug of which are procainamide and hydralazine [98]. Exposure to UV light, especially UVB, is a well known environmental factor that can induce apoptosis of keratinocytes and the release of pro-inflammatory cytokines. This provides a mechanism for the exposure of self antigens to the immune system and provokes autoimmunity [99]. Several dietary factors are implicated in the pathogenesis of SLE. Possible beneficial compounds are also reported such as vitamin E, vitamin A, fish oils (omega-3 polyunsaturated fatty acids) and evening primrose oil. On the other hand, excess calories, excess protein, high fat, zinc, and iron appear to be related to the risk of SLE [100, 101]. Some Epstein-Barr virus (EBV) proteins share sequence homologies with SLE autoantigens that may crossreact 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 [102]. In addition, the exposure to environmental estrogens through the consumption of meat and milk products or by utilization in clinical practice of the contraception and post-menopausal hormone replacement therapy (HRT) may induce or unmask SLE [9, 102]. Additionally, SLE is a predominant female disease, sex hormones seem to play an important role in the disease onset/perpetuation. Therefore, excessive estrogenic but insufficient androgenic hormonal activity in both men and women with SLE might be responsible for the alteration of the immune responses. These findings suggest a role for endogenous sex hormones in disease predisposition [9, 103].

SLE and Immune System

The principal functions of an immune system are to recognize a wide variety of pathogens and to destroy those pathogens while tolerating the tissues of the host organism. The immune system of mammals has two main arms, the innate immune system and the adaptive immune system. The innate immune system, consisting of cells such as neutrophils, macrophages, mast cells and natural killer (NK) cells, is constantly poised and ready to attack invading pathogens. This rapidity is, however, counterbalanced by a lack of both specificity and memory. By contrast, the adaptive immune system needs priming to eradicate pathogens. Although it is slower, the adaptive immune system has greater accuracy to enable recognition of specific pathogens by virtue of its vast receptor diversity. Most importantly, it has memory and upon subsequent infection with the same pathogen, and can rapidly respond and clear the invaders [104].

As described above, SLE is characterized by B cell hyperactivity and production of various autoantibodies that result in formation of immune complex, which is an important contributor to the development of SLE. Many reports, however, suggested that T cells are present in target tissues of SLE and produce pro-inflammatory cytokines or

upregulate cell-to-cell adhesion, finally leading to apoptosis and inflammation of target tissues. Therefore, both SLE B cells and T cells play a central role in the pathogenesis of SLE [1].

B Cells Abnormalities in SLE

Several reports propose a primary role for B cells in SLE. The importance of primary B cell abnormalities is strongly supported by genetic studies. B cell signaling defects in Fc receptor (FcγRIIb) leads to a lupus-like phenotype in mice [105]. In human SLE, polymorphisms in FcγRIIb may facilitate the generation and activation of autoreactive B cells and these effects may predispose to the development of SLE [106]. Alterations in B cell longevity also can lead to lupus-like phenotypes. In mice with overexpressing of B-cell activating factor (BAFF), a key cytokine that promotes B cell survival, development of a lupus-like phenotype mediated by autoantibody overproduction was reported [107]. Moreover, elevated BAFF serum levels have been found in human SLE and this rising correlates with serum IgG and autoantibody levels [108]. Altered signals of B cells such as CD80/CD86, CD19 and CD22 also might be involved in autoimmunity [109, 110]. Additionally, somatic hypermutation of immunoglobulin variable regions, abnormality of receptor editing, antigen-derived autoantibody production and defect of idiotypic networks could be associated with SLE development [111-114].

T Cells Abnormalities in SLE

The major role of T cells in SLE is confirmed by the findings of a blockade of disease development after elimination of T cells in lupus mice [115]. In addition, athymic mice do not develop SLE [116]. As mentioned previously, SLE is characterized by the production of pathogenic autoantibodies secreting from auto-reactive B cells, and it is widely believed that T cells provide help to autoantibody-producing B cells. An escape from immunological tolerance by T cells may have a central role in the pathogenesis of

SLE. The followings are some key defects in T cell responses which may be involved in SLE.

- *Antigen Processing and Presentation*

In SLE, during apoptosis, autoantigens can be modified by protease cleavage (by caspases or granzyme B) or by oxidative cleavage, these self-antigens modifications might reveal cryptic or neoepitopes to the immune system, and these modifications could have important effects on breaking T cell tolerance [10]. The histone-derived peptide H2B, H3 and H4 stimulated T cells from SLE patient allow them to help B cells lead to the production of autoantibodies [11]. 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 [10].

- *T Cell Effector Surface Molecules and Co-stimulation*

SLE T cells with many different surface phenotypes including classic helper T cells ($CD4^+CD8^-$ α/β TCR), $CD4^+CD8^-$ γ/δ TCR cells, $CD4^-CD8^+$ α/β TCR cells, and CD1-restricted double-negative natural killer T cells can provide help for autoantibody production [12-14]. Overexpression of lymphocyte function antigen-1 (LFA-1) decreased the threshold for T cell activation, allowing cells to respond to self-MHC class II molecules presenting inappropriate antigens [15]. Overexpression of CD40L leading to prolong co-stimulation that sustains autoantibody producing B cells [16]. Furthermore, overexpression of adhesion molecules such as soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1) has been reported to occur in SLE [17].

- *Apoptosis and Apoptotic Clearance*

Normally, apoptosis must be appropriately controlled for avoidance of autoreactivity. Regulation of apoptosis in human SLE T cells is reported to be impaired. T cells from peripheral blood of some SLE patients show decreased intracellular synthesis of TNF-α, which could result in undesirable survival of autoreactive cells [18]. SLE T cells resist anergy and apoptosis by upregulating *cox-2* and *bcl-2* expression, both of which encoding anti-apoptotic proteins [19-21]. Increased apoptosis, however, has been reported in SLE [22]. This accelerated apoptosis is likely to provide more autoantigens to stimulate the immune system. In addition, impaired clearance of apoptotic materials has been reported in the pathogenesis of SLE. Macrophages from SLE patients are defective in the phagocytosis of autologous apoptotic materials [23]. Therefore, the impaired clearance apoptotic materials might serve as immunogen for the induction of autoreactive lymphocytes and as antigen for immune complex formation.

- *Regulatory T cells*

Regulatory T cells (Treg) play an important role in peripheral immune tolerance and in the prevention of pathogenic autoimmunity. Defects in the reduced number of Treg cells or suppressive functions of Treg cells are linked to various autoimmune diseases. Studies in SLE found that the number of $CD4^{+}CD25^{+}$ Tregs decreased in active SLE but have normal phenotype and function [24-26], whereas some investigators reported that suppressive functions of Treg cells were poor during active SLE [27]. Therefore, it is still controversial whether Treg plays a role in SLE.

- *T helper-associated cytokines*

Aberrations in cytokine profiles or cytokine signaling could alter thresholds for immune responses, resulting in presentation of self-antigens and the following misdirection of pro-inflammatory adaptive immunity against self, which is often observed in autoimmune diseases [117]. Cytokine expression profiles have been studied extensively in patients with SLE. Previous studies have shown that serum concentrations

of Th2 cytokines, IL-6 [28] and IL-10 [29], were elevated, while Th1 cytokines, IL-2 and IFN-γ [30], decreased in active SLE. However, some other studies have shown that serum Th1 cytokines such as IL-12 [31], TNF-α [32] and IFN-γ [33] were significantly higher in SLE patients, as compared with controls. Increased expression of Th1 cytokines followed by induction of Th2 cytokines have been reported in murine SLE model [118]. These differences imply that the T helper cytokine response in SLE is highly complex and requires further investigation.

T Helper 17 (Th17) Cells : the Newest Member of the T Helper Cell Family

The major components of adaptive immune system are $CD4^+$ T cells or T helper (Th) cells. Initially, two polarized forms of T helper effectors, namely type 1 (Th1) and type 2 (Th2), were identified in both mice and humans [119, 120]. The differentiation of a naive T helper cell into a specific lineage (Figure 1) is directed by signals from antigen-presenting cells in response to the type of pathogens they have encountered. Interferon (IFN)–γ and IL-12 initiate the differentiation of Th1 cells that are characterized by high production of IFN-γ which is essential for clearing intracellular pathogens. Aberration in Th1 responses are thought to be important for driving autoimmune diseases and chronic inflammation. In another effector lineage, IL-4 triggers the differentiation of Th2 cells that are important in host defense against helminth infection and in helping B cells to produce antibodies. In some circumstances, however, Th2 response is linked to asthma and allergies. Th1–Th2 subsets showed specific gene profiles and specific signaling requirements for their phenotypes and effector functions. Signal transducer and activator of transcription (STAT) proteins are the key signaling transcription elements in the T helper subset differentiation pathway (Figure 1). STAT-1 and STAT-4 play major roles in maintaining and amplifying the Th1 response [121, 122]. Similarly, STAT-6 activation is necessary for Th2 development [123]. The lineage-specifying transcription factors for Th1 and Th2 are T box family protein expressed in T cells (T-bet) [124] and

GATA-binding protein 3 (GATA-3) [125], respectively. The effector cytokines that are subsequently produced by Th1 and Th2 cells (i.e., IFN-γ and IL-4) can potentially feed back to amplify Th1 and Th2 cells and further enhance differentiation of the respective T cell subset. Moreover, IFN-γ and IL-4 antagonize each other on different levels, and thus, Th1 and Th2 development is considered mutually exclusive [126-129].

Recently, the Th1/Th2 paradigm has been expanded, following the discovery of a third subset of effector T helper cells that mainly produce IL-17 and exhibit effector functions distinct from Th1 and Th2 cells. This lineage of T helper effector is called Th17. The primary function of Th17 cells appears to be the clearance of pathogens that are not adequately handled by Th1 or Th2 cells, mainly by recruitment of neutrophils and triggering inflammation. Th17 cells are, however, potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions. In recent years, we have witnessed an accumulation of information on this new T cell subset. The cytokines necessary for its differentiation and expansion have been identified [34, 37-39, 130-132] and the key transcription factors that are involved in its generation have been elucidated [133, 134], firmly establishing Th17 cells as an independent T helper cell lineage in human and mice.

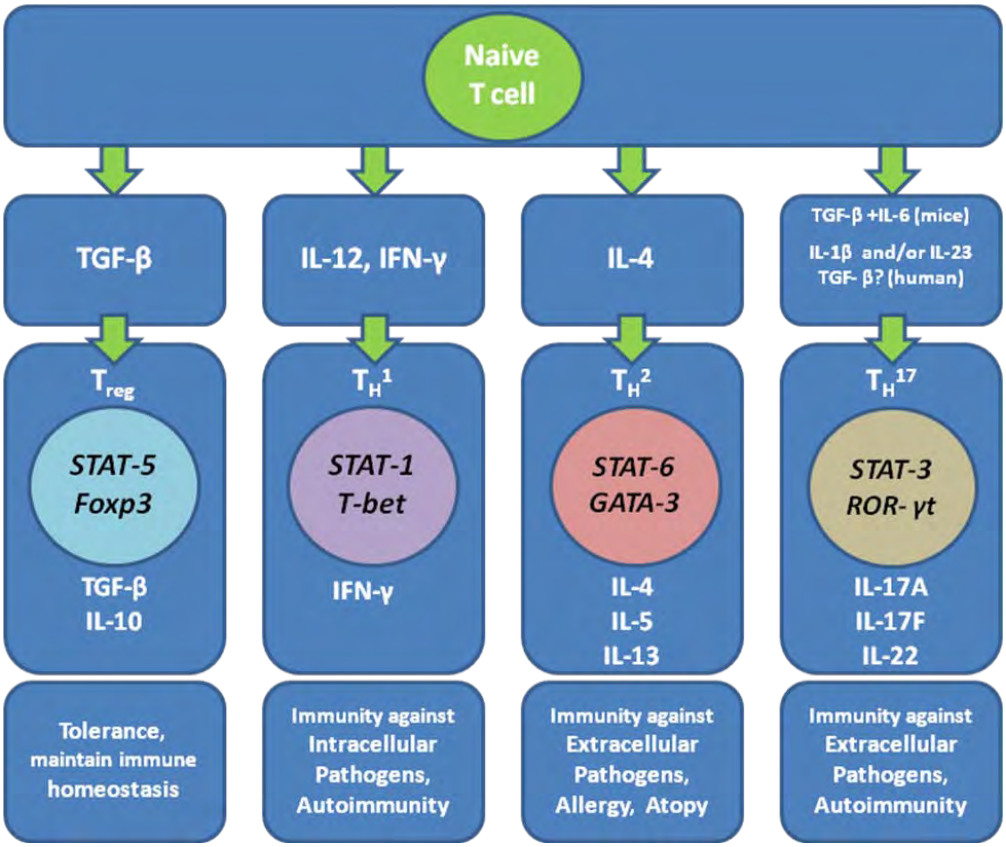


Figure 1. **Subsets of Effector T Helper Cells.** Antigen and specific cytokine signals induce the differentiation of naive T cells into various subsets of T helper cells (Th1, Th2 and Th17). While these subsets produce specific patterns of cytokines that induce immunity, regulatory T cells (Treg), naturally occurring $Foxp3^{+}$Treg cells and induced $Foxp3^{+}$ Treg cells secrete anti-inflammatory mediators such as IL-10 and TGF-β that maintain immune tolerance and immune homeostasis.

Modified from [135]

Discovery of the Th17 Cells

The discovery of the Th17 lineage came from study on mouse models of autoimmunity. Two prototypical autoimmune models, experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA), that have previously been associated with Th1 responses, due to the fact that disease development was ablated by treatment with neutralizing antibodies specific for IL-12p40 or gene-targeted mice deficient in the p40 subunit of IL-12 [136-138]. This association, however, came into question with the discovery a new IL-12 family member, IL-23 [139]. IL-23 shares the IL-12p40 subunit with IL-12, but it is paired with a distinct subunit IL-23p19, instead of IL-12p35. Thus, it became unclear whether protective effects using neutralizing antibodies described above were truly due to inhibition of IL-12 or may involve IL-23.

To resolve this paradox, Cua *et al.* revisited the development of EAE and CIA using mice deficient in IL-12 (*Il12p35*$^{-/-}$), IL-23 (*Il23p19*$^{-/-}$), or both (*Il12p40*$^{-/-}$) [132]. It was found that mice deficient in IL-23 (*Il23p19*$^{-/-}$, *Il12p40*$^{-/-}$) were protected from EAE and CIA, while mice lacking IL-12 (*Il12p35*$^{-/-}$) only remained susceptible. Thus, it appears that IL-23, not IL-12, is importantly related to autoimmunity in these models. On studying the pathogenic role of IL-23, Murphy *et al.* analyzed the cytokine production profiles of effector CD4^{+}T cells in CIA [140]. It was shown that mice deficient in IL-23p19 had normal Th1 responses, but did not produce IL-17, whereas mice deficient in IL-12p35 showed an increased number of IL-17-producing CD4^{+} T cells in inflamed tissues.

IL-17 was known as a T cell–derived cytokine that is highly expressed during autoimmune disorders and is able to activate epithelial cells during inflammatory responses. Consistent with these findings, mice deficient in IL-17 were resistant to EAE or CIA, whereas overexpression of IL-17 exacerbated the diseases [141, 142]. The relationship between IL-23 and IL-17 was established after the study that activation of effector and memory T cells in the presence of IL-23 resulted in IL-17 production [52] and this induction was blocked by IL-12 or IFN-γ. It was proposed that IL-23 promoted a

T cell subset distinct from Th1 cells that expressed IL-17. Passive transfer of these IL-23–generated IL-17–producing $CD4^+$ T cells was sufficient to induce EAE [34]. It became clear that the IL-23/IL-17 axis, rather than the IL-12/IFN-γ axis, was important for EAE disease development. Then, the concept of a new T helper cell subset was established and these cells were then called T helper 17 cells (Th17).

Definition of Th17 Cells

Th17 is a newly discovered subset of T helper cells that produces the signature cytokines interleukin (IL)-17 (IL-17A), IL-17F and IL-22 [34-36]. These cells contribute to host defense against extracellular pathogen, mainly at mucosal surface [38, 61, 62]. Their activities, however, are also pivotal in the development of autoimmune diseases under pathologic conditions [143].

Unique Cytokine Environment for Human Th17 Development

After the identification of differentiation factors of mouse Th17 cells (Table 3), several groups sought to determine whether human Th17 cell development follows similar cytokine requirements as its murine counterpart. Initial reports on identifying factors for human Th17 differentiation suggested that, in contrast to mice, the combination of TGF-β and IL-6 was not capable to generate human Th17 cells [40, 41, 144, 145]. Instead of TGF-β, a combination of IL-1β or IL-23 was sufficient to drive Th17 differentiation from circulating human naive T cells without the addition of exogenous TGF-β [40-42]. This *in vitro*–derived Th17 cells express Th17 lineage-specific transcription factors RORγt, the signature cytokines, IL-17, IL-22, IL-17F, IL-26, surface marker CCL20, CCR6. The studies that followed, however, reported that TGF-β is required for the differentiation of human Th17 cells, like for murine Th17 cells [43-45]. Volpe *et al*. [44] reported that the cytokine cocktail composed of TGF-β, IL-23, IL-1β, and IL-6 are required for Th17 differentiation from umbilical cord blood cells or

circulating naive $CD4^+$ T cells. On the other hand, Manel *et al.* [43] proposed that the combination of TGF-β, IL-23, and IL-1β was finest for Th17 differentiation of naive cord blood $CD4^+$ T cells and that IL-6 and IL-21 were not required for Th17 cell development. In contrast, Yang *et al.* [45] proposed that the combination of TGF-β and IL-21 activated the development of Th17 cells that produced IL-17. Manel *et al.* [43], however, did not detect production of IL-17 under the similar conditions. Finally, the recent work of Cosmi *et al.* [46] suggested that human Th17 cells could derive from umbilical cord blood $CD161^+CD4^+$ T cell precursors in the absence of exogenous TGF-β in the presence of both IL-1β and IL-23. Moreover, another study from the same group proposed that TGF-β is not essential for human Th17 development but instead indirectly provides to Th17 cell expansion through inhibition of Th1 cells [47]. Interestingly, the recent report have shown that TGF-β was not required for Th17 differentiation but enhanced production of IL-17 induced by IL-1β, IL-23, and IL-6 [146]. Nevertheless, TGF-β was essential for expression of the key transcription factor *RORC*, the closest human relative of murine RORγt [47]. Molecular mechanisms leading to differentiation and maturation of murine and human Th17 cells are shown in Figure 2.

Table 3. The Factors Involved in the Differentiation of the Murine Th17 Subset.

Differentiation Factors	Self-amplification Factors	Expansion/ Maintenance Factors	Inhibitory Factors
• TGF-β + IL-6 [37-39] • TGF-β + IL-21 [130, 131, 149]	• IL-21 [135, 143]	• IL-23 [147, 148]	• IL-12 [35, 36] • IFN-γ [35, 36] • IL-4 [35, 36] • IL-27 [150, 151]

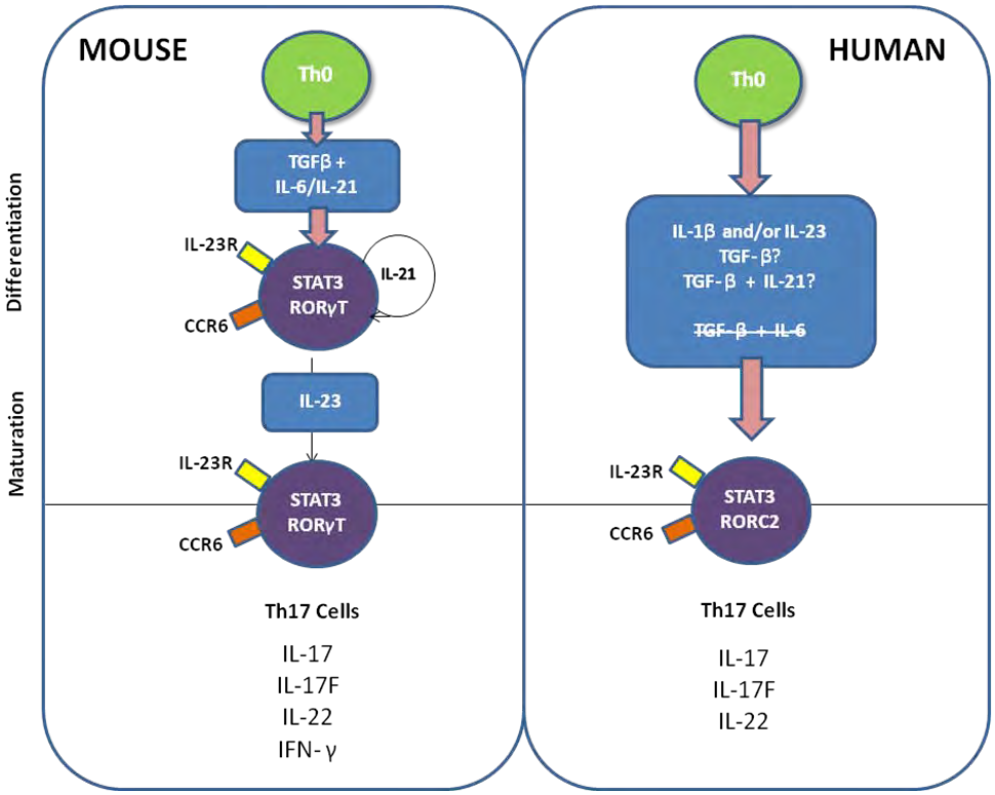


Figure 2. **Molecular Mechanisms of Differentiation and Maturation of Murine and Human Th17 Cells.** In the murine system the combination of TGF-β and IL-6 or IL-21, is a vital mediator driving the activation of STAT3, the upregulation of RORγt, and the subsequent differentiation of naive $CD4^+$ T cells into Th17 cells. Importantly, IL-6 and IL-21 induce expression of IL-23R on these cells. IL-23 is central to the effector function of pathogenic Th17 cells. In human subjects the combination of TGF-β and IL-6 is not able to induce Th17 lineage commitment from circulating or cord blood naive $CD4^+$ T cells. IL-1β and IL-23 consistently emerge as critical factors in the induction of human TH17 cell development.

Modified from [143]

The Signature Cytokine of Th17 Cells

- Interleukin (IL)-17 or IL-17A

IL-17A was originally cloned and described by Rouvier *et al.* and named CTLA8 [152]. It was subsequently renamed IL-17 and, more recently, IL-17A. IL-17A is the founding member of the IL-17 family of cytokines, which has five other family members, designated IL-17A-F and the IL-17 receptors family consists of five receptors (IL-17RA–IL-17RE) [147]. The most closely related members of the ligand family are IL-17A and IL-17F, both of which are produced mainly by activated T cells [153] and bind to the same receptors (IL-17RA and IL-17RC) [154]. IL-17RA is expressed broadly and mediates its effects through a number of immune and non-immune cells (particularly endothelial and epithelial cells) [52]. While IL-17RC can not signal in the absence of IL-17RA, its expression is low in hematopoietic tissues but high in cells of the liver, kidney, thyroid, prostate and joints [155, 156]. The predominant function of IL-17A is thought to be a pro-inflammatory mediator through a variety of mechanisms as summarized in Figure 3. It is now known that several cell types are capable of producing IL-17A other than $CD4^+$ T cells. The $\gamma\delta$ T cells [53], $CD8^+$ memory T cells [54, 55], NK T cells [56, 57], NK cells [58], eosinophils [59], neutrophils [54] and monocytes [60] can also be a source of IL-17A. Nevertheless, the predominant source of IL-17A remains the $CD4^+$ T cell population [51].

- Interleukin (IL)-17F

Among the IL-17 cytokines family, IL-17F shares the greatest amino acid homology (55%) with IL-17A. Both IL-17A and IL-17F are produced by Th17 cells, whereas the other IL-17 family members, IL-17B, IL-17C, and IL-17D, are produced by non-T cell sources. IL-17A and IL-17F can give rise to three forms of IL-17A homodimers, IL-17F homodimers and IL-17A-IL-17F heterodimers [157]. Notably, IL-17A and IL-17F share similar functions in terms of their ability to induce chemokines that are important in neutrophil recruitment and activation [158]. Among the three forms, IL-17A

homodimers show the greatest potency in inducing chemokine expression in epithelial cells, followed by IL-17A–F heterodimers and IL-17F homodimers [157]. As described above, IL-17A and IL-17F bind to a receptor complex composed of at least two IL-17RA and IL-17RC subunits. The binding affinities of IL-17A and IL-17F are also quite different with respect to the individual IL-17RA and IL-17RC subunits [156], indicating that each cytokine target different cells.

- Interleukin (IL)-22

Besides IL-17A and IL-17F, IL-22 is another Th17 effector cytokine. IL-22 was first identified as an IL-10-related T cell-derived inducible factor (IL-TIF) [159]. It binds to the IL-22 receptor comprising IL-22Rα and IL-10R2 (ubiquitously expressed on all cells). IL-22 is exclusively produced by hematopoietic cells such as T cells, while in contrast, the expression of IL-22Rα is restricted to epithelial cells and epithelial-derived cells and some fibroblasts, in certain tissue including the skin, pancreas, small and large intestine, and lung [160]. Thus, immune cells are not target cells of IL-22 [161, 162]. On the other hand, IL-22 elicits strong responses from many epithelial cells [163]. Data from many studies implicate the potential roles of IL-22 in host defense, inflammation and tissue repair [164-166]. Firstly, IL-22 induces pro-inflammatory responses, such as the production of cytokines, chemokines, and acute-phase proteins, from many cell types. Secondly, IL-22 drives the production of many antimicrobial peptides, including β-defensins, S100-family proteins, and regenerating-gene (Reg)-family proteins. Gene expression study in keratinocytes treated with IL-22 shown uncovered a large group of genes involved in tissue-repair and wound-healing [165, 167]. Furthermore, IL-22 also stimulates proliferation, abnormal differentiation, and migration of various epithelial cells *in vitro* [164, 165, 168]. Collectively, these data strongly support a role of IL-22 in host defense and epithelial-barrier function.

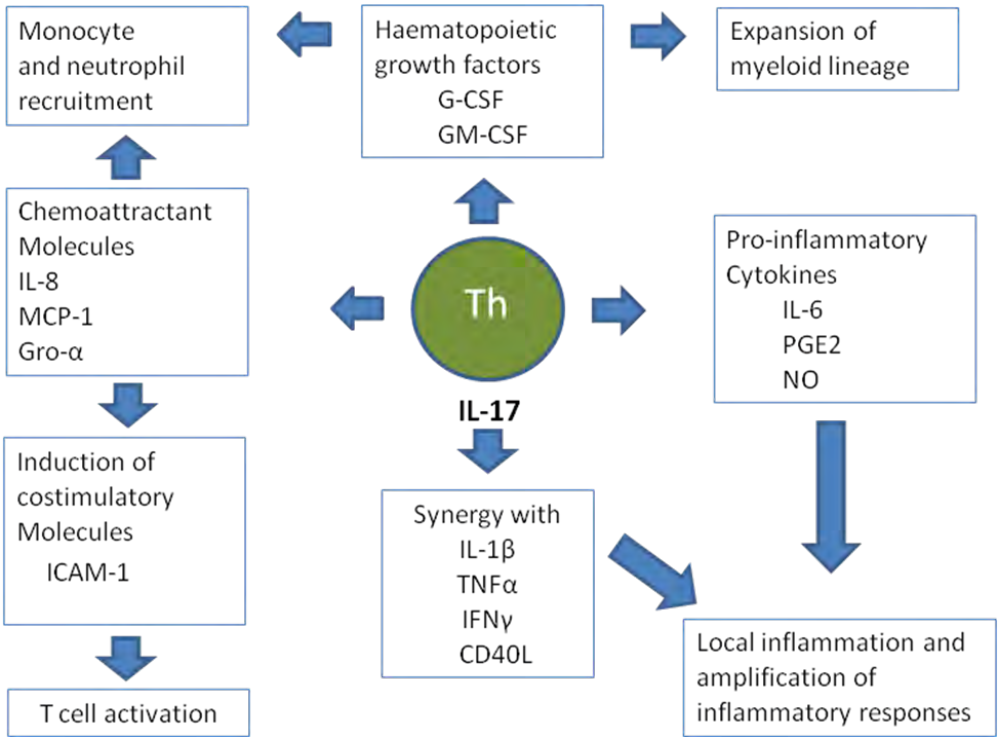


Figure 3. The Biological Function of IL-17A. IL-17A activates production of IL-6, nitric oxide and prostaglandin E_2 (PGE_2), while synergy with other inflammatory cytokines such as IL-1β, TNF-α, IFN-γ and CD40 ligand (by increasing surface levels of CD40) leads to progression and amplification of local inflammation. IL-17A induces the recruitment of neutrophils and monocytes to sites of inflammation through the chemoattractant mediators IL-8, monocyte chemoattractant protein (MCP)-1 and growth-related protein (Gro)-α while enhancing production of hematopoietic growth factors, such as granulocyte-colony stimulating factor (G-CSF) and granulocyte-macrophage (GM)–CSF, which promote the growth and maturation of the recruited myeloid cells. Furthermore, IL-17A acts as a bridge between the innate and adaptive immune response by augmenting the induction of co-stimulatory molecules such as ICAM-1 by other cytokines, thereby supporting T cell activation.

Modified from [51]

Key Transcription Factors in Th17 Cells

In addition to a unique cytokine expression profile, the T helper cell lineages are defined by lineage-specific transcription factors (Table 4). Th17 cells were identified as an independent lineage since neither Th1 transcription factors, such as STAT-1, STAT-4 and T-bet, nor Th2, such as STAT-6 and GATA-3, was found to be expressed by Th17 cells. The orphan nuclear receptor RORγt (RORC in human) is the key transcription factor that regulates the differentiation of Th17 lineage [42, 133]. The RORγt induces transcription of the genes encoding IL-17 and IL-17F in naïve $CD4^+$ T helper cells and is required for their expression in response to IL-6 and TGF-β, the cytokines known to induce IL-17 [133]. Although RORγt plays a central role in the development of Th17 cells, RORγt-deficient $CD4^+$ T cells did not completely abolish Th17 cytokine expression, suggesting that other transcription factors may contribute to the Th17 differentiation. In fact, the combination of IL-6 and TGF- β induce another closely related transcription factor RORα that can also control IL-17A and IL-17F production. Furthermore, RORα and RORγt co-expression synergistically led to greater Th17 differentiation. On the other hand, double deficiencies of both RORα and RORγt entirely impaired Th17 generation *in vitro* and *in vivo* [169].

In microarray analysis of *in vitro* driven Th17 cells revealed a third transcription factor uniquely expressed in Th17; the aryl hydrocarbon receptor (AhR), a ligand activated transcription factor also known as the dioxin receptor [170]. AhR deficiency was shown to impair, but not prevent, the generation of IL-17 producing T cells [170, 171]. Most remarkably, AhR-deficient Th17 cells completely failed to express one of the signature cytokines, IL-22, whereas it was induced upon ligand activation of AhR [170].

In addition to cytokines and master transcription factors, T helper cells differentially utilize STATs protein, which couple cytokine receptors with gene expression. STAT3, activated by both IL-6 and IL-23, plays a critical role in Th17 development. Although STAT3 mediates multiple cytokine signaling events, the use of

a T cell-specific deletion of STAT3 firmly established it as the transcription factor responsible for both RORα and RORγt induction in response to IL-6 and IL-23 [134].

Table 4. Unique Transcriptional Programme of T Helper Cells Lineages.

	Th1 Lineage	Th2 Lineage	Treg Lineage	Th17 Lineage
Transcription Activators	STAT-4 STAT-1 [121, 122]	STAT-6 [123]	STAT-5 [172]	STAT-3 [134]
Transcription Factors	T-bet [124]	GATA-3 [125]	Foxp3 [173, 174]	RORγt [42, 133] RORα [169] AhR [170, 171]

Surface Receptors on Th17 Cells

The pattern of chemokine and cytokine receptor expression on human Th17 cells has been investigated extensively [43, 48-50]. In accordance with the robust effect of IL-23, IL-17-mediated responses require IL-23 to maintain and expand differentiated Th17 cells, and the IL-23 receptor is specifically expressed on Th17 cells. The studies in IL-23R-GFP knock-in reporter mice, however, showed that in addition to Th17 cells, a subset of myeloid cells express IL-23R and respond to IL-23 by producing IL-17 and IL-22 [175]. Like most cytokine receptors, it is difficult to purify Th17 cells on the basis of IL-23R expression since it is expressed at low levels. Another selection marker, the

chemokine receptor CCR6, is reported to be a predominant marker for Th17 cells. Within the human T cells memory compartment, IL-17 was found to be produced only by $CD4^+$ T cells expressing CCR6 [48-50].

Th17 cells and Infection

Although the discovery and description of Th17 cells are mainly associated with autoimmune diseases, not all Th17 cells functions are harmful. Th17 cells play a crucial role in host defense against certain pathogen. Both IL-17A and IL-17F are induced in several models of infections, telling the involvement of this Th17 subset [176]. IL-17 promotes neutrophils and other myeloid cells recruitment at the site of infection by upregulates specific chemokines and pro-inflammatory cytokines. This cellular recruitment is the important process in many infections. For example, mice deficient in IL-17 or IL-17RA are susceptible to pulmonary infection with *Klebsiella pneumonia*e [177], and similarly, mice deficient in IL-17RA are seriously susceptible to *Candida albicans* [178]. The responsibility of IL-17 is clearly important in supporting innate responses, but other Th17-associated cytokines, such as IL-17F and IL-22, also appear to be essential for control of infections. In two studies, using *K. pneumonia* [62] and *Citrobacter rodentium* infection [61], determined that IL-23–dependent production of IL-22 is essential for full protection of the host. Furthermore, IL-17F also plays a nonredundant role in protection against *C. rodentium* [179]. Thus, IL-17, IL-17F, and IL-22 perform synergistically on the epithelium to combat bacterial infections and emerge as important factors in defense against pathogens.

Th17 Cells and Autoimmunity

While Th1 and Th2 cells have long been known to control cellular and humoral immunity, Th17 cells have been identified only recently as a T helper lineage that regulates inflammation via production of distinct cytokines such as interleukin (IL)-17.

Some of the Th17–associated cytokines are found at higher levels in serum and affected tissues in human diseases and in animal models. The crucial function of this T cell subset in the induction and development of murine autoimmune diseases has been confirmed and its actual role in human autoimmune diseases is now supported by a large body of evidence as summarized in Table 5.

Table 5. Evidence for a Role of Th17 Cells in Immune-Mediated Diseases [180].

● Rheumatic diseases	
Psoriasis	++
Rheumatoid arthritis	++
Systemic sclerosis	?
Systemic lupus erythematosus	?
● Non-rheumatic autoimmunity	
Multiple sclerosis	+
Autoimmune myocarditis	?
Type I diabetes	?
Autoimmune thyroiditis	?
● Asthma and allergic diseases	
Asthma	++
Atopic dermatitis	+
Contact hypersensitivity	+
● Other immune-mediated diseases	
Inflammatory bowel disease	++
Periodontal disease	++

++, there is substantial and convincing evidence for a role for Th17 cells in both humans and animal models; +, there is good evidence for a role for Th17 cells, either in humans or animal models, but more conclusive studies are needed; ?, there is very limited evidence suggestive of a possible role for Th17 cells, but no conclusion can be drawn without substantial additional works.

Psoriasis is a chronic autoimmune disease affecting mainly the skin. Both Th1 and Th17 cells are implicated in the pathogenesis, as there are elevated levels of both Th1 and Th17-associated cytokines in serum and lesional skin [42, 167, 181-183]. The role of IL-17 in the pathogenesis of psoriasis has been more confirmed by increased expression of RORC, IL-6, IL-1β, and IL-23 in psoriatic skin as compared with noninvolved skin or skin from healthy individuals [42]. The strong evidence for the role of Th17 cells in psoriasis comes from a study which found that antibody-mediated blockade of the shared IL-23/IL-12 p40 subunit is an effective treatment [63]. Interestingly, Ortega *et al.* observed a higher frequency of $CD8^{+}IL\text{-}17^{+}$ T lymphocytes in psoriasis plaque biopsies from psoriasis patients compared to skin biopsies from healthy subjects [184].

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease that may affect many tissues and organs, but mainly attacks the joints. It has long been classified as a Th1-mediated disease but is now also thought to be a primarily Th17-driven disease [185]. Primary evidence for a pathogenic role of IL-17 in RA came from studies demonstrating that IL-17 was increased in the sera and synovial fluids of RA patients [64-66]. Like psoriasis, there is also increased IL-22 and IL-23 in the synovium of RA patients [186, 187].

Multiple sclerosis (MS) is a chronic autoimmune disease that predominantly affects the white matter of the central nervous system. There is a strong association between IL-17 and MS [180], but information on the role of IL-17 in this autoimmune disease is incomplete. Studies in mice have shown that Th17 cells play a vital role in EAE, the mouse model of MS. It is not clear, however, whether IL-17 mediates its pathogenic effects directly or via recruitment of other immune cells in humans with MS. Kebir *et al.* demonstrated that human blood–brain barrier endothelial cells from MS patients express the receptors for IL-17 and IL-22, and shown that IL-17 and IL-22 disrupt blood brain barrier tight junctions leading to increased transmigration of

$CD4^+$ T cells [67]. Accordingly, a main pathological mechanism in MS could be IL-17-mediated destruction of the blood brain barrier allowing easier access of myelin-specific T cells to this immune privileged site. Moreover, Tzartos *et al.* observed a higher frequency of $CD4^+IL\text{-}17^+$ and $CD8^+IL\text{-}17^+$ T lymphocytes in areas of active lesion (73%) compared with inactive lesions (17%) of the brain. These observations suggest that both $CD4^+$ cells and $CD8^+$ IL-17-producing T cells play an essential role in MS pathophysiology [68].

Inflammatory bowel disease (IBD) is a collective term that refers to chronic, autoimmune, inflammatory diseases of the bowel, mainly ulcerative colitis and Crohn's disease. Possibly the strongest evidence for the pathological role of Th17 cells came from studies of inflammatory bowel disease [188, 189]. A large cohort study of European populations of different ethnicities found that a single nucleotide polymorphism in a non-coding region of the IL-23R was significantly associated with both Crohn's disease and ulcerative colitis [190]. Additionally, high IL-17 level is found in sera and colonic biopsies of Crohn's disease patients [69, 70]. IL-22 is also overexpressed by colonic $CD4^+$ T cells in patients with IBD compared to healthy controls [191].

Th17 Cells and SLE

Although SLE has been considered classically an autoantibody- and immune complex-driven disease, recent study exhibits that IL-17 is involved in different aspects of SLE pathogenesis. Because of IL-17 is a potent pro-inflammatory cytokine, along with the effects it exerts in a variety of cells, it is possible that its unregulated production has indeed widespread effects in animals and patients with lupus (Table 6).

Table 6. Interleukin (IL)-17 in Murine Models and patients with Systemic Lupus Erythematosus.

	Experimental Evidence and Outcomes	References
Murine Models		
BXD2	Increased numbers of IL-17-producing T cells provide help to B cells and stimulate spleen germinal centre formation. IL-17 over-expression enhanced disease; IL-17R blockade reduced its intensity	[192]
SNF1	Increased numbers of IL-17^{+} cells	[193]
Ets-1 knock-out	Enhanced differentiation of naive T cells into Th17 cells	[194]
Human		
Chinese	High level of plasma IL-17 but no correlation with SLEDAI	[71]
Japanese	No increased serum IL-17 and *IL-17* mRNA expression was also undetectable in peripheral blood in patients with SLE	[72]
Chinese	High level of plasma IL-17 and correlated with SLEDAI, higher number of IL-17A secreting cells in SLE patients	[73]

In human SLE, many studies have reported potential roles for T-cell-derived cytokines in human SLE pathogenesis, but only recently that the data involving IL-17 in SLE become available. Initial studies by Wong *et al.* in 2000, reported that the levels of IL-17 as measured by enzyme linked immunosorbent assay (ELISA), were significantly higher in the plasma of a cohort of 36 Chinese SLE patients than normal subjects. The investigators did not, however, detect a significant correlation between the levels of IL-17 and the score of SLEDAI [71]. In that same year, Kurasawa *et al.* reported some evidence arguing against the role of IL-17 in human SLE. The investigators reported that the levels of IL-17 as measured by ELISA, did not increase in the sera of a cohort of 9 Japanese SLE patients compared with those in normal subjects. Instead, elevation of IL-17 in serum was characteristic of systemic sclerosis patients.

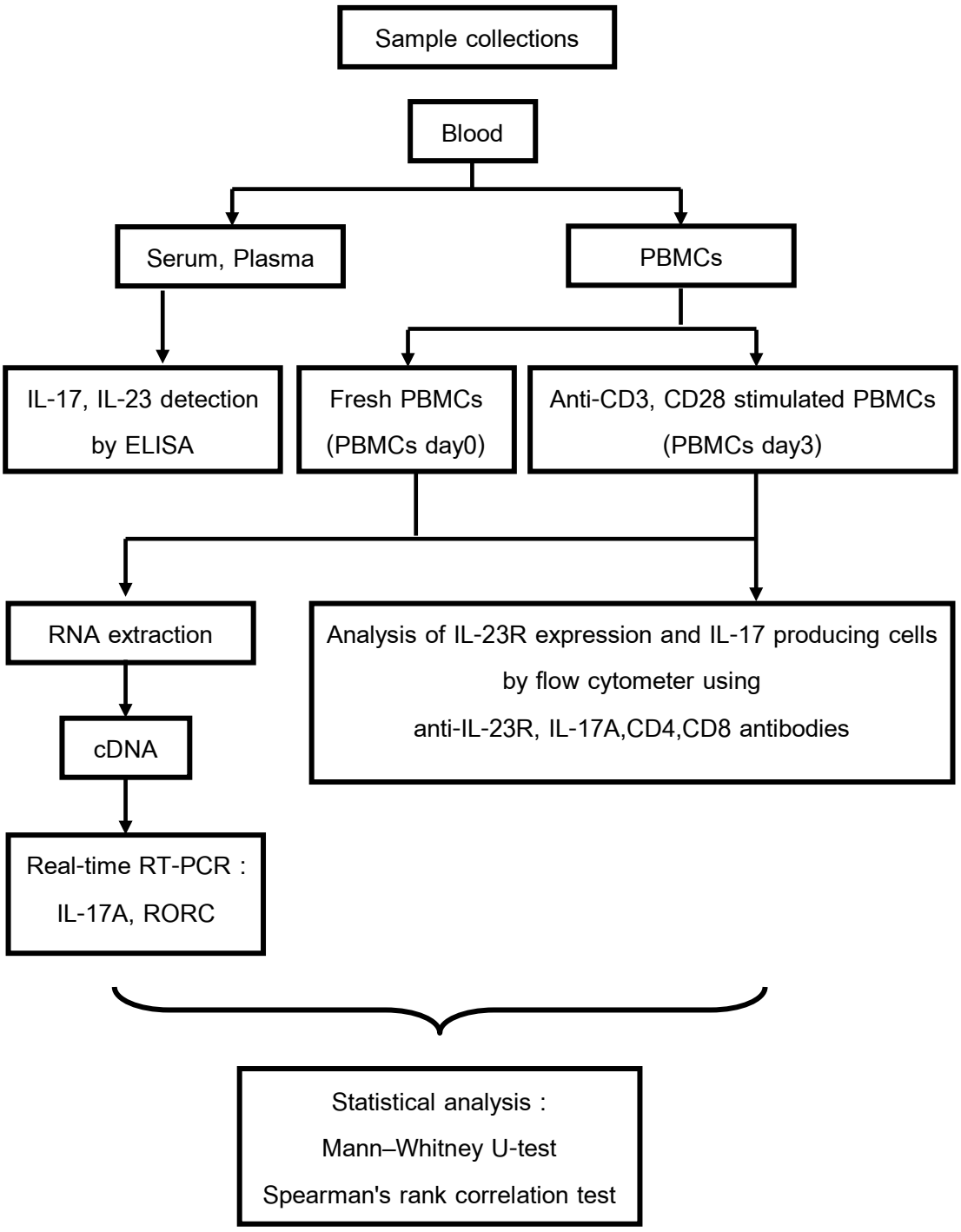
In addition, the *IL-17* mRNA expression as determined by RT-PCR, was also undetectable in peripheral blood lymphocytes (PBL) from 14 Japanese SLE patients [72]. Eight years after these initial reports, in 2008, Wong *et al.* reported that the levels of IL-17 as measured by ELISA, significantly increased in the plasma of a cohort of 80 Chinese SLE patients (40 SLE patients with renal disease and 40 SLE patients without renal disease) compared with those in normal subjects. Moreover, the investigators also found a significant positive correlation between the plasma level of IL-17 and the disease activity (SLEDAI) in SLE patients without renal disease. Thus, IL-17 could play a pathological role in the development of auto-inflammatory response in SLE patients with severe disease. The investigators further examined the number of IL-17A secreting cells in PBMCs using ELISPOT assay, upon 24 h activation by phorbol-myristate acetate (PMA) and ionomycin *ex vivo*. The results revealed that SLE patients showed significantly higher number of IL-17A secreting cells than that in normal subjects. This group, furthermore, investigated the involvement of IL-23 in the IL-23/IL-17 auto-inflammatory axis in SLE, they observed that the *ex vivo* production of IL-17 upon anti-CD3 and anti-CD28 antibody activation in the presence of IL-23 from PBMCs was significantly higher in SLE patients than normal subjects. Thus, these studies presented evidence on the direct involvement of the IL-23/IL-17 inflammatory axis, in the auto-inflammatory responses in SLE [73].

In conclusion, there is increasing evidence in both human and mouse models that IL-17 and Th17 cells play a role in SLE progression. However, there is also evidence that IL-17 and Th17 cells is not always necessary for disease to occur. Because SLE is known to be heterogeneous, it is likely that different mechanisms of pathogenesis occur in different patient populations. Thus, the exact role of IL-17 and Th17 cells in SLE autoimmune disease need further investigation.

CHAPTER IV

MATERIALS AND METHODS

Research Outline



Patients and Normal Subjects

Twenty-nine Thai SLE patients, 28 females and 1 male, age ranging between 15 and 50 years (mean ± SD 33.72 ± 11.12 years), were recruited from King Chulalongkorn Memorial hospital and Bhumibol Adulyadej Hospital (Bangkok, Thailand). Diagnosis of SLE was established according to the Revised American College of Rheumatology (ACR) criteria (Table1), and disease activity was evaluated by the SLE disease activity index (SLEDAI) 2000 score (Table2). Active SLE disease was defined as a SLEDAI-2K score ≥ 6 and inactive SLE disease was defined as a SLEDAI-2K score < 6 [195]. The SLE patients were classified into two groups: 13 SLE patients with active SLE disease (active group) and 16 SLE patients with inactive SLE disease (inactive group). These SLE patients were on treatment with Prednisolone 14.23 ± 17.16 mg daily, Cyclophosphamide 2.79 ± 7.67 mg daily, Azathioprine 20.31 ± 31.02 mg daily, Mycophenolate mofetil 20.08 ± 99.98 mg daily, or in combination as shown in Table 7. Ten normal subjects recruited from King Chulalongkorn Memorial hospital were included as healthy control (mean age 26.6± 1.58 years old, 10 females). This study was approved by the Ethics Committee for Human Research of the Faculty of Medicine, Chulalongkorn University, and informed consents were obtained from all subjects.

PBMCs Isolation and Serum Collections

Seventeen milliliters (ml) of venous peripheral blood from patients and normal subjects were collected in ACD blood collection tubes (BD Pharmingen Corp, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque Isoprep (Robbins Scientific Corporation, CA, USA) at the ratio of 3:4 (v/v; Ficoll-Hypaque reagent : whole blood). This solution was centrifuged at 2,200 rpm for 30 minutes at 25°C. After density-gradient centrifugation, separated PBMCs in interface by specific density were collected and washed twice in 10 ml of RPMI-1640 Medium (GIBCO, Germany) by

centrifugation at 1,500 rpm for 10 minutes at 25°C. PBMCs were checked for viability and adjusted to 1 × 10^6 cells/ml by hemacytometer chamber for following experiments.

Four milliliters of venous peripheral blood from each patients and normal subjects were collected in serum collection tubes (BD Pharmingen Corp, NJ, USA). The blood was allowed to clot for 30 minutes to 1 hour at room temperature and centrifuged at 3,000 rpm for 10 minutes at 25°C. The serum were collected in 1.5 ml sterile tubes and kept at -70°C until used for IL-17 and IL-23 detection by ELISA as described later.

PBMCs Cultures

PBMCs (1×10^6 cells/ml) were divided into two portions. First portions were harvested for an immediate analysis (day 0), another part of PBMCs were incubated (1 × 10^6 cells/ml/well) in 24-well plates (NUNC™, NY, USA) which were pre-coated with anti-CD3 and anti-CD28 monoclonal antibodies (BD Pharmingen Corp, CA, USA) (both at 1 µg/ml), in RPMI-1640 medium (GIBCO, Germany) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Germany), 100 U/mL penicillin (GIBCO, Germany), 100 µg/ml streptomycin (GIBCO, Germany) and non-essential amino acids (GIBCO, Germany) for 72 hours at 37°C in a 5% CO_2 environment (day 3). After incubation, PBMCs were harvested by gentle repetitive pipetting and subjected to antibody staining and flow cytometric analysis. RNA from PBMCs were isolated and used in quantitative real time RT-PCR.

Antibodies

Allophycocyanin-conjugated anti-CD4 (anti-CD4–APC), peridinin chlorophyll protein (PerCP)-conjugated anti-CD8 (anti-CD8–PerCP) from BD Biosciences (CA, USA), biotinylated anti-IL-23 receptor from R&D Systems (MN, USA) and streptavidin-FITC conjugated from BD Biosciences (CA, USA) were used for cell surface staining.

Staining for intracellular cytokines was performed with Phycoerythrin (PE) anti-IL-17A purchased from eBioscience (CA, USA).

PBMCs Stimulation for Intracellular Cytokine Staining

PBMCs were cultured (1×10^6 cells/well) in 96-well plates (NUNC™, NY, USA) with 25 µg/ml Phorbol-12-myristate-13-acetate (PMA) (Merck KGaA, Germany) and 10 µg/ml ionomycin (Merck KGaA, Germany) in different wells for the PBMCs from each SLE patients and normal subjects, in the presence of 10 µg/ml brefeldin A (Merck KGaA, Germany). The incubator was set at 37°C in a 5% CO_2 environment. After 4 hours of culture, the contents of the well were transferred to 1.5 ml sterile tubes. One ml of PBS were added. PBMCs were then centrifuged at 1500 rpm for 10 minutes at 4°C. The PBMCs were collected and subjected to surface and intracellular staining with monoclonal antibodies.

Surface and Intracellular Staining with Antibodies

After stimulation, the cells were resuspended in 100 µl of staining buffer (PBS supplemented with 0.02% sodium azide (Sigma-Aldrich, MO, USA) and 1% bovine serum albumin (GIBCO , Germany)). The conjugated antibodies (mAbs) CD4–APC, CD8-PerCP, biotinylated anti-IL-23R and Streptavidin-FITC were added and cells were incubated at 4°C in dark for 30 minutes for cell surface staining. After cell surface staining, the cells were washed with 1 ml of staining buffer by centrifugation at 1,500 rpm for 10 minutes at 4°C. The cells were fixed in 300 µl of fixation buffer (4% paraformaldehyde (Sigma-Aldrich, MO, USA) in PBS) for 20 minutes at room temperature in dark. After cell fixation, the cells were washed with 1 ml of staining buffer by centrifugation at 1,500 rpm for 10 minutes at 4°C. One milliliters of permeabilization buffer (PBS supplemented with 0.04% sodium azide (Sigma-Aldrich, MO, USA), 0.1% bovine serum albumin (GIBCO, Germany) and 0.1% saponin) was added to the cells

and they were incubated for 10 minutes at room temperature in dark. The cells were centrifuged at 1,500 rpm for 10 minutes at 4°C and resuspended in 100 µl of permeabilization buffer, incubated for 30 minutes at 4°C in dark with anti-IL-17-PE. The cells were washed with 1 ml of permeabilization buffer by centrifugation at 1500 rpm for 10 minutes at 4°C, and then resuspended in 300 µl of 1% paraformaldehyde in dark before subjecting to flow cytometric analysis.

Flow Cytometric Analysis

Cell samples were analyzed with a four-color FACS Calibur analyzer (BD Biosciences, CA, USA). Analysis was performed with Summit software 5.0 (Dako, Denmark). For each sample, at least 10,000 events were acquired in a stored live lymphocyte gate. Representative FACS plots and gating strategy is shown in Figure 4.

RNA Extraction and Complementary DNA Synthesis

PBMCs from day 0 and day 3 were harvested and washed twice in 10 ml of PBS. One ml of TRIzol® Reagent (Invitrogen Corporation, CA, USA) was added, followed by repetitive pipetting. We incubated the homogenized samples for 10 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Two hundred µl of chloroform were added, 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 tubes. RNA was precipitated by mixing with 500 µl of isopropyl alcohol and incubated for 10 minutes at room temperature and then centrifuged at 12,000 g for 10 minutes at 4°C. Wash the RNA pellet once with 75% ethanol and centrifuged at 7,500 g for 5 minutes at 4°C. At the end of the procedure, RNA pellet was air-dried for 5 minutes, dissolved subsequently with RNase-free water (QIAGEN, Germany) and stored at -80°C. RNA concentration was determined using absorbance value from a spectrophotometer for complementary DNA (cDNA) synthesis.

The first strand cDNA was then synthesized from 250 ng of total RNA using Taqman® Reverse Transcriptase Reagent (Applied Biosystems Inc, CA, USA). Each RNA sample were prepared by mixing 10x RT buffer 3 µl, 25 mM $MgCl_2$ 6.6 µl, 10 mM dNTP 2 µl, 50 µM random hexamer primer 0.5 µl, 20 U/µl RNase inhibitor 0.6 µl, 50 U/µl superscript reverse transcriptase 0.25 µl and RNase-Free water was added to the reaction to bring the total volume to 30 µl. 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 (MA, USA), and cDNA was kept at -20°C.

Semi-Quantitative Real-time RT-PCR

A real time RT-PCR assay was developed for detection and quantification of *IL-17A* and *RORC* transcripts using *β-actin* housekeeping transcripts as internal control. PCR Primers were indicated in Table 8. Each PCR was set up for 20 µl reaction volume. PCR amplification was performed with 2x QuantiTect SYBR® Green PCR Master Mix with 0.31 µM primers, 24 ng cDNA and nuclease-free water according to the manufacturer's protocol (QIAGEN, Germany). PCR amplification included an initial activation at 95°C for 15 minutes, denaturation at 95°C for 15 seconds, annealing at 55°C (*β-actin* and *RORC*) or 57°C (*IL-17A*) for 30 seconds and extension at 72°C for 30 seconds followed by repeating for 40 cycles (*β-actin*) or 50 cycles (*IL-17A* and *RORC*) . The levels of mRNA were measured by a Light Cycler machine (Roche Molecular Biochemicals, IN, USA). Levels of mRNA were expressed as threshold cycle (C_T) and used comparative C_T method for analysis. For relative quantification, the expression of *IL-17A* and *RORC* as target genes were normalized by expression of *β-actin* (housekeeping gene) as endogenous reference and relative to a calibrator. Finally, the amount of target was given by $2^{-\Delta\Delta CT}$.

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

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

Enzyme Linked Immunosorbent Assay (ELISA)

Sera from SLE patients and normal subjects were collected and kept at -70°C until use. The IL-17A and IL-23 level in sera from SLE patients and normal subjects were determined using Human IL-17A and Human IL-23 ELISA Ready-SET-Go! (eBioscience, CA, USA). The procedure was carried out by Best Protocols™ (eBioscience, CA, USA). Briefly, The Corning Costar ELISA plates were coated with 100 µl/well of capture antibody. The plates were sealed and incubated overnight at 4°C. After incubation, the plates were washed 5 times with 300 µl/well of Wash Buffer (allowing time for soaking 1 minute). For blocking, 200 µl of 1X Assay Diluent were added into each well and the plates were incubated at room temperature for 1 hour and the washing step was repeated. To prepare the top standard solution, twenty µl of standard solution were added to 10 ml of assay diluent. One hundred µl/well of standard was added to the appropriate wells and perform 2-fold serial dilutions of the top standards to make the standard curve. One hundred µl/well of samples were added to the appropriate wells then seal the plate and incubate at 4°C overnight. After incubation, wells were washed extensively. After the washing step, 100 µl/well of detection antibody were added into each wells and the plates were sealed and was incubated at room temperature for 1 hour. One hundred µl/well of avidin-HRP were added into each wells and the plates were incubate at room temperature for 30 minutes. After incubation, the plates were washed 7 times. One hundred µl/well of substrate solution were added into each well and the plates were incubated at room temperature for 15 minutes. For stop reaction, 50 µl of Stop Solution were added into each well. The absorbance was immediately read at 450 nm using Multiskan EX primary EIA v.2.1-0 (Thermo Fisher Scientific, MA, USA)

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 4 software (GraphPad Software, CA, USA). Differences among groups were compared using the Mann–Whitney U-test. Spearman's rank correlation test was used to assess the correlations of two variables. Probability values (p) < 0.05 were considered as statistically significance. All probabilities were two tailed.

Table 7. Characteristics of SLE Patients Included in This Study.

No. Patients	Stage	Age (years)	Sex	Treatments	Doses (mg/d)	SLEDAI score	Clinical Features
1	inactive	26	F	-	-	4	PI
2	inactive	38	F	PRD, AZT	5, 50	0	-
3	inactive	49	F	PRD, AZT	35, 12.5	4	RA, MU
4	inactive	50	F	PRD, AZT	5, 25	1	FE
5	inactive	22	F	PRD	80	5	RA, MU, FE
6	inactive	19	F	PRD	2.5	0	-
7	inactive	34	F	PRD, CPM	10, 17	4	PU
8	inactive	24	F	PRD	5	4	AR
9	inactive	40	F	-	-	4	PU
10	inactive	36	F	PRD	5	0	-
11	inactive	29	F	PRD, AZT	10, 50	4	PU
12	inactive	25	F	PRD, CPM	7.5, 25	0	-
13	inactive	17	F	PRD, CPM	10, 25	4	PU
14	inactive	25	F	PRD	10	2	MU
15	inactive	47	F	PRD, AZT	7.5, 100	5	PU, LP
16	inactive	47	F	-	-	0	-
17	active	32	F	PRD, AZT	5, 100	8	HE, PU
18	active	33	F	-	-	8	HE, PU
19	active	38	M	PRD, MMF	2.5, 2000	8	HE, PU
20	active	48	F	PRD	15	30	SZ, UC, HE, PU, PI, PE, LC, IDB
21	active	47	F	PRD, AZT	5, 25	11	MY, HE, PL, LP
22	active	50	F	PRD	5	8	AR, PU
23	active	23	F	PRD, AZT	30, 50	29	VA, AR, HE, PU, PI, RA, MU, LP
24	active	15	F	PRD	30	11	PU, AL, PL, FE, TC, LP
25	active	43	F	PRD, AZT	5, 50	8	VA
26	active	27	F	PRD, MMF	5, 500	13	HE, PU, AL, MU, LP
27	active	45	F	PRD	20	12	HE, PU, PI
28	active	21	F	PRD, AZT	20, 25	16	VA, PU, MU, LC
29	active	28	F	PRD	15	6	PU, LC

PRD, prednisolone; AZT, azathioprine; MMF, mycophenolate mofetil; CPM, cyclophosphamide; SZ, seizure; VA, vasculitis; AR, arthritis; MY, myositis; UC, urinary casts; HE, hematuria; PU, proteinuria; PI, pyuria; RA, rash; AL, alopecia; MU, mucosal ulcers; PL, pleurisy; PE, pericarditis; LC, low complement; IDB, Increased DNA binding; FE, fever; TC, thrombocytopenia; LP, leucopenia

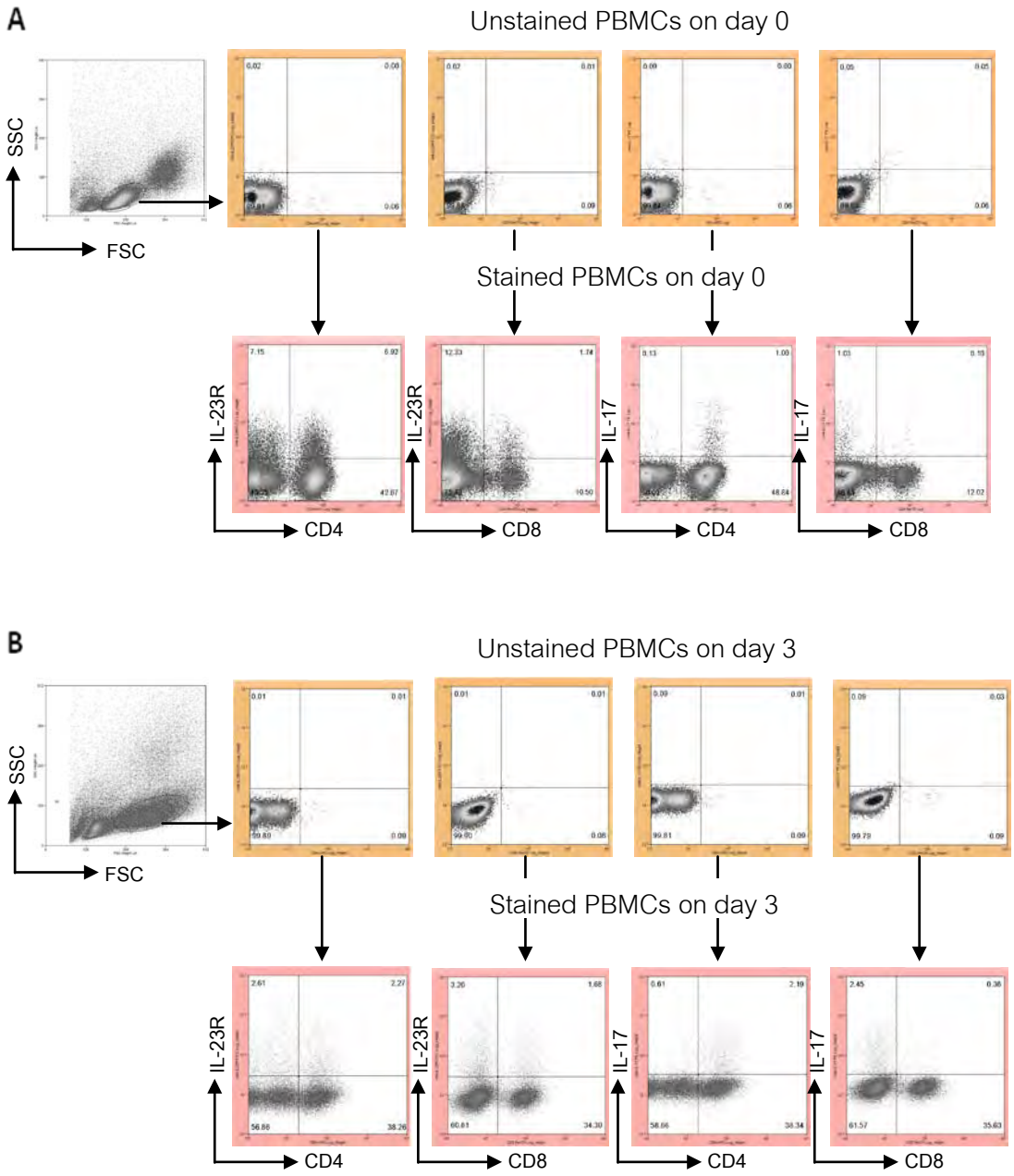


Figure 4. Representative FACS Plots and Gating Strategy Used in This Study.

PBMCs were gated on small lymphocytes on the basis of their profiles of forward and side scatters. The gated populations were analyzed in CD4 vs. IL-23R, CD8 vs. IL-23R, CD4 vs. IL-17 and CD8 vs. IL-17 scatter plot. Values of negative controls (Unstained PBMCs) were below 0.1% of total gated population. Negative values were subtracted from the test values, and values of ≥ 0.1% were considered positive. (A) Gating strategy used in PBMCs on day 0. (B) Gating strategy used in PBMCs on day 3.

Table 8. List of primers used for analysis of the *IL-17A*, *RORC* and *β-actin* gene.

Primers	Sequence (5' → 3')	Product Size
IL-17A [196]		95 bp
- Forward	AATCTCCACCGCAATGAGGA	
- Reverse	ACGTTCCCATCAGCGTTGA	
RORC (this study)		115 bp
- Forward	CCGAGATGCTGTCAAGTTC	
- Reverse	CTTGACCACTGGTTCCTGTT	
β-actin [197]		380 bp
- Forward	ACCAACTGGGACGACATGGAGAA	
- Reverse	GTGGTGGTGAAGCTGTAGCC	

CHAPTER V

RESULTS

1. SLE Patients and Normal Subjects

Twenty-nine SLE patients (13 in active stage and 16 in inactive stage) and ten normal subjects were recruited for this study. Characteristics of each individual and the experimental analysis undertaken for all subjects are summarized in Table 9. In flow cytometric analysis, all subjects were included. For semi-quantitative real-time RT-PCR analysis, RNA quality obtained is a limitation, and, thus, we included only the samples that the threshold cycle (C_t) values for *β-actin* (internal control) was less than 30. For this reason, nineteen SLE patients (7 active and 12 inactive patients) and eight normal were included in the semi-quantitative real-time RT-PCR analysis in freshly isolated PBMCs (day 0) and in PBMCs cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3). For serum cytokine detection by ELISA, only sera from twenty-eight SLE patients (12 active and 16 inactive SLE patients) and nine normal subjects were included in the analysis because of the missing samples. In addition, we have included the analysis of plasma cytokine detection by ELISA later in the experimental design. Therefore, new subjects were recruited for this analysis, including twenty-two SLE patients (11 active and 11 inactive SLE patients) and ten normal subjects. The characteristics of the newly recruited subjects were summarized in Table 10.

No. Samples	Stage	Age (years)	Sex	Treatments	Doses (mg/day)	SLEDAI-2K Scores	Clinical Features	Flow Cytometric Analysis	Serum Cytokine Levels Detection		Quantitative Real-time RT-PCR Analysis	
									Serum IL-17A	Serum IL-23	Day0 *β-actin* Ct	Day3 *β-actin* Ct
Normal1	normal	28	F	-	-	-	-	✓	✓	✓	23.19	20.45
Normal2	normal	27	F	-	-	-	-	✓	✓	✓	22.21	18.07
Normal3	normal	24	F	-	-	-	-	✓	✓	✓	23.56	18.90
Normal4	normal	25	F	-	-	-	-	✓	✓	✓	23.1	19.05
Normal5	normal	25	F	-	-	-	-	✓	✓	✓	22.94	19.88
Normal6	normal	25	F	-	-	-	-	✓	✓	✓	22.54	19.72
Normal7	normal	24	F	-	-	-	-	✓	✓	✓	32.38	22.72
Normal8	normal	28	F	-	-	-	-	✓	✓	✓	23.24	18.6
Normal9	normal	24	F	-	-	-	-	✓	✓	✓	22.48	20.12
Normal10	normal	26	F	-	-	-	-	✓	×	×	32.29	25.11
SLE1	inactive	26	F	-	-	4	PI	✓	✓	✓	21.54	20.15
SLE2	inactive	38	F	PRD, AZT	5, 50	0	-	✓	✓	✓	21.95	20.99
SLE3	inactive	49	F	PRD, AZT	35, 12.5	4	RA, MU	✓	✓	✓	22.06	20.66
SLE4	inactive	50	F	PRD, AZT	5, 25	1	FE	✓	✓	✓	20.99	20.93
SLE5	inactive	22	F	PRD	80	5	RA, MU, FE	✓	✓	✓	20.45	19.28
SLE6	inactive	19	F	PRD	2.5	0	-	✓	✓	✓	26.17	22.71

Table 9. (Continue)

No. Samples	Stage	Age (years)	Sex	Treatments	Doses (mg/day)	SLEDAI-2K Scores	Clinical Features	Flow Cytometric Analysis	Serum Cytokine Levels Detection		Quantitative Real-time RT-PCR Analysis	
									Serum IL-17A	Serum IL-23	Day0 *β-actin* Ct	Day3 *β-actin* Ct
SLE7	inactive	34	F	PRD, CPM	10, 17	4	PU	✓	✓	✓	26.56	22.53
SLE8	inactive	24	F	PRD	5	4	AR	✓	✓	✓	40.00	40.00
SLE9	inactive	40	F	-	-	4	PU	✓	✓	✓	32.22	32.90
SLE10	inactive	36	F	PRD	5	0	-	✓	✓	✓	31.32	23.25
SLE11	inactive	29	F	PRD, AZT	10, 50	4	PU	✓	✓	✓	31.58	24.68
SLE12	inactive	25	F	PRD, CPM	7.5, 25	0	-	✓	✓	✓	27.09	23.55
SLE13	inactive	17	F	PRD, CPM	10, 25	4	PU	✓	✓	✓	26.76	23.52
SLE14	inactive	25	F	PRD	10	2	MU	✓	✓	✓	27.88	23.20
SLE15	inactive	47	F	PRD, AZT	7.5, 100	5	PU, LP	✓	✓	✓	27.87	23.44
SLE16	inactive	47	F	-	-	0	-	✓	✓	✓	25.75	21.21
SLE17	active	32	F	PRD, AZT	5, 100	8	HE, PU	✓	✓	✓	22.13	18.88
SLE18	active	33	F	-	-	8	HE, PU	✓	✓	✓	22.60	19.91
SLE19	active	38	M	PRD, MMF	2.5, 2000	8	HE, PU	✓	✓	✓	21.17	19.15
SLE20	active	48	F	PRD	15	30	SZ, UC, HE, PU, PI, PE, LC, IDB	✓	✓	✓	21.79	19.67
SLE21	active	47	F	PRD, AZT	5, 25	11	MY, HE, PL, LP	✓	✓	✓	27.58	22.57
SLE22	active	50	F	PRD	5	8	AR, PU	✓	✓	✓	30.08	25.68
SLE23	active	23	F	PRD, AZT	30, 50	29	VA, AR, HE, PU, PI, RA, MU, LP	✓	✓	✓	28.05	31.28

Table 9. (Continue)

No. Samples	Stage	Age (years)	Sex	Treatments	Doses (mg/day)	SLEDAI-2K Scores	Clinical Features	Flow Cytometric Analysis	Serum Cytokine Levels Detection		Quantitative Real-time RT-PCR Analysis	
									Serum IL-17A	Serum IL-23	Day0 *β-actin* Ct	Day3 *β-actin* Ct
SLE24	active	15	F	PRD	30	11	PU, AL, PL, FE, TC, LP	✓	✓	✓	32.00	23.63
SLE25	active	43	F	PRD, AZT	5, 50	8	VA	✓	✓	✓	31.32	22.92
SLE26	active	27	F	PRD, MMF	5, 500	13	HE, PU, AL, MU, LP	✓	×	×	30.80	23.00
SLE27	active	45	F	PRD	20	12	HE, PU, PI	✓	✓	✓	34.92	23.04
SLE28	active	21	F	PRD, AZT	20, 25	16	VA, PU, MU, LC	✓	✓	✓	24.73	22.91
SLE29	active	28	F	PRD	15	6	PU, LC	✓	✓	✓	27.19	20.44

Table 9. Characteristics of Individual Subjects that were Recruited for Flow Cytometric Analysis, Serum Cytokine Detection by ELISA and Semi-Quantitative Real-time RT-PCR Analysis. Twenty-nine SLE patients (13 active and 16 inactive SLE patients) and ten normal subjects were recruited for this study. F, female; M, male; PRD, prednisolone; AZT, azathioprine; MMF, mycophenolate mofetil; CPM, cyclophosphamide; SZ, seizure; VA, vasculitis; AR, arthritis; MY, myositis; UC, urinary casts; HE, hematuria; PU, proteinuria; PI, pyuria; RA, rash; AL, alopecia; MU, mucosal ulcers; PL, pleurisy; PE, pericarditis; LC, low complement; IDB, Increased DNA binding; FE, fever; TC, thrombocytopenia; LP, leucopenia; ✓, subjects were recruited; ×, subjects were excluded. Note that we included only the samples that the *β-actin* (internal control) threshold cycle (C_t) values of less than 30 (both on day 0 and day 3) for semi-quantitative real-time RT-PCR analysis.

No. Samples	Stage	Age (years)	Sex	Treatments	Doses (mg/day)	SLEDAI-2K Scores	Clinical Features	No. Samples	Stage	Age (years)	Sex	Treatments	Doses (mg/day)	SLEDAI-2K Scores	Clinical Features
Normal A	normal	25	F	-	-	-	-	SLE G	inactive	32	F	PRD	2.5	4	PU
Normal B	normal	28	F	-	-	-	-	SLE H	inactive	ND	F	PRD	2.5	5	PU
Normal C	normal	24	F	-	-	-	-	SLE I	inactive	33	F	PRD, MMF	1.25, 1000	4	PU
Normal D	normal	24	F	-	-	-	-	SLE J	inactive	43	F	-	-	4	PU
Normal E	normal	24	F	-	-	-	-	SLE K	inactive	35	F	PRD	3.75	4	PU
Normal F	normal	24	M	-	-	-	-	SLE L	active	31	F	PRD, MMF	5, 1500	12	HE, PU, PI
Normal G	normal	24	F	-	-	-	-	SLE M	active	34	M	PRD, CPM	25, 1000	16	UC, HE, PU, PI
Normal H	normal	24	F	-	-	-	-	SLE N	active	37	M	-	-	12	UC, PU, PI
Normal I	normal	24	F	-	-	-	-	SLE O	active	ND	F	PRD	10	12	HE, PU, PI
Normal J	normal	33	M	-	-	-	-	SLE P	active	ND	F	PRD, MMF	1.25, 1500	8	PU, PI
SLE A	inactive	42	F	PRD, MMF	1.25, 500	4	PI	SLE Q	active	ND	F	PRD	3.75	8	HE, PU
SLE B	inactive	25	F	PRD	2.5	4	PU	SLE R	active	ND	F	PRD	10	8	HE, PU
SLE C	inactive	39	F	PRD	2.5	0	-	SLE S	active	45	F	PRD	1.25	12	HE, PU, PI
SLE D	inactive	ND	F	PRD	1.25	4	PI	SLE T	active	48	F	PRD, AZT	1.25, 100	8	UC, HE
SLE E	inactive	17	F	PRD, AZT, MMF	2.5, 75, 8	4	PU	SLE U	active	24	F	PRD	10	12	UC, PU, PI
SLE F	inactive	41	F	PRD, MMF	3.75, 1500	4	HE	SLE V	active	37	F	PRD	15	12	UC, HE, PU

Table 10. Characteristics of Individual Subjects that were Recruited for Plasma Cytokine Detection by ELISA. Twenty-two SLE patients (11 active SLE patients and 11 inactive SLE patients) and ten normal subjects were recruited for this study. F, female; M, male; PRD, prednisolone; AZT, azathioprine; MMF, mycophenolate mofetil; CPM, cyclophosphamide; SZ, seizure; VA, vasculitis; AR, arthritis; MY, myositis; UC, urinary casts; HE, hematuria; PU, proteinuria; PI, pyuria; RA, rash; AL, alopecia; MU, mucosal ulcers; PL, pleurisy; PE, pericarditis; LC, low complement; IDB, Increased DNA binding; FE, fever; TC, thrombocytopenia; LP, leucopenia; ND, no data.

2. The Percentages of IL-23R$^+$CD4$^+$ T cells, IL-23R$^+$CD8$^+$ T cells, IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$T cells in PBMC from SLE Patients and Normal Subjects

T cells abnormalities have been reported in the pathogenesis of SLE. The exact T cells subpopulations that set off inflammation in SLE, however, are not known. For that reason, twenty-nine SLE patients (13 active and 16 inactive SLE patients) and ten normal subjects were recruited for this study. Because IL-23 has been shown to play an important role in the phenotypic maintenance and proliferation of IL-17 producing T cells, and possibly in their differentiation (at least in human), we therefore examined the expression of IL-23R on CD4$^+$and CD8$^+$ T cells. Using flow cytometry, we observed a higher percentages of IL-23R$^+$CD4$^+$ T cells (Figure 5A) and IL23R$^+$CD8$^+$T cells (Figure 6A) in PBMCs from SLE patients (the inactive group, the active group, total SLE patients combining inactive and active groups), in comparison with those from normal subjects both on recently isolated PBMCs (day 0) (Figure 5A; left and 6A; left), and in PBMCs cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3) (Figure 5A; right and 6A; right). The increase in percentages was statistically significant (p<0.05 by Mann–Whitney U-test for all comparisons). There was, however, no difference between the active and the inactive groups. Representative flow cytometric profiles of SLE patients and normal subjects were shown in Figure 5B and 6B, respectively. This result suggested that increased IL-23R$^+$ T cells may be one of the features of SLE patients.

Next, since IL-17A has been shown to play an important role in many autoimmune and inflammatory diseases, we analyzed the production of IL-17A from T cells by the same technique. When we studied freshly isolated cells (day 0), the percentages of IL-17$^+$CD4$^+$ T cells in normal subjects (Figure 7A: left) were significantly higher than those of the inactive and the total SLE patients (p = 0.0219 and p = 0.0197, respectively), whereas there was no difference between the normal and the active group (p = 0.0772). The percentages of IL-17$^+$CD8$^+$ T cells (Figure 8A: left) showed no

differences in all groups (p = 0.2800, p = 0.7802 and p =0.4122 for the inactive, the active and the total SLE groups in comparison with normal subjects, respectively). In cells cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3), we observed a significantly higher percentages of IL-17$^+$CD8$^+$ T cells in SLE than in normal subjects (p<0.05 for all comparisons) (Figure 8A; right). We also found a higher percentages of IL-17$^+$CD4$^+$ T cells in SLE patients compared with normal subjects, but this difference did not reach statistical significance (p>0.05) (Figure 7A; right). Furthermore, there was no difference between the active and the inactive group. Representative flow cytometric profiles for SLE patients and normal subjects were shown in Figure 7B and 8B, for the IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$ T cells, respectively.

In addition, we compared the percentages of IL-17$^+$ T cells at day 3 vs. at day 0 by dividing those from day 3 by day 0 from individual sample and the results were presented as the fold increase in IL-17$^+$ T cells. These analyses revealed the fold increase in the percentage of IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$ T cells from SLE patients (the inactive group, the active group, total SLE patients combining inactive and active groups) were significantly higher than those of the normal subjects (p<0.05 for all comparisons) (Figure 9A and 9B, respectively).

Interestingly, we observed positive relationships between the percentages of IL-23R$^+$CD4$^+$ T cells and those of IL-17$^+$CD4$^+$ T cells on day 3 in the active and the total SLE group (r = 0.7692, p = 0.0021 and r = 0.5601, p = 0.0016, respectively) (Figure 10A and 10C, respectively). Moreover, a significant positive relationships between the percentages of IL-23R$^+$CD8$^+$ T cells and the percentages of IL-17$^+$CD8$^+$ T cells on day 3 was also found in the active and the total SLE groups (r = 0.5714, p = 0.0413 and r = 0.4833, p = 0.0079, respectively) (Figure 10B and 10D, respectively).

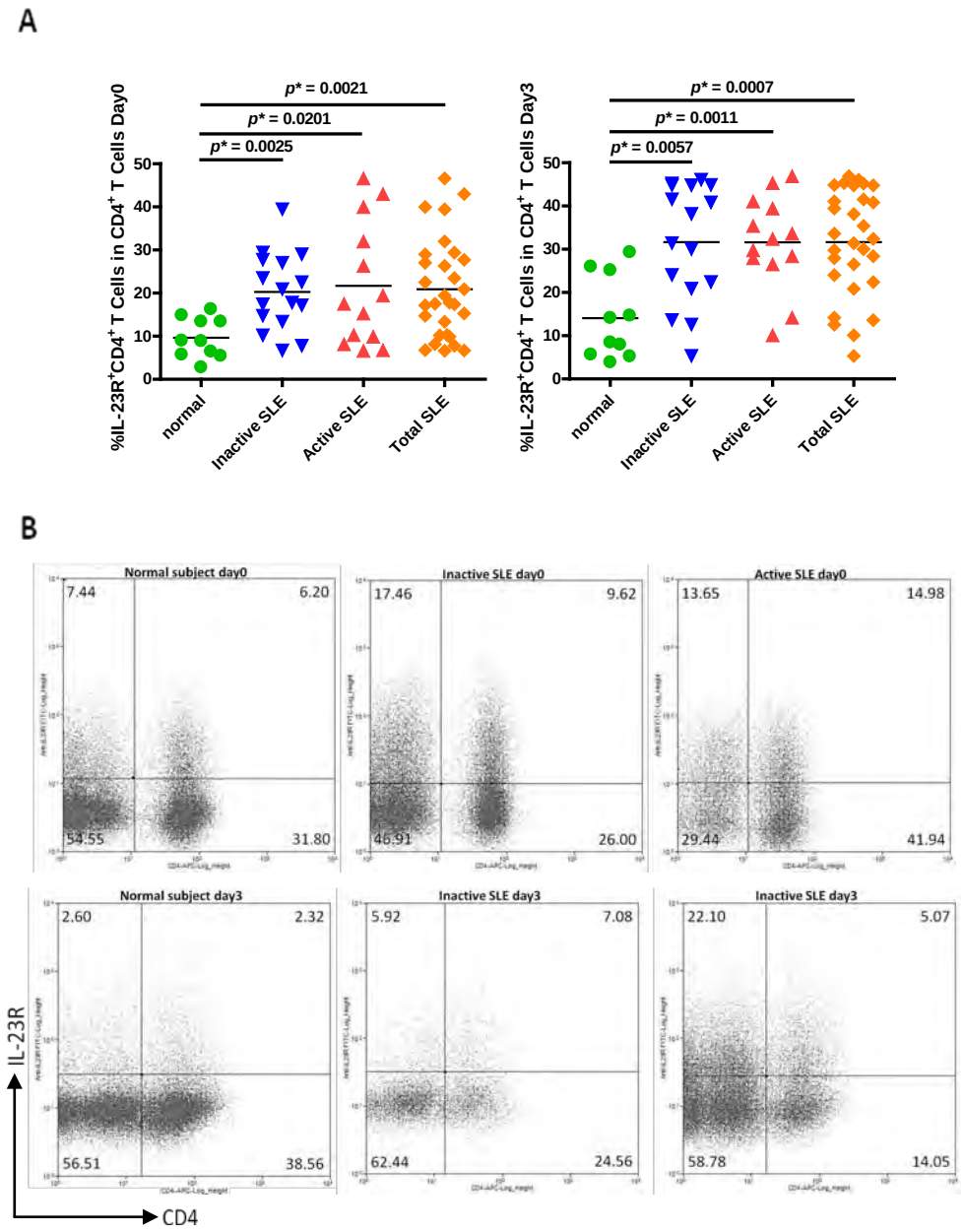


Figure 5. **The Percentages of IL-23R$^+$CD4$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects.** Percentages of IL-23R$^+$CD4$^+$ T cells (A) in PBMCs from the inactive, the active SLE patients and normal subjects on day 0 (A:left) and day 3 (A:right). The horizontal bars show the means values. The percentages of IL-23R$^+$CD4$^+$ T cells were calculated from total CD4$^+$ T cell after lymphocyte gating. Representative flow cytometric profiles of IL-23R$^+$CD4$^+$ T cells (B) in PBMCs from the inactive (day 0: upper middle row, day 3: lower middle row), the active (day 0: upper right, day 3: lower right) and normal subjects (day 0: upper left, day 3: lower left).

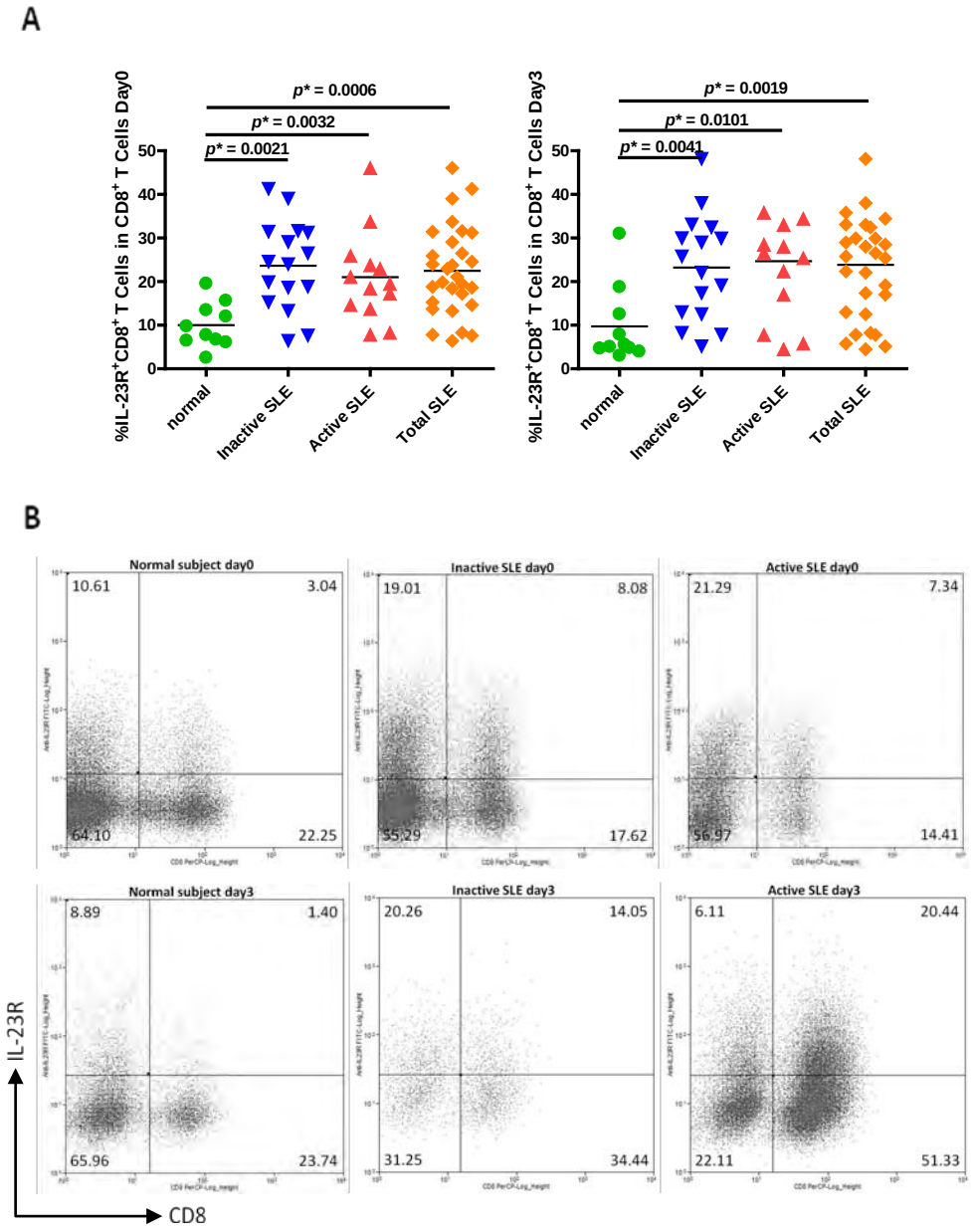


Figure 6. The Percentages of IL-23R^{+}CD8^{+} T Cells in PBMCs from SLE Patients and Normal Subjects. Percentages of IL-23R^{+}CD8^{+} T cells (A) in PBMCs from the inactive, the active SLE patients and normal subjects on day 0 (A: left) and day 3(A: right). The horizontal bars show the means values. The percentages of IL-23R^{+}CD8^{+} T cells were calculated from total CD8^{+} T cell after lymphocyte gating. Representative flow cytometric profiles of IL-23R^{+}CD8^{+} T cells (B) in PBMCs from the inactive (day 0: upper middle row, day3: lower middle row), the active (day 0: upper right, day 3: lower right) and normal subjects (day 0: upper left, day 3: lower left).

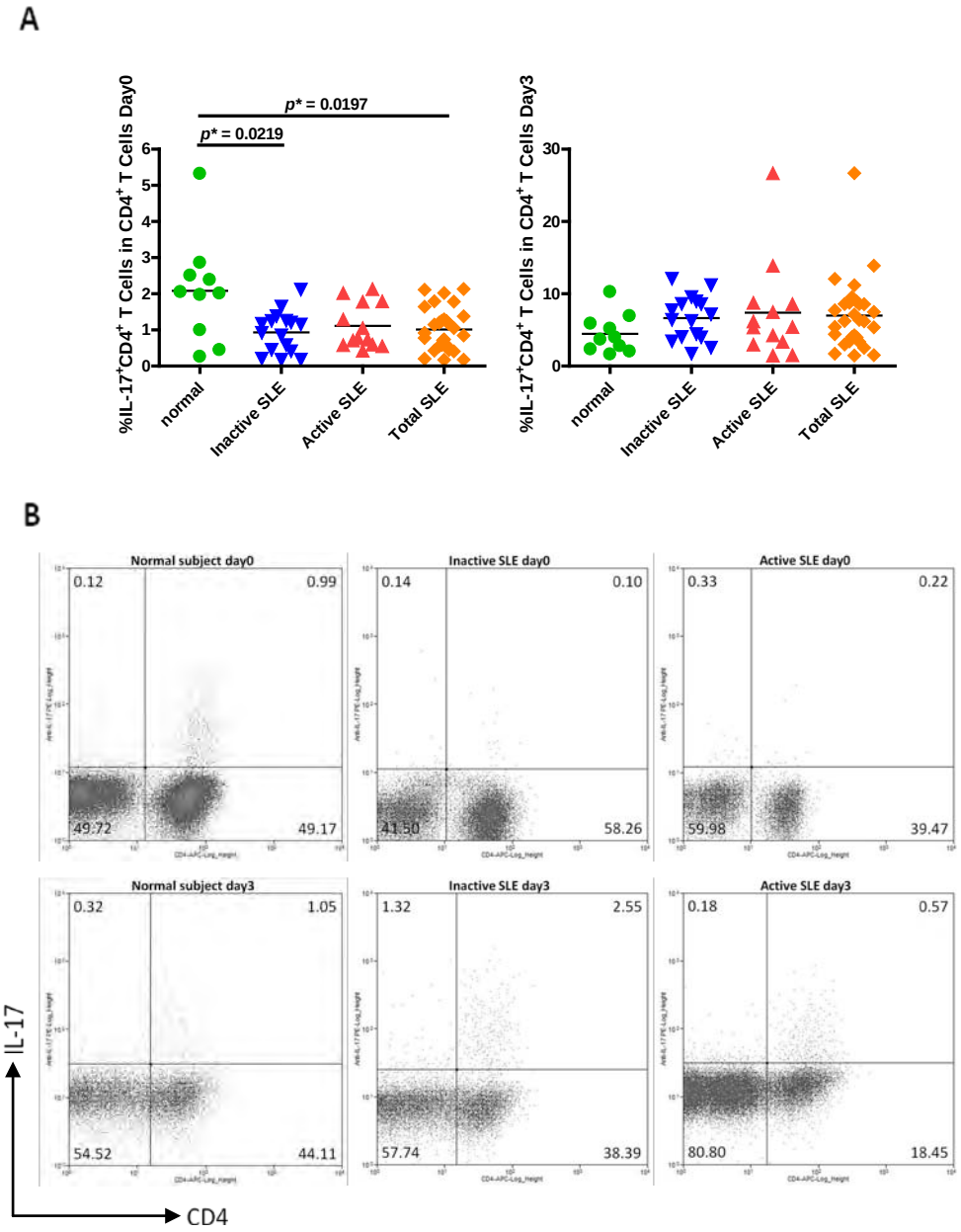


Figure 7. The Percentages of IL-17^{+}CD4^{+} T Cells in PBMCs from SLE Patients and Normal Subjects. Percentages of IL-17^{+}CD4^{+} T cells (A) in PBMCs from the inactive, the active SLE patients and normal subjects on day 0 (A: left) and day 3(A: right). The horizontal bars show the means values. The percentages of IL-17^{+}CD4^{+} T cells were calculated from total CD4^{+} T cell after lymphocyte gating. Representative flow cytometric profiles of IL-17^{+}CD4^{+} T cells (B) in PBMCs from the inactive (day 0: upper middle row, day 3: lower middle row), the active (day 0: upper right, day 3: lower right) and normal subjects (day 0: upper left, day 3: lower left).

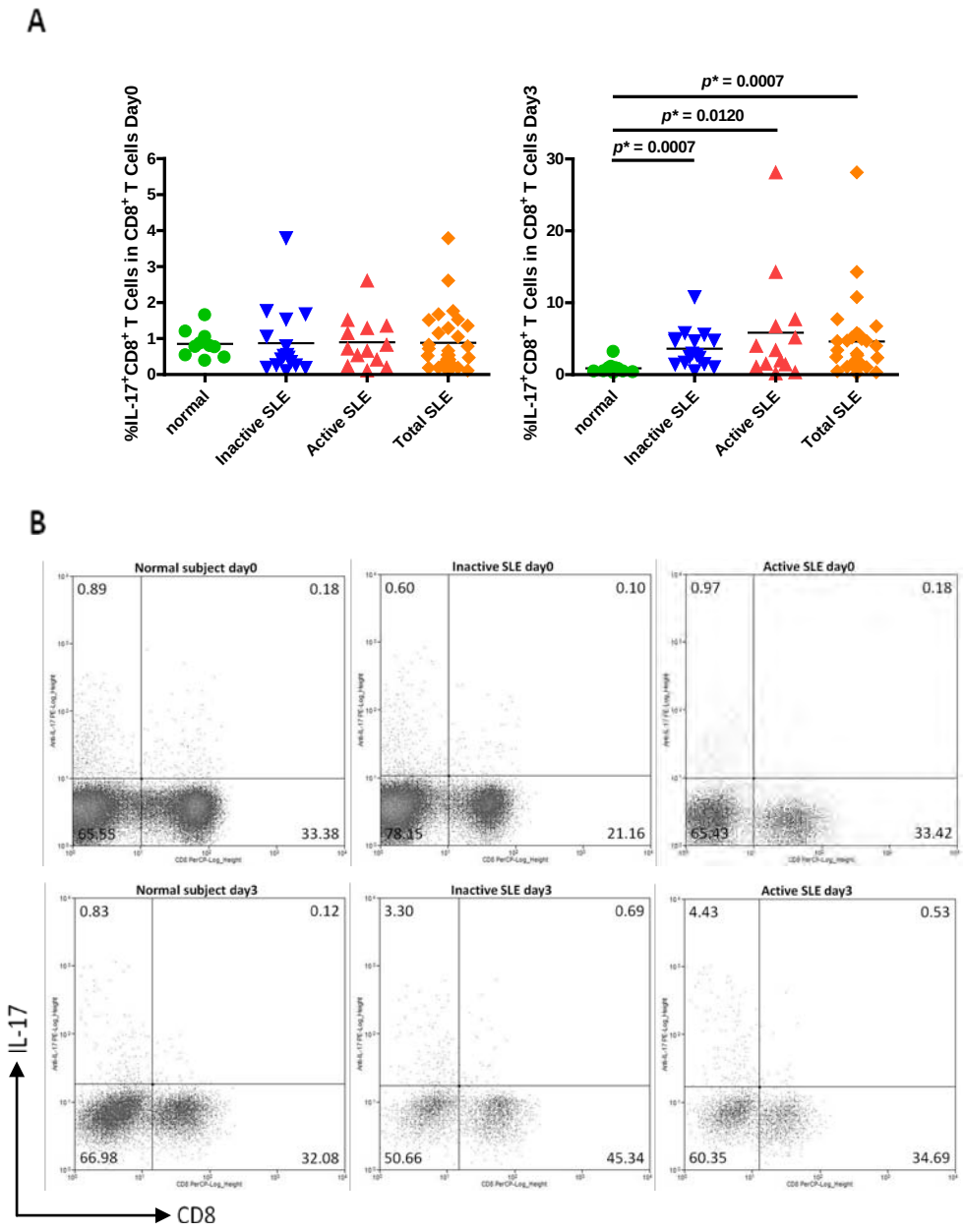


Figure 8. **The Percentages of IL-17$^+$CD8$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects.** Percentages of IL-17$^+$CD8$^+$ T cells (A) in PBMCs from the inactive, the active SLE patients and normal subjects on day 0 (A: left) and day 3(A: right). The horizontal bars show the means values. The percentages of IL-17$^+$CD8$^+$ T cells were calculated from total CD8$^+$ T cell after lymphocyte gating. Representative flow cytometric profiles of IL-17$^+$CD8$^+$ T cells (B) in PBMCs from the inactive (day 0: upper middle row, day 3: lower middle row), the active (day 0: upper right, day 3: lower right) and normal subjects (day 0: upper left, day 3: lower left).

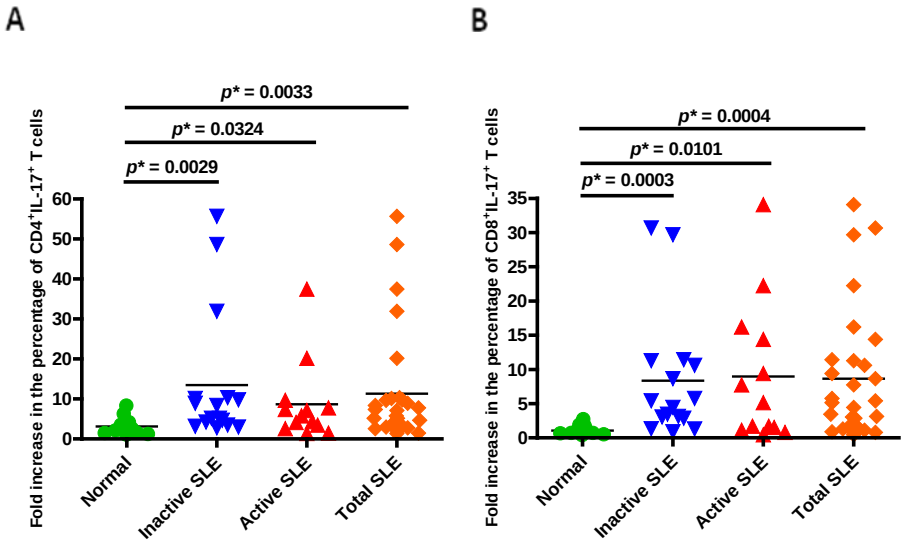


Figure 9. **Fold Increase in the Percentage of IL-17^{+}CD4^{+} T cells and IL-17^{+}CD8^{+} T cells from SLE Patients and Normal Subjects.** The percentages of IL-17^{+} T cells at day 3 was divided by day 0 from individual sample and the results were presented as the fold increase. (A) The fold increase in the percentage of IL-17^{+}CD4^{+} T cells in PBMCs from the inactive, the active SLE patients and normal subjects. (B) The fold increase in the percentage of IL-17^{+}CD8^{+} T cells in PBMCs from the inactive, the active SLE patients and normal subjects. The horizontal bars show the means values. Mann–Whitney U-test was used to analyze these data.

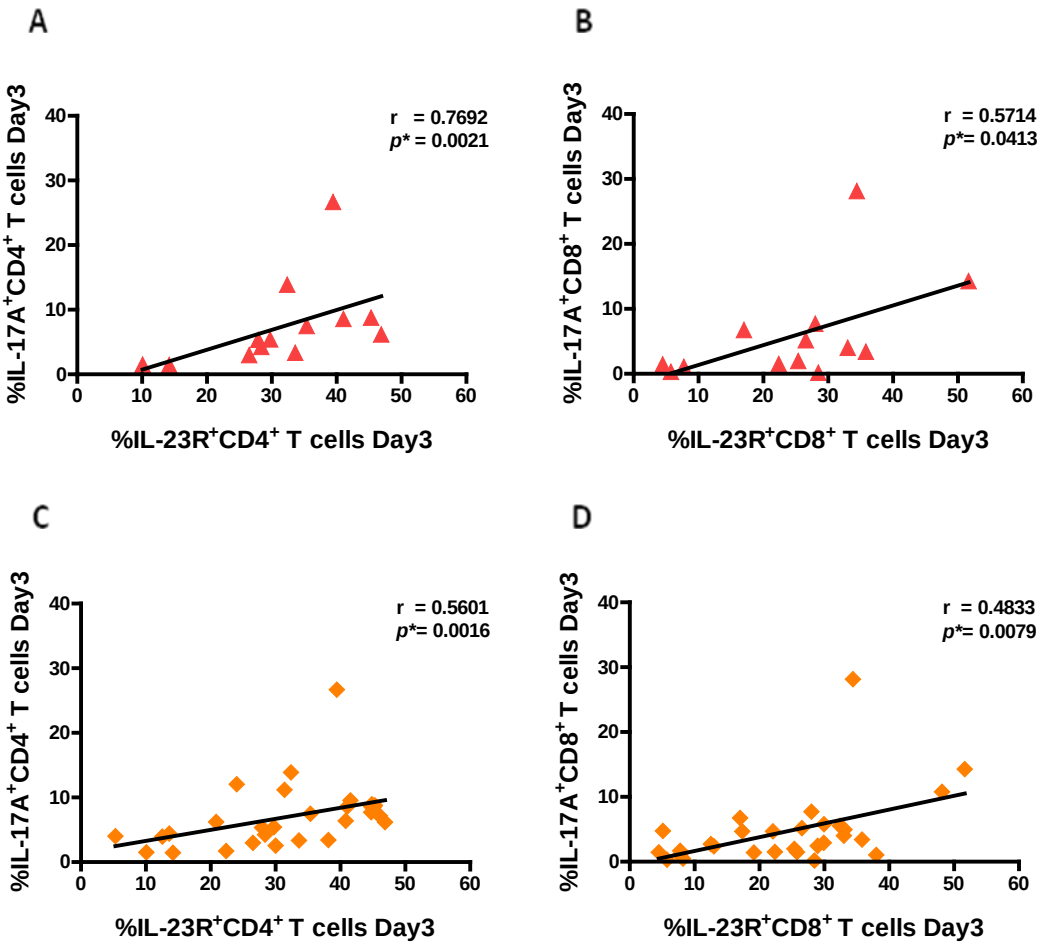


Figure 10. Correlations between the Percentages of IL-23R$^+$ T Cells and the Percentages of IL-17$^+$ T Cells. (A, C) A relationships between the percentages of IL-23R$^+$CD4$^+$ T cells and the percentages of IL-17$^+$CD4$^+$ T cells on day 3 in the active (A) and the total SLE (C) group (r = 0.7692, p = 0.0021 and r = 0.5601, p = 0.0016, respectively), (B, D) A relationships between the percentages of IL-23R$^+$CD8$^+$ T cells and the percentages of IL-17$^+$CD8$^+$ T cells on day 3 was also found in the active (B) and the total SLE (D) group (r = 0.5714, p = 0.0413 and r = 0.4833, p = 0.0079, respectively). Spearman's correlation test was used to analyze these data.

3. Detection of IL-17A and IL-23 Levels in Serum and Plasma from SLE Patients and the Normal Subjects

An important role of IL-17A in autoimmunity has been supported by many studies and IL-23 is critical for differentiation and maintaining the phenotype of IL-17 producing T cells. Since increased percentages of $CD4^+$ and $CD8^+$ T cells with IL-23R and IL-17A were detected in SLE patients, we wonder whether IL-17A and IL-23 were higher in sera from SLE patients. For detection of IL-17A and IL-23 levels in serum, sera from twenty-eight SLE patients (12 active and 16 inactive SLE patients) and nine normal subjects were assayed by ELISA. In this experiment, serum IL-17A level was only detectable in two active SLE patients (5.12 and 6.78 pg/ml, respectively) and was undetectable in the sera of all the normal subjects and other SLE patients (Figure 11). The sensitivity of the ELISA test kit for IL-17A and IL-23 used in this study are 4 and 15 pg/ml, respectively. The serum IL-23 level was undetectable in all subjects (Figure 12). Since previous studies [71, 73] reported increased IL-17A in the plasma of SLE patients, we further investigated whether we could detect IL-17A using plasma samples. We newly recruited subjects of twenty-two SLE patients (11 active and 11 inactive SLE patients) and ten normal subjects (Table 11). Plasma IL-17A and IL-23 levels were similarly examined by ELISA. For IL-17A detection in plasma, the level was detectable in only one active and one inactive SLE patients (5.75 and 8.50 pg/ml, respectively) but it was undetectable in all normal subjects and other SLE patients (Figure 13). For IL-23 detection in plasma, all samples yielded no detectable results (Figure 14).

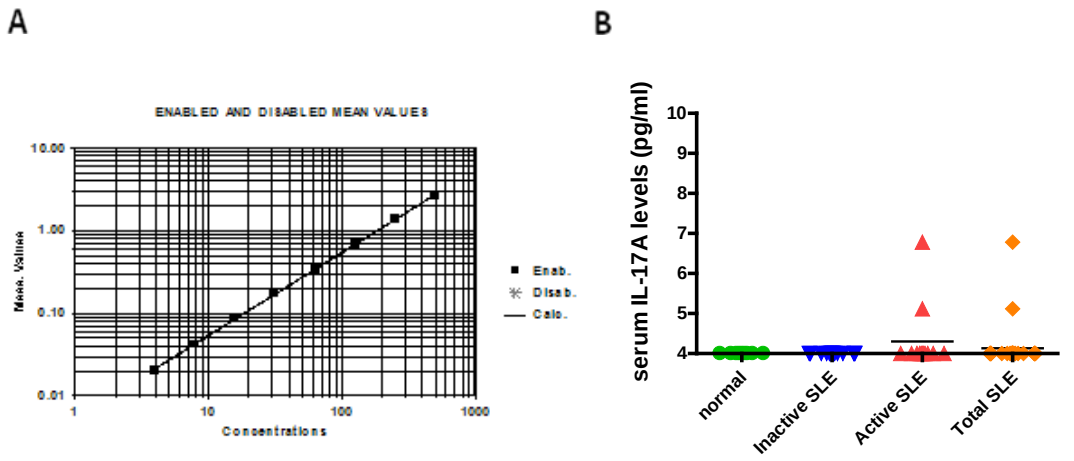


Figure 11. **Detection of Serum IL-17A Levels in SLE Patients and Normal Subjects by ELISA.** (A) Standard curve of human IL-17A test kit used in this study; the standard curve range: 4-500 pg/ml. The minimum significant detection level of the assay was 4pg/ml. (B) Summarized ELISA results of serum IL-17A are shown. The serum IL-17A level was only detectable in two active SLE patients (5.12 and 6.78 pg/ml, respectively)

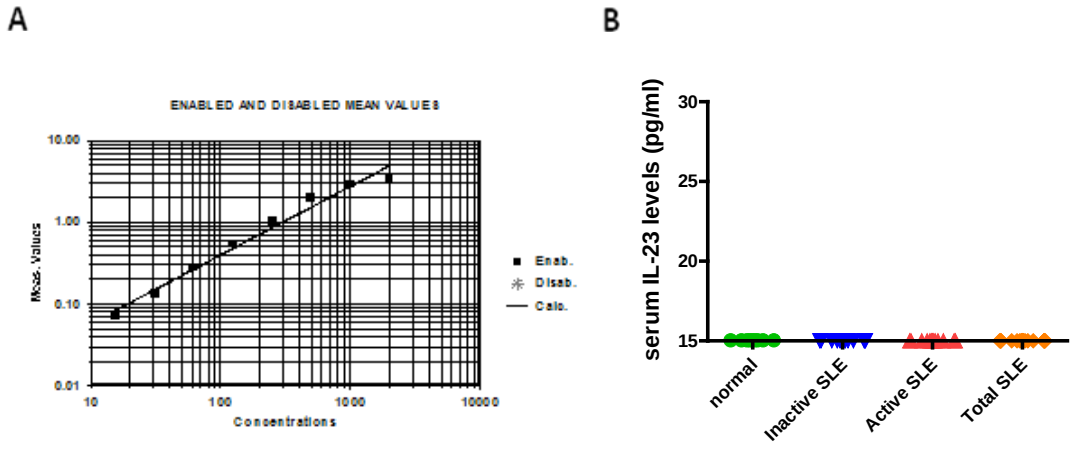


Figure 12. **Detection of Serum IL-23 levels in SLE Patients and Normal Subjects by ELISA.** (A) Standard curve of human IL-23 ELISA test kit used in this study; the standard curve range: 15-2000 pg/ml. The minimum significant detection level of the assay was 15 pg/ml. (B) Summarized ELISA results of serum IL-23 are shown. The serum IL-23 level was undetectable in all subjects.

No. Samples	Stage	Treatments	Doses (mg/day)	SLEDAI-2K scores	Clinical Features	Plasma Cytokine Levels (pg/ml)		No. Samples	Stage	Treatments	Doses (mg/day)	SLEDAI-2K scores	Clinical Features	Plasma Cytokine Levels (pg/ml)	
						IL-17A	IL-23							IL-17A	IL-23
Normal A	normal	-	-	-	-	UD	UD	SLE G	inactive	PRD	2.5	4	PU	UD	UD
Normal B	normal	-	-	-	-	UD	UD	SLE H	inactive	PRD	2.5	5	PU	UD	UD
Normal C	normal	-	-	-	-	UD	UD	SLE I	inactive	PRD, MMF	1.25, 1000	4	PU	UD	UD
Normal D	normal	-	-	-	-	UD	UD	SLE J	inactive	-	-	4	PU	UD	UD
Normal E	normal	-	-	-	-	UD	UD	SLE K	inactive	PRD	3.75	4	PU	UD	UD
Normal F	normal	-	-	-	-	UD	UD	SLE L	active	PRD, MMF	5, 1500	12	HE, PU, PI	UD	UD
Normal G	normal	-	-	-	-	UD	UD	SLE M	active	PRD, CPM	25, 1000	16	UC, HE, PU, PI	UD	UD
Normal H	normal	-	-	-	-	UD	UD	SLE N	active	-	-	12	UC, PU, PI	UD	UD
Normal I	normal	-	-	-	-	UD	UD	SLE O	active	PRD	10	12	HE, PU, PI	5.75	UD
Normal J	normal	-	-	-	-	UD	UD	SLE P	active	PRD, MMF	1.25, 1500	8	PU, PI	UD	UD
SLE A	inactive	PRD, MMF	1.25, 500	4	PI	UD	UD	SLE Q	active	PRD	3.75	8	HE, PU	UD	UD
SLE B	inactive	PRD	2.5	4	PU	UD	UD	SLE R	active	PRD	10	8	HE, PU	UD	UD
SLE C	inactive	PRD	2.5	0	-	6.50	UD	SLE S	active	PRD	1.25	12	HE, PU, PI	UD	UD
SLE D	inactive	PRD	1.25	4	PI	UD	UD	SLE T	active	PRD, AZT	1.25, 100	8	UC, HE	UD	UD
SLE E	inactive	PRD, AZT, MMF	2.5, 75, 8	4	PU	UD	UD	SLE U	active	PRD	10	12	UC, PU, PI	UD	UD
SLE F	inactive	PRD, MMF	3.75, 1500	4	HE	UD	UD	SLE V	active	PRD	15	12	UC, HE, PU	UD	UD

Table 11. Individual Characteristics and Summary of the Results of Samples that were Recruited for Plasma Cytokine Detection by ELISA.

Twenty-two SLE patients (11 active patients and 11 inactive SLE patients) and ten normal subjects were recruited for this study. F, female; M, male; PRD, prednisolone; AZT, azathioprine; MMF, mycophenolate mofetil; CPM, cyclophosphamide; urinary casts; HE, hematuria; PU, proteinuria; PI, pyuria; UD, undetectable.

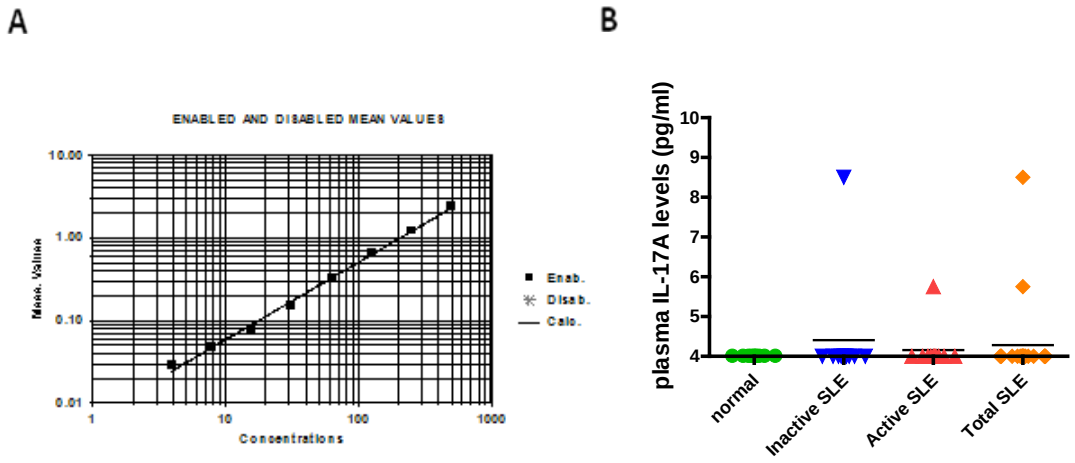


Figure 13. **Detection of Plasma IL-17A Levels in SLE Patients and Normal Subjects by ELISA.** (A) Standard curve of human IL-17A ELISA test kit used in this study; the standard curve range: 4-500 pg/ml. The minimum significant detection level of the assay was 4 pg/ml. (B) Summarized ELISA results of plasma IL-17A are shown. The plasma IL-17A level was detectable in one active and one inactive SLE patient (5.75 and 8.50, respectively).

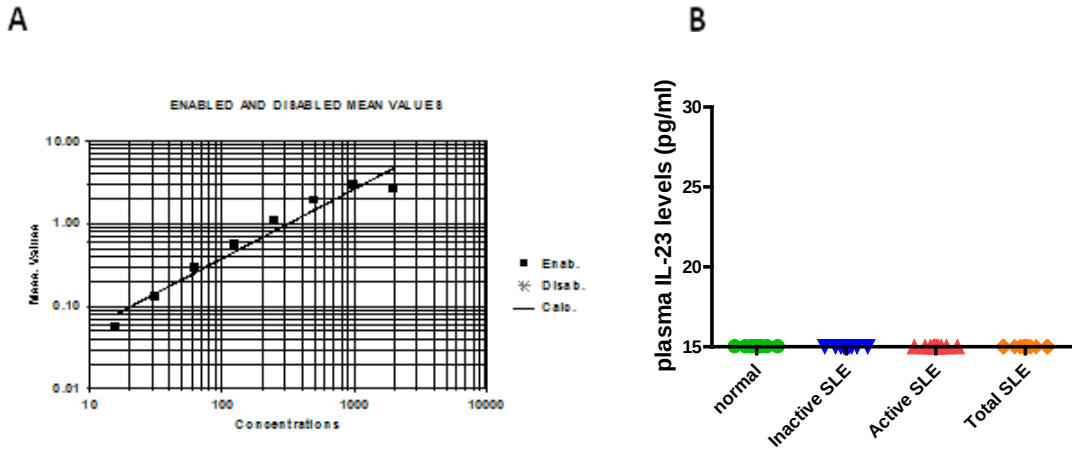


Figure14. **Detection of Plasma IL-23 Levels in SLE Patients and Normal Subjects by ELISA.** (A) Standard curve of human IL-23 ELISA test kit used in this study; the standard curve range : 15-2000 pg/ml. The minimum significant detection level of the assay was 15 pg/ml. (B) Summarized ELISA results for plasma IL-23 are shown. The plasma IL-23 level was undetectable in all subjects.

4. Semi-Quantitative Real-time RT-PCR Analysis for *IL-17A* in PBMCs from SLE Patients and Normal Subjects

IL-17A has been shown to play an important role in many autoimmune and inflammatory diseases. Semi-Quantitative real-time RT-PCR for *IL-17A* analysis using SYBR® Green was carried out to detect *IL-17A* transcripts. Nineteen SLE patients (7 active and 12 inactive SLE patients) and eight normal subjects were included. The level of *IL-17A* mRNA expression in freshly isolated PBMCs (day 0) was undetectable in all subjects (*IL-17A* threshold cycle (C_t) values was more than 45, data not shown)

Next, we investigated the level of *IL-17A* mRNA expression in PBMCs cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3) by using the same technique. The level of *IL-17A* mRNA expression in PBMCs at day 3 from the SLE patients were higher than those of the normal subjects, but did not reach statistical significance (p = 0.0826, p=0.7789 and p = 0.2883 for the inactive, the active and the total SLE patients, respectively, in comparison with normal subjects) (Figure 15).

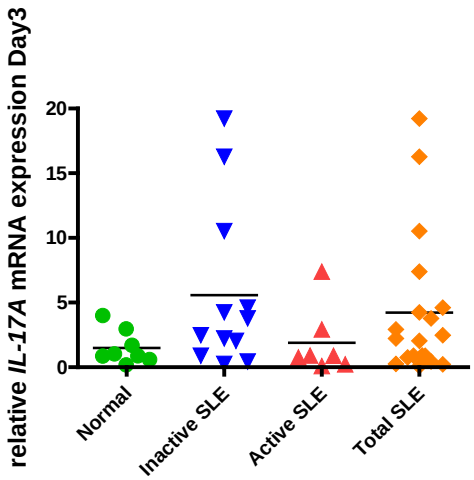


Figure 15. **Semi-Quantitative Real-time RT-PCR Analysis for *IL-17A* in PBMCs at Day 3 from SLE Patients and Normal Subjects.** The level of *IL-17A* mRNA expression at day 3. Each dot represented the level of *IL-17A* mRNA from each subject and the horizontal bars show the means values of the relative *IL-17A* expression level in SLE patients and normal subjects. Mann–Whitney U-test was used to analyze these data.

5. Semi-Quantitative Real-time RT-PCR Analysis for *RORC* in PBMCs from SLE Patients and Normal Subjects

An essential role of the orphan nuclear receptor RORγt/RORC (mice/humans) in controlling Th17 differentiation and IL-17 expression has been reported. Therefore, expression of mRNA encoding *RORC* in PBMC, both in freshly isolated PBMCs (day 0) and in PBMCs cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3), was investigated by semi-quantitative real-time RT-PCR using SYBR® Green. Nineteen SLE patients (7 active and 12 inactive SLE patients) and eight normal subjects were tested. The comparisons of *RORC* mRNA expression in PBMC among the inactive group, the active group, total SLE patients combining inactive and active groups, and normal subject are shown in Figure 16. The level of *RORC* mRNA expression in freshly isolated PBMCs (day 0) from total SLE patients groups was significantly lower when compared with those of the normal subjects (p=0.0003, p=0.0003 and p<0.0001 for the inactive, the active and the total SLE patients, respectively, in comparison with normal subjects) (Figure 16A). In PBMCs which were cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3), the level of *RORC* mRNA expression was significantly lower in the active (p=0.0093) and the total SLE (p=0.0316), when compared with those of the normal subjects (Figure 16B). However, the level of *RORC* mRNA expression day 3 in PBMCs from the inactive SLE group was also lower when compared with those of the normal subjects, but did not reach statistical significance (p>0.05) (Figure 16B).

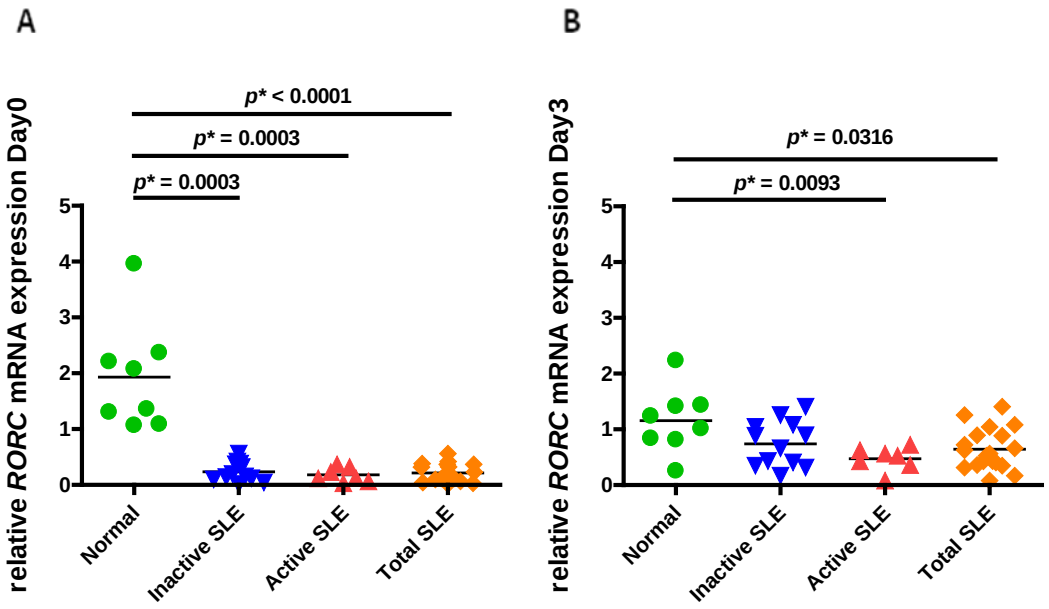


Figure 16. **Semi-Quantitative Real-time RT-PCR Analysis for *RORC* Expression in PBMCs at Day 0 and Day 3 from SLE Patients and Normal Subjects.** (A) The level of *RORC* mRNA expression at day 0 was summarized and each dot represented the *RORC* mRNA level of each subject. The horizontal bars show the mean values. (B) The level of *RORC* mRNA expression at day 3 was summarized and each dot represented the *RORC* mRNA level of each subject. The horizontal bars show the mean values. Mann–Whitney U-test was used to analyze these data.

6. Correlations of the Percentages of IL-23R$^+$CD4$^+$ T Cells, IL-23R$^+$CD8$^+$ T Cells, IL-17$^+$CD4$^+$T Cells and IL-17$^+$CD8$^+$T Cells with the Relative mRNA Expression of *IL-17A* and *RORC* in PBMCs from SLE Patients and Normal Subjects.

The results from flow cytometric analysis, serum cytokine detection by ELISA and quantitative real-time RT-PCR analysis were summarized in Table 12. The results from plasma cytokine detection by ELISA is shown in Table 11.

We analyzed the correlations of the percentages of IL-23R$^+$CD4$^+$ T cells, IL-23R$^+$CD8$^+$ T cells, IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$ T cells (from flow cytometric analysis) with the relative *IL-17A* mRNA expression and the relative *RORC* mRNA expression. As described above, we selected each sample that the threshold cycle (C_t) values of *β-actin* (internal control) were less than 30. For this reason, data from nineteen SLE patients (7 active and 12 inactive SLE patients) and eight normal subjects were analyzed in this study. The results from flow cytometric analysis in PBMCs on day 0 were paired with those from the semi-quantitative real-time RT-PCR analysis in PBMCs on day 0, and the similar pairing was done for the results of PBMCs at day 3. Spearman's correlation test was used to analyze these data.

We determined the correlation of the results from flow cytometric analysis with the results from semi-quantitative real-time RT-PCR analysis for *IL-17A* using Spearman's correlation analysis (Figure 17). As mentioned earlier, the level of *IL-17A* mRNA expression in freshly isolated PBMCs (day 0) was undetectable in all subjects (IL-17A threshold cycle (C_t) values was more than 45). Therefore, we determined this correlation from the results in only PBMCs on day 3. From the data sets from PBMCs on day 3, we found a significant positive correlations between the percentages of IL-23R$^+$CD4$^+$T cells and the relative *IL-17A* mRNA expression in PBMCs on day3 in the active and the total SLE patients ($r = 0.8571$, $p = 0.0238$ and $r = 0.6123$, $p = 0.0053$, respectively) (Figure17 A; ▲ = the active SLE patients and ◆ = the total SLE patients, respectively) Moreover, we found a significant positive correlations between the

percentages of IL-17^{+}CD4^{+}T cells and the relative *IL-17A* mRNA expression in PBMCs on day 3 in the normal subjects, the inactive and the total SLE patients (r = 0.8095, *p* = 0.0218; r = 0.6434, *p* = 0.0240 and r = 0.6298, *p* = 0.0039, respectively) (Figure 17C; ● = the normal subjects, ▼ = the inactive SLE patients and ◆ = the total SLE patients, respectively). In addition, we observed a significant positive correlations between the percentages of IL-17^{+}CD8^{+} T cells and the relative *IL-17A* mRNA expression in PBMCs on day 3 in the normal subjects (r = 0.8810, *p* = 0.0072) (Figure 17D; ●= the normal subjects).

The correlation between the results from flow cytometric analysis and the results from the semi-quantitative real-time RT-PCR analysis for *RORC* were shown in Figure 18. We did not find any correlation of the two pairs of data set from all subjects in PBMCs on day 0 (Figure 18; A-D). A significant positive correlations, however, was observed between the percentages of IL-23R^{+}CD4^{+} T cells and the relative *RORC* mRNA expression on day 3 in the active and the total SLE patients (r = 0.7857, *p* = 0.0480 and r = 0.5544, *p* = 0.0138, respectively) (Figure 18E; ▲ = the active SLE patients and ◆ = the total SLE patients, respectively). A significant positive correlations was also found between the percentages of IL-17^{+}CD4^{+} T cells and the relative *RORC* mRNA expression on day 3 in the inactive, the active and the total SLE group (r = 0.6853, *p* = 0.0139; r = 0.7857, *p* = 0.0480 and r = 0.6772, *p* = 0.0014, respectively) (Figure 18G; ▼ = the inactive SLE patients, ▲ = the active SLE patients and ◆ = the total SLE patients, respectively).

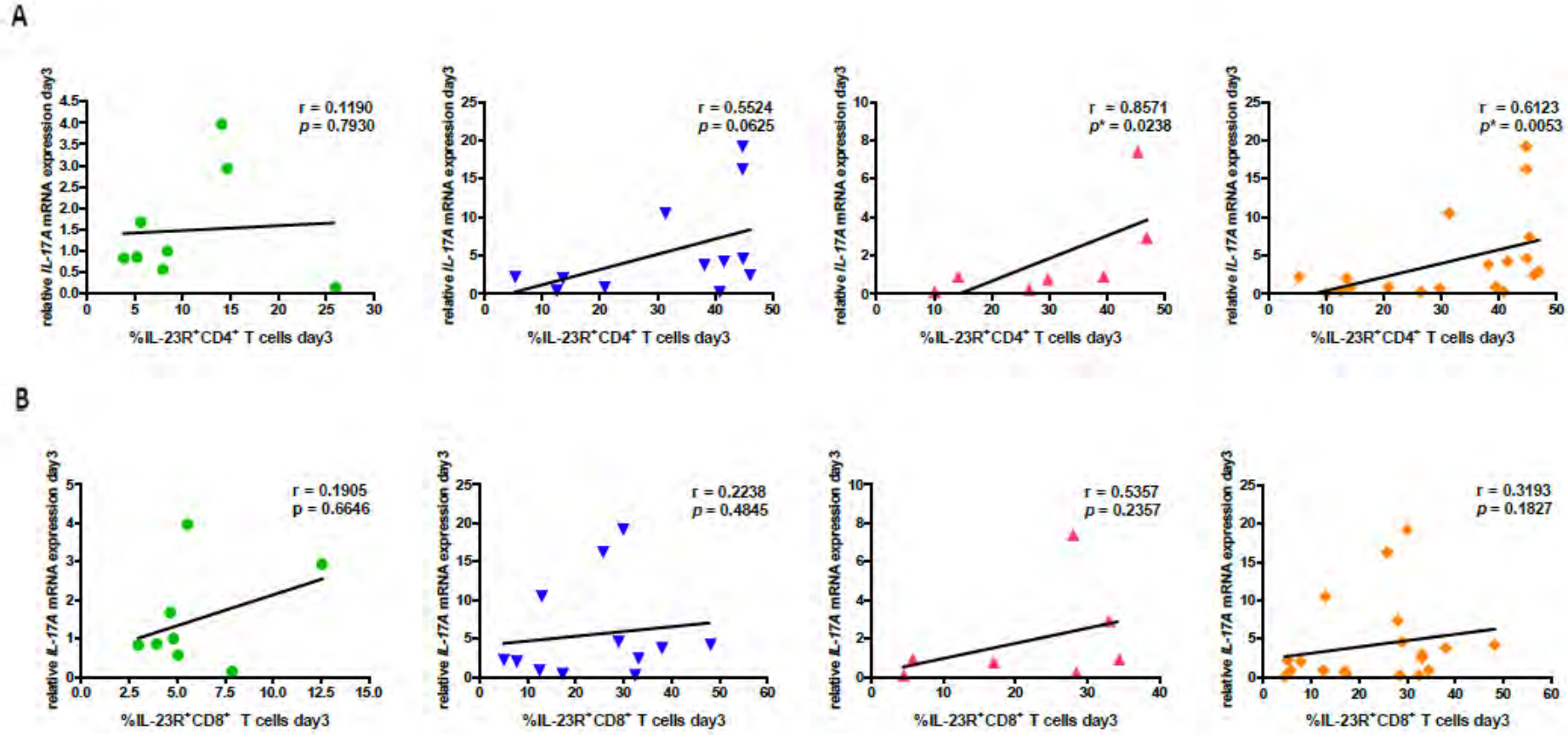
No. Samples	Stage	Flow Cytometric Analysis (% T cells)								Serum Cytokine Levels Detection (pg/ml)		Quantitative Real-time RT-PCR Analysis		
		Day0				Day3						IL-17A	RORC	
		IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-17A	IL-23	Day3	Day0	Day3
Normal1	normal	9.03	9.77	5.32	1.65	14.13	5.57	5.88	1.11	UD	UD	3.96	2.08	1.42
Normal2	normal	8.90	7.76	2.39	1.05	26.00	7.89	2.33	0.47	UD	UD	0.13	1.09	0.26
Normal3	normal	2.82	2.57	2.86	0.76	5.68	4.67	4.00	0.50	UD	UD	1.66	1.07	2.24
Normal4	normal	5.76	6.46	1.00	0.77	7.99	5.08	2.03	0.26	UD	UD	0.56	2.21	1.24
Normal5	normal	16.32	12.02	2.05	0.87	5.27	3.97	5.18	0.81	UD	UD	0.85	1.31	0.84
Normal6	normal	13.46	13.48	1.97	0.80	3.91	3.01	2.78	0.47	UD	UD	0.82	1.36	1.015
Normal7	normal	14.90	15.63	0.45	0.48	25.20	18.76	3.69	0.88	UD	UD	ND		
Normal8	normal	5.47	6.09	2.51	1.20	14.70	12.57	10.25	3.19	UD	UD	2.92	2.37	0.81
Normal9	normal	6.48	6.71	2.01	0.54	8.46	4.83	6.94	0.91	UD	UD	0.99	3.96	1.44
Normal10	normal	13.43	19.58	0.26	0.39	29.37	31.02	1.63	0.37	UD	UD	ND		
SLE1	inactive	6.67	6.40	1.20	0.47	20.84	12.52	6.23	2.72	UD	UD	0.89	0.32	0.35
SLE2	inactive	27.01	31.44	0.45	0.20	13.61	7.80	4.40	1.68	UD	UD	2.05	0.56	1.41
SLE3	inactive	27.73	41.27	0.20	0.19	40.81	32.42	6.38	5.55	UD	UD	0.25	0.10	0.43
SLE4	inactive	29.39	31.61	0.17	0.35	41.54	48.15	9.54	10.79	UD	UD	4.24	0.15	1.26
SLE5	inactive	17.41	24.56	1.15	0.42	5.33	5.13	4.00	4.74	UD	UD	2.23	0.14	0.31
SLE6	inactive	22.54	29.06	0.41	0.10	38.16	38.01	3.41	1.04	UD	UD	3.77	0.05	0.41

Table 12. (Continue)

No. Samples	Stage	Flow Cytometric Analysis (% T cells)								Serum Cytokine Levels Detection (pg/ml)		Quantitative Real-time RT-PCR Analysis		
		Day0				Day3						IL-17A	RORC	
		IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-17A	IL-23	Day3	Day0	Day3
SLE7	inactive	17.76	26.50	1.38	3.80	46.07	33.11	7.14	4.96	UD	UD	2.47	0.37	0.66
SLE8	inactive	39.46	39.00	0.58	0.55	22.38	8.23	1.71	0.49	UD	UD	ND		
SLE9	inactive	17.16	18.62	0.92	0.26	30.00	19.14	2.54	1.42	UD	UD	ND		
SLE10	inactive	14.73	13.27	0.18	0.24	45.20	29.91	8.59	2.93	UD	UD	ND		
SLE11	inactive	10.18	19.86	1.17	1.05	24.02	22.10	12.06	4.69	UD	UD	ND		
SLE12	inactive	23.47	18.83	1.64	0.52	44.76	25.77	7.71	1.50	UD	UD	16.28	0.19	0.90
SLE13	inactive	13.35	24.00	1.25	1.77	31.40	12.99	11.18	2.37	UD	UD	10.52	0.42	1.08
SLE14	inactive	28.97	31.22	0.85	1.68	44.76	29.95	8.57	5.78	UD	UD	19.23	0.12	0.88
SLE15	inactive	20.88	15.28	2.11	0.79	44.85	28.98	8.94	2.46	UD	UD	4.61	0.38	1.04
SLE16	inactive	7.763	7.61	1.25	1.50	12.54	17.36	3.96	4.67	UD	UD	0.43	0.02	0.17
SLE17	active	10.28	7.83	2.02	0.11	26.52	28.48	3.00	0.19	UD	UD	0.23	0.23	0.53
SLE18	active	39.97	18.45	1.05	0.72	14.17	5.75	1.45	0.33	UD	UD	0.90	0.37	0.43
SLE19	active	31.97	23.75	2.13	0.54	45.32	28.02	8.77	7.71	UD	UD	7.39	0.32	0.72
SLE20	active	42.98	46.08	0.71	0.83	39.45	34.43	26.70	28.15	UD	UD	0.90	0.12	0.63
SLE21	active	15.28	17.24	1.79	2.61	46.90	33.00	6.19	4.00	UD	UD	2.92	0.12	0.56
SLE22	active	46.59	25.89	0.78	0.23	33.61	26.56	3.36	5.17	UD	UD	ND		
SLE23	active	6.77	8.25	1.78	1.52	32.40	51.64	13.88	14.29	UD	UD	ND		
SLE24	active	8.14	14.63	0.55	1.36	27.92	7.75	5.35	1.09	6.78	UD	ND		

No. Samples	Stage	Flow Cytometric Analysis (% T cells)								Serum Cytokine Levels Detection (pg/ml)		Quantitative Real-time RT-PCR Analysis		
		Day0				Day3						IL-17A	RORC	
		IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-17A	IL-23	Day3	Day0	Day3
SLE25	active	9.89	19.49	0.43	0.19	41.05	22.37	8.58	1.50	UD	UD	ND		
SLE26	active	26.32	33.75	0.60	1.15	28.38	25.38	4.26	1.98	UD	UD	ND		
SLE27	active	17.50	22.94	1.29	0.66	35.39	35.82	7.49	3.41	5.12	UD	ND		
SLE28	active	19.45	21.06	0.74	0.42	29.76	17.02	5.42	6.74	UD	UD	0.76	0.06	0.36
SLE29	active	6.58	13.74	0.58	1.29	10.09	4.50	1.51	1.44	UD	UD	0.10	0.03	0.08

Table 12. **Summary of the Results of the Subjects that were Recruited for Flow Cytometric Analysis, Serum Cytokine Detection by ELISA and Semi-Quantitative Real-time RT-PCR Analysis.** Twenty-nine SLE patients (13 active and 16 inactive SLE patients) and ten normal subjects were recruited for flow cytometric analysis. For detection of IL-17A and IL-23 levels in serum, twenty-eight SLE patients (12 active and 16 inactive SLE patients) and nine normal subjects were assayed. Note that only the samples that the *β-actin* (internal control) threshold cycle (C_t) values of less than 30 was included for semi-quantitative real-time RT-PCR analysis. UD, undetectable; ND, no data.


A
relative IL-17A mRNA expression day3
r = 0.1190
p = 0.7930
%IL-23R+CD4+ T cells day3
r = 0.5524
p = 0.0625
%IL-23R+CD4+ T cells day3
r = 0.8571
p* = 0.0238
%IL-23R+CD4+ T cells day3
r = 0.6123
p* = 0.0053
%IL-23R+CD4+ T cells day3
B
relative IL-17A mRNA expression day3
r = 0.1905
p = 0.6646
%IL-23R+CD8+ T cells day3
r = 0.2238
p = 0.4845
%IL-23R+CD8+ T cells day3
r = 0.5357
p = 0.2357
%IL-23R+CD8+ T cells day3
r = 0.3193
p = 0.1827
%IL-23R+CD8+ T cells day3

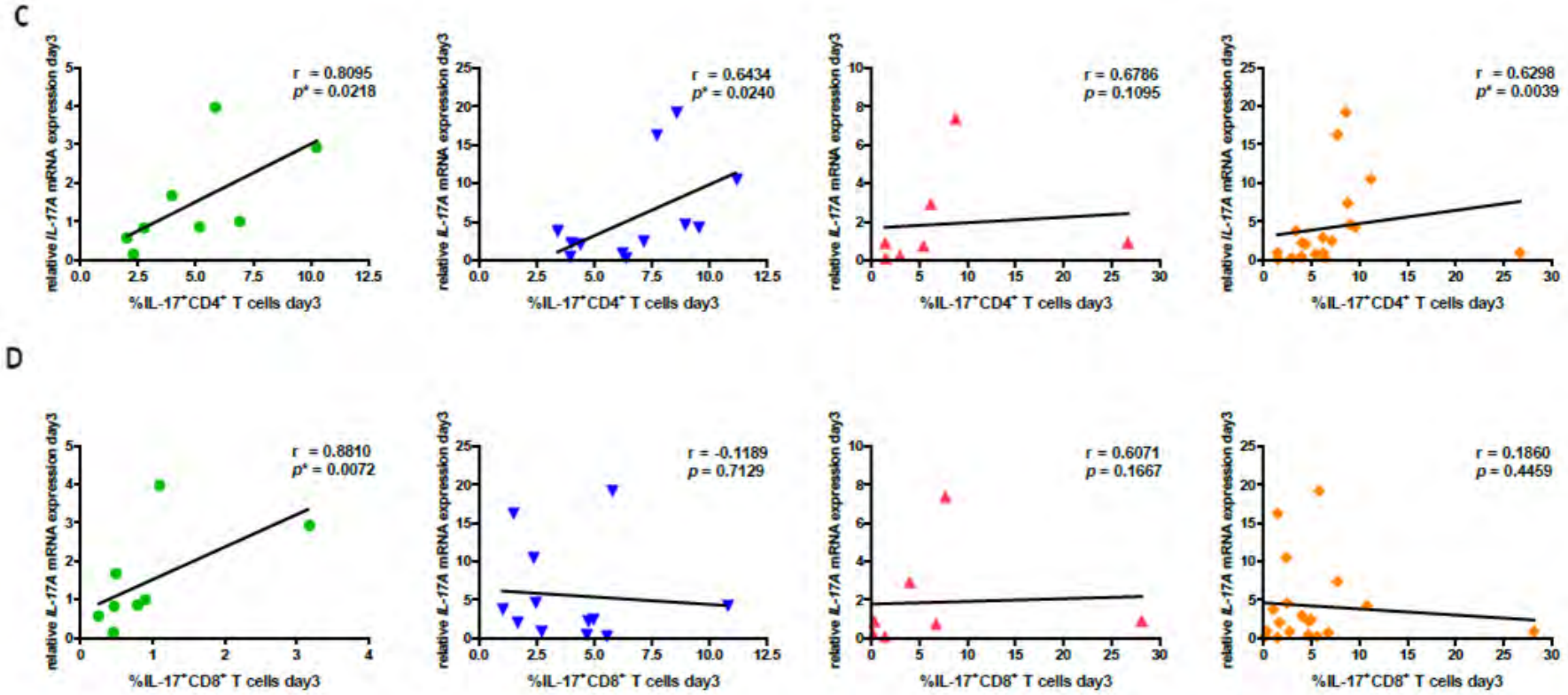


Figure 17. Correlation between Relative *IL-17A* Expression and the Results from Flow Cytometric Analysis. The relative *IL-17A* mRNA expression in PBMC at day 3 was plotted against the followings: (A) %IL-23R$^+$CD4$^+$T cells at day 3, (B) %IL-23R$^+$CD8$^+$T cells at day 3, (C) %IL-17$^+$CD4$^+$T cells at day 3 and (D) %IL-17$^+$CD8$^+$T cells at day 3. Spearman's correlation test was used to analyze these data. (● = the normal subjects, ▼ = the inactive SLE patients, ▲ = the active SLE patients and ◆ = the total SLE patients)

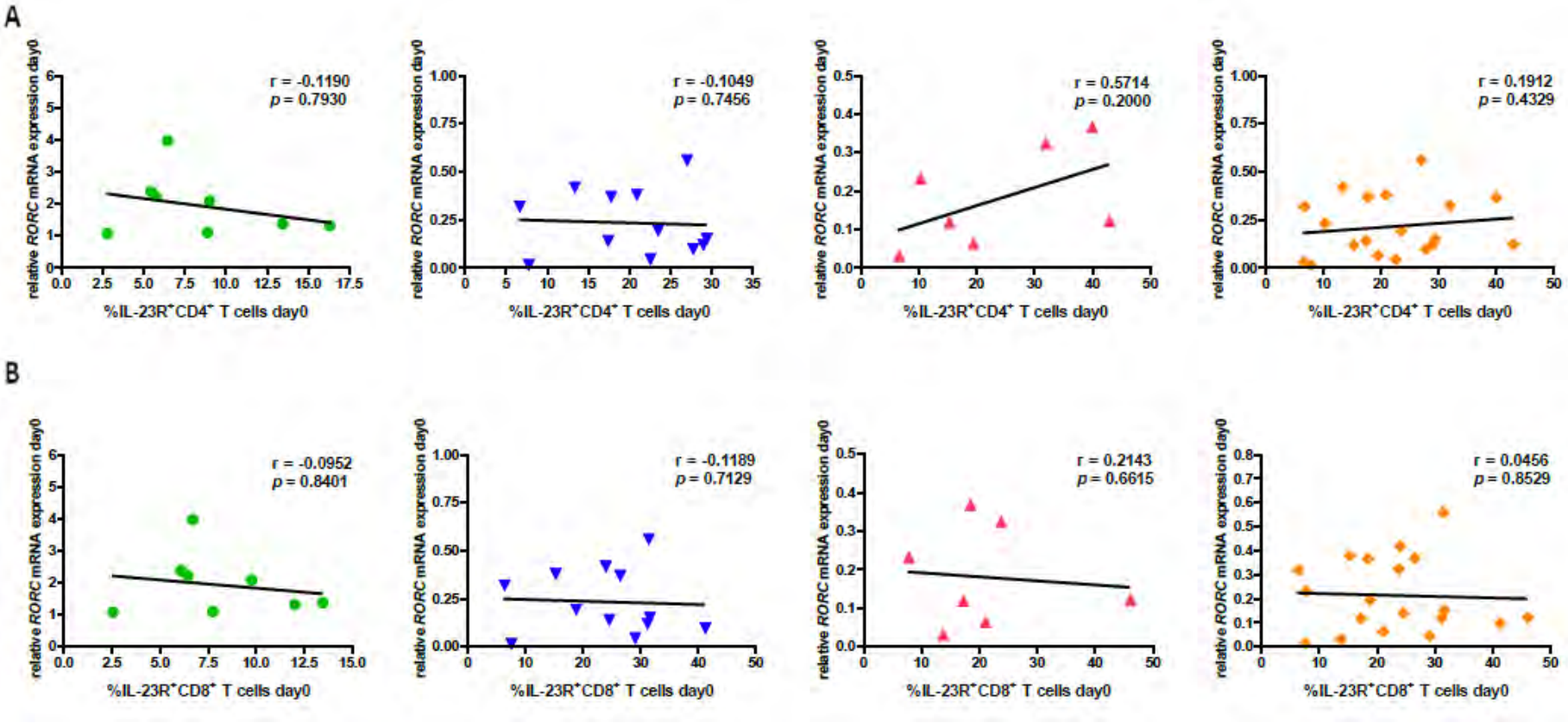

A
relative RORC mRNA expression day0
r = -0.1190
p = 0.7930
%IL-23R+CD4+ T cells day0
relative RORC mRNA expression day0
r = -0.1049
p = 0.7456
%IL-23R+CD4+ T cells day0
relative RORC mRNA expression day0
r = 0.5714
p = 0.2000
%IL-23R+CD4+ T cells day0
relative RORC mRNA expression day0
r = 0.1912
p = 0.4329
%IL-23R+CD4+ T cells day0
B
relative RORC mRNA expression day0
r = -0.0952
p = 0.8401
%IL-23R+CD8+ T cells day0
relative RORC mRNA expression day0
r = -0.1189
p = 0.7129
%IL-23R+CD8+ T cells day0
relative RORC mRNA expression day0
r = 0.2143
p = 0.6615
%IL-23R+CD8+ T cells day0
relative RORC mRNA expression day0
r = 0.0456
p = 0.8529
%IL-23R+CD8+ T cells day0

Figure 18 (Continue)

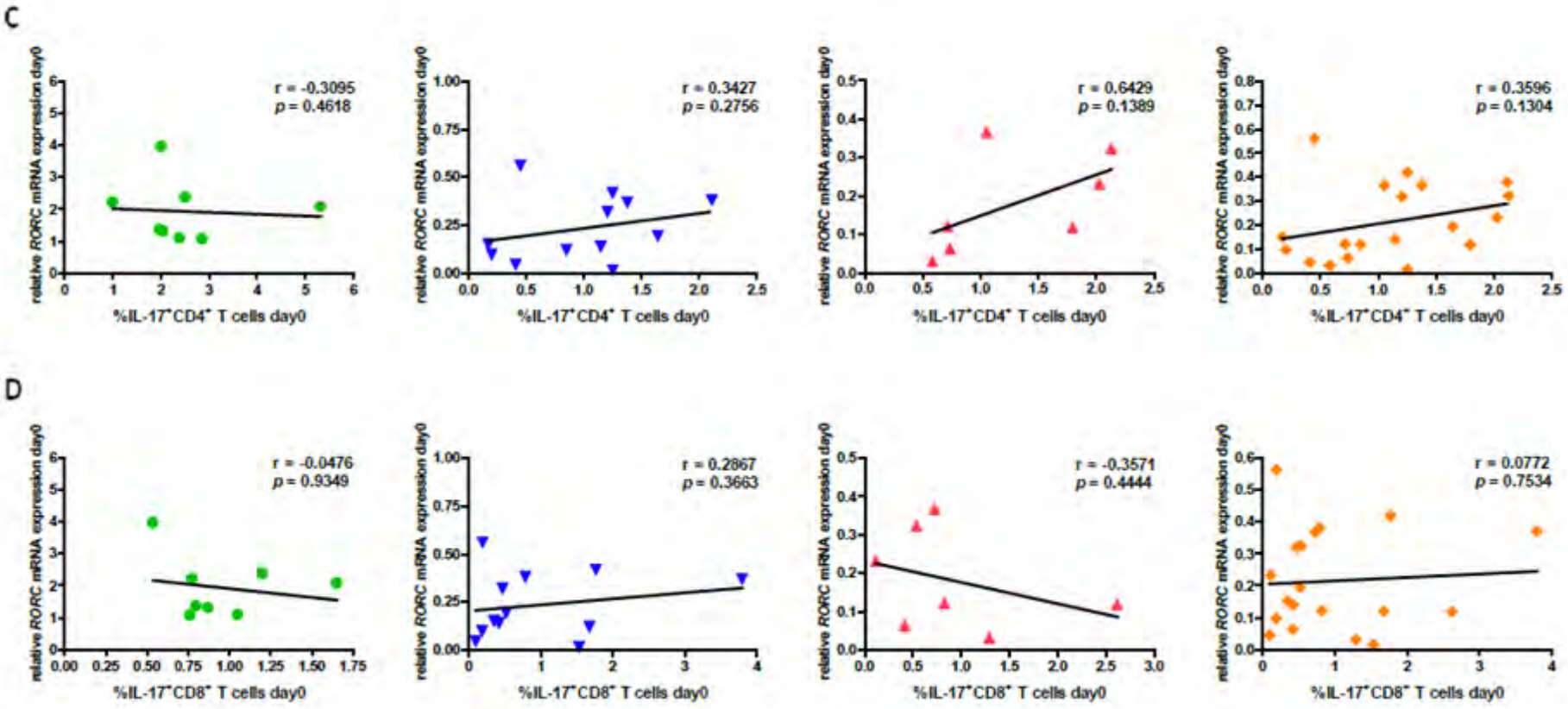
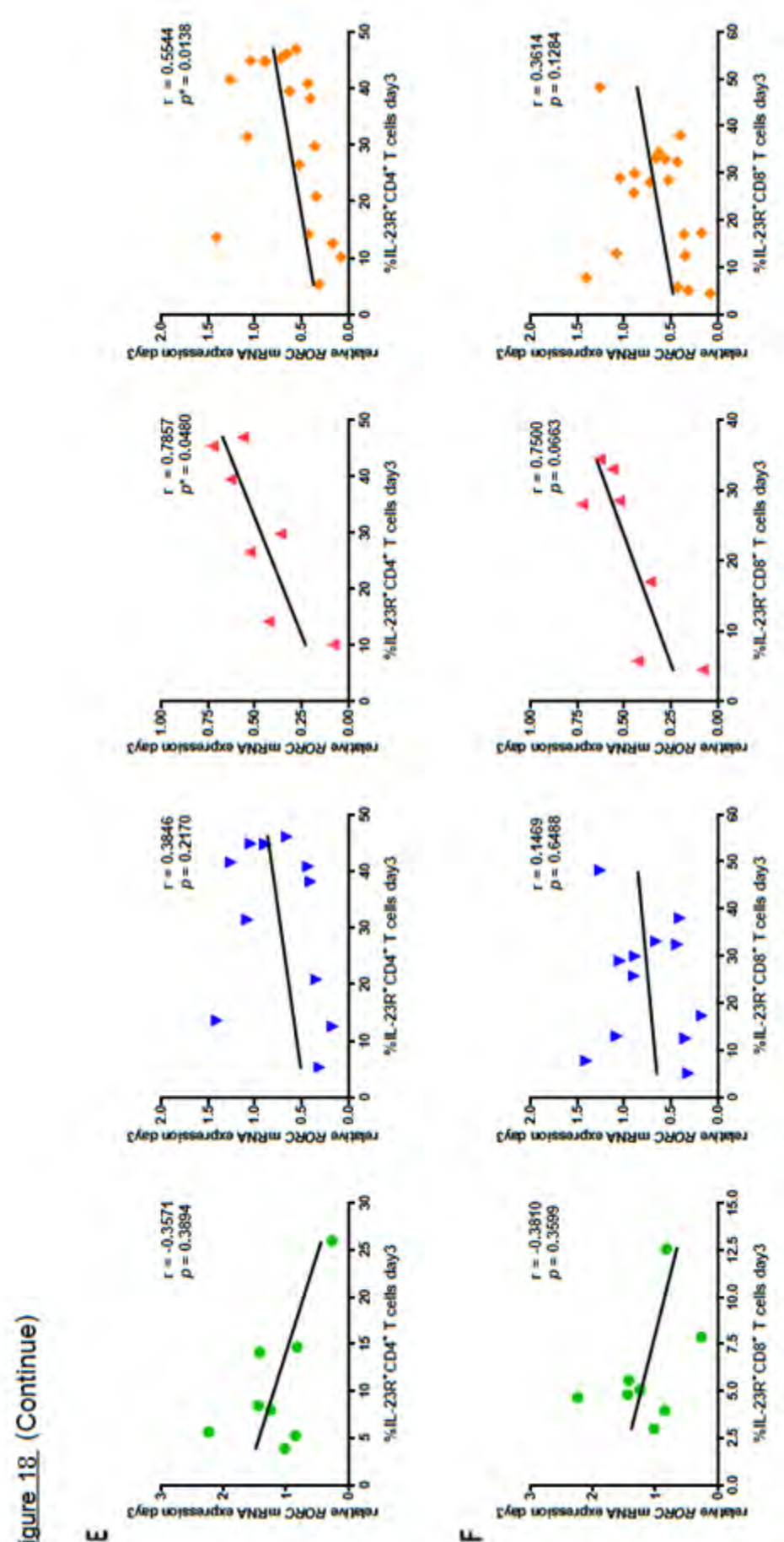


Figure 18 (Continue)



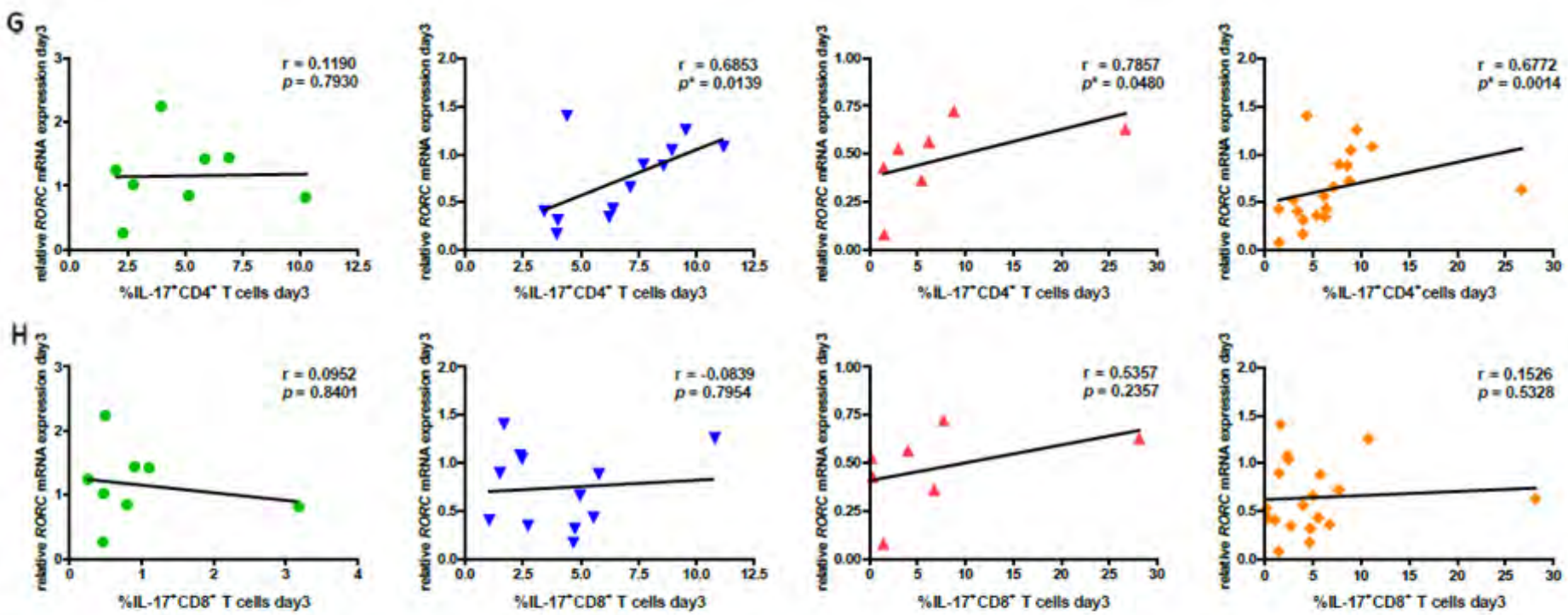
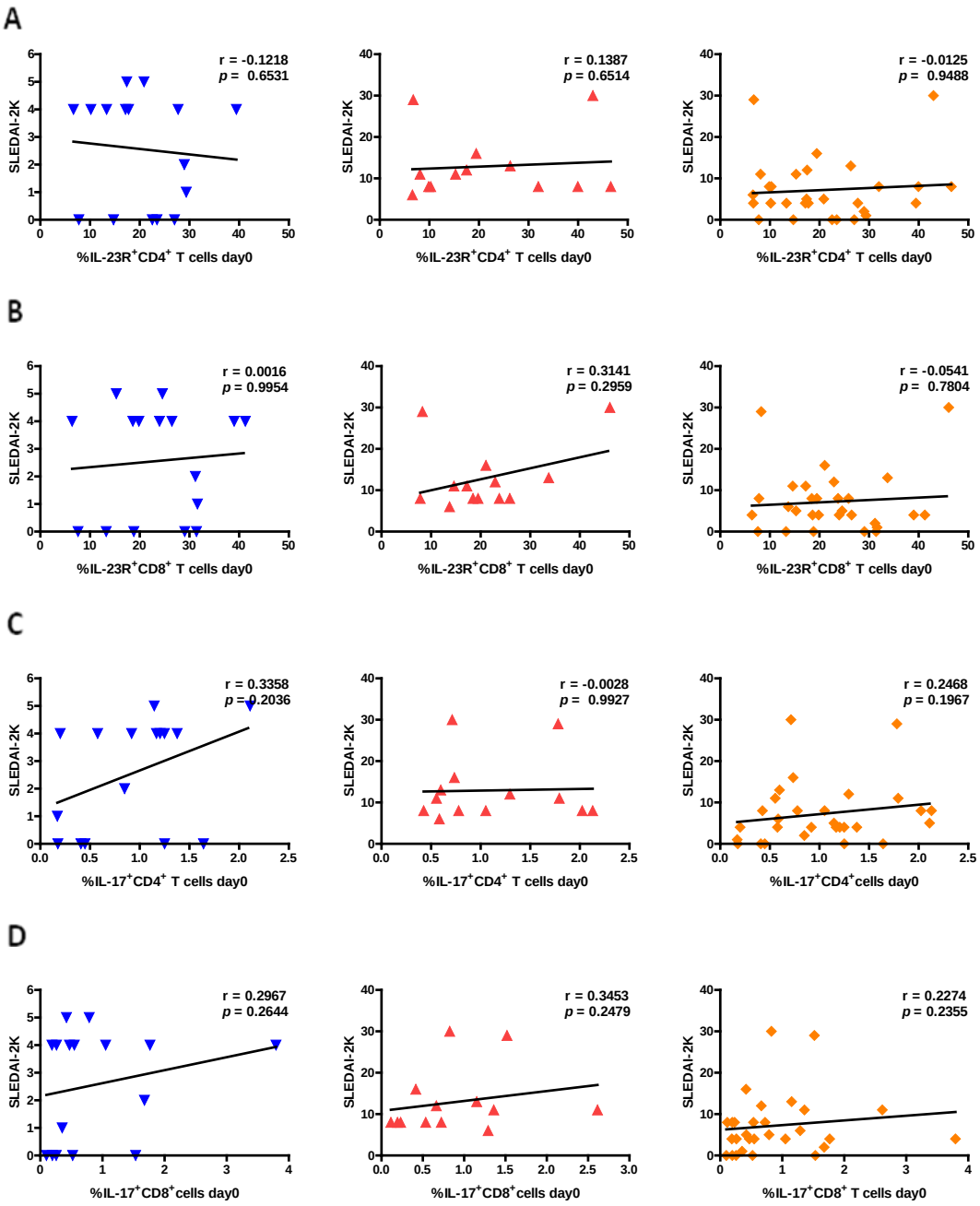


Figure 18. Correlation between Relative *RORC* Expression and the Results from Flow Cytometric Analysis. The relative *RORC* mRNA expression in PBMC at day 0 was plotted against the followings: (A) %IL-23R$^+$CD4$^+$ T cells at day 0, (B) %IL-23R$^+$CD8$^+$ T cells at day 0, (C) %IL-17$^+$CD4$^+$ T cells at day 0 and , (D) %IL-17$^+$CD8$^+$ T cells at day 0. The relative *RORC* mRNA expression in PBMC at day 3 was plotted against the followings: (E) %IL-23R$^+$CD4$^+$ T cells at day 3, (F) %IL-23R$^+$CD8$^+$ T cells at day 3, (G) %IL-17$^+$CD4$^+$ T cells at day 3 and (H) %IL-17$^+$CD8$^+$ T cells at day 3. Spearman's correlation test was used to analyze these data sets. (● = the normal subjects, ▼ = the inactive SLE patients, ▲ = the active SLE patients and ◆ = the total SLE patients)

7. Correlation with the SLEDAI-2K Scores

The SLEDAI-2K score was accepted by physicians as an assessment of the clinical status of SLE patients and represented disease activity. The correlations of the percentages of IL-23R$^+$CD4$^+$ T cells, IL-23R$^+$CD8$^+$ T cells, IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$ T cells in PBMCs from SLE patients (both on day 0 and day 3) with the scores of SLEDAI-2K using Spearman's correlation analysis were determined. We did not find any correlation of the percentages of IL-23R$^+$CD4$^+$ T cells, IL-23R$^+$CD8$^+$ T cells, IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$ T cells in PBMC from the inactive and the total SLE patients (both on day 0 and day 3) with the scores of SLEDAI-2K (Figure 19; A-H; ▽ = the inactive SLE patients and ◆ = the total SLE patients). The percentages of IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$ T cells in PBMC on day 3 from the active SLE patients, however, significantly correlated with the scores of SLEDAI-2K ($r = 0.6056$, $p = 0.0283$ and $r = 0.6085$, $p = 0.0273$, respectively) (Figure 19G, middle row and 19H, middle row, respectively). In addition, we observed a positive correlations between the percentages of IL-23R$^+$CD8$^+$cells in PBMCs on day 3 and the scores of SLEDAI-2K in the active SLE patients, but did not reach statistical significance ($r = 0.5462$, $p = 0.0535$) (Figure 19F, middle). We further explored the correlation between *IL-17A* mRNA level (on day 3 only) and *RORC* mRNA level (both on day 0 and day 3) with the scores of SLEDAI-2K, but did not find any correlation of *IL-17A* and *RORC* mRNA expression with the scores of SLEDAI-2K (Figure 20).


A
r = -0.1218
p = 0.6531
SLEDAI-2K
%IL-23R+CD4+ T cells day0
r = 0.1387
p = 0.6514
SLEDAI-2K
%IL-23R+CD4+ T cells day0
r = -0.0125
p = 0.9488
SLEDAI-2K
%IL-23R+CD4+ T cells day0
B
r = 0.0016
p = 0.9954
SLEDAI-2K
%IL-23R+CD8+ T cells day0
r = 0.3141
p = 0.2959
SLEDAI-2K
%IL-23R+CD8+ T cells day0
r = -0.0541
p = 0.7804
SLEDAI-2K
%IL-23R+CD8+ T cells day0
C
r = 0.3358
p = 0.2036
SLEDAI-2K
%IL-17+CD4+ T cells day0
r = -0.0028
p = 0.9927
SLEDAI-2K
%IL-17+CD4+ T cells day0
r = 0.2468
p = 0.1967
SLEDAI-2K
%IL-17+CD4+cells day0
D
r = 0.2967
p = 0.2644
SLEDAI-2K
%IL-17+CD8+cells day0
r = 0.3453
p = 0.2479
SLEDAI-2K
%IL-17+CD8+cells day0
r = 0.2274
p = 0.2355
SLEDAI-2K
%IL-17+CD8+ T cells day0

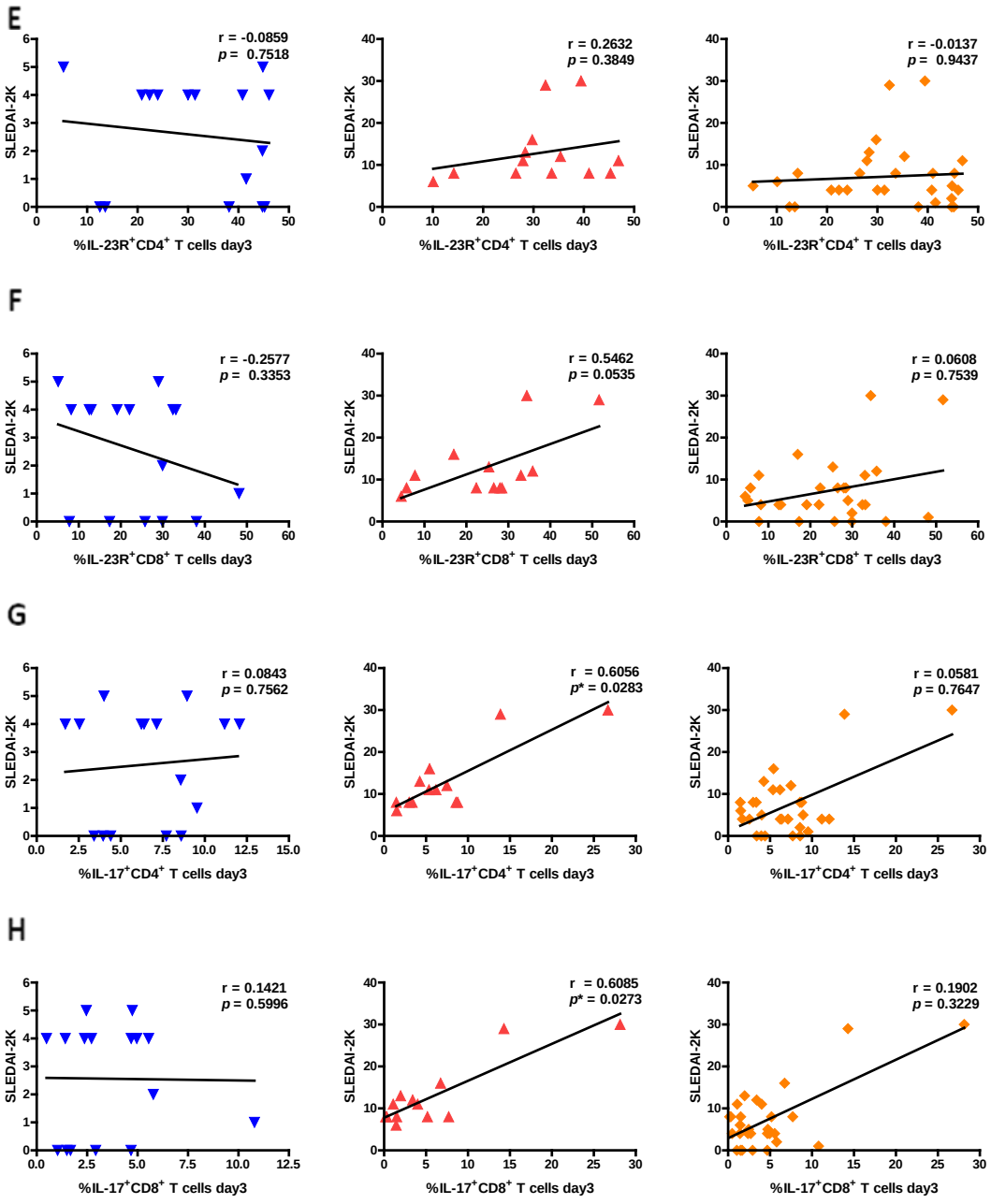


Figure 19. Correlation Analyses between the Scores of SLEDAI-2K and the Percentages of IL-23R$^+$CD4$^+$ T Cells, IL-23R$^+$CD8$^+$ T Cells, IL-17$^+$CD4$^+$ T Cells and IL-17$^+$CD8$^+$ T Cells in PBMCs from 29 SLE Patients. The scores of SLEDAI-2K was plotted against the following: the percentages of IL-23R$^+$CD4$^+$ T cells (A; day 0, E; day 3), IL-23R$^+$CD8$^+$ T cells (B; day 0, F; day 3), IL-17$^+$CD4$^+$ T cells (C; day 0, G; day 3) and IL-17$^+$CD8$^+$ T cells (D; day 0, H; day 3) Spearman's correlation test was used to analyze these data. (▼ = the inactive SLE patients, ▲ = the active SLE patients and ◆ = the total SLE patients).

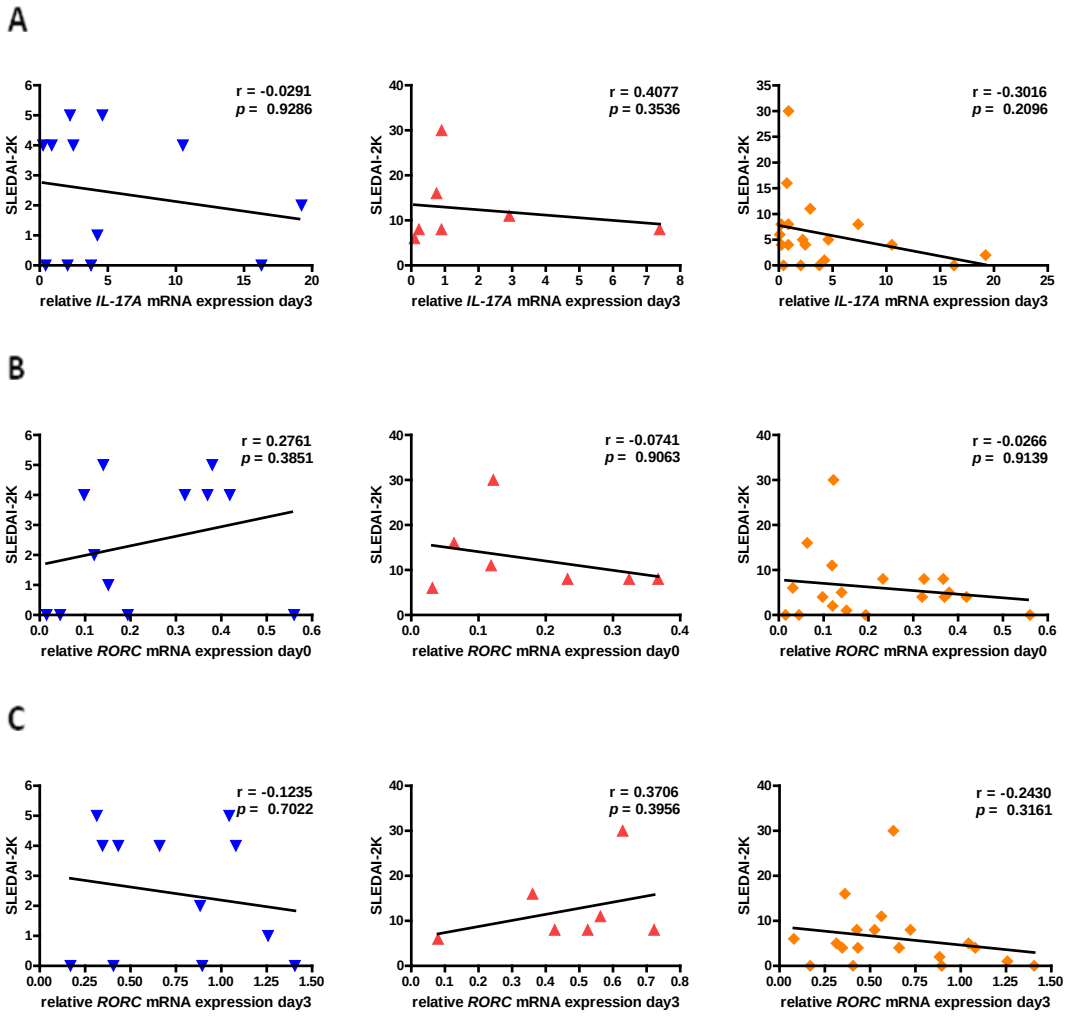


Figure 20. **Correlation Analyses between the Scores of SLEDAI-2K and Relative mRNA Expression in PBMCs from 19 SLE Patients.** The scores of SLEDAI-2K was plotted against the following: (A) the relative *IL-17A* mRNA expression day 3, (B) the relative *RORC* mRNA expression day 0, the relative *RORC* mRNA expression day 3 and (C) (▼ = the inactive SLE patients, ▲ = the active SLE patients and ◆ = the total SLE patients). Spearman's correlation test was used to analyze these data.

CHAPTER VI

DISCUSSION

SLE is a severe systemic autoimmune disease of which the exact pathogenesis remains elusive. The complicated involvement of inflammatory mediators including cytokines seems to be fundamental to the aberrant immune system of SLE [117, 198]. Traditionally, imbalance in the cytokines produced by Th1 and Th2 is believed to play an important role in the pathogenesis of SLE. However, reports on the Th1/Th2 imbalance in SLE have been inconsistent [28-33]. More recent findings have identified Th17 which is another T helper subset that mainly produces the pro-inflammatory cytokine IL-17A [34-36]. Since IL-17 and its related cytokines function in pro-inflammatory manner, it is not surprising that a large body of evidence suggests that Th17 and Th17–associated cytokines have a crucial role in the development of a wide range of autoimmune disorders, such as psoriasis [42, 63], rheumatoid arthritis [64-66], multiple sclerosis [67, 68], inflammatory bowel disease [69, 70] and recent study demonstrated that IL-17A is involved in SLE pathogenesis [71-73].

In this study, we report for the first time that the frequency of $CD4^+$ and $CD8^+$ T cells in SLE patients which expressed IL-23R, the receptor for IL-23 which is important in the maturation process of IL-17 producing T cells, was significantly higher than those in the normal subjects (Figure 5 and 6) both in freshly isolated PBMCs (day 0) and PBMCs receiving *ex vivo* stimulation by anti-CD3 and CD-28 antibodies (day 3). Our results are consistent with the studies reported by Yang *et al*. [199] that showed higher *IL-23R* mRNA expression levels in freshly isolated PBMC from SLE patients, compared with those from normal subjects. Although it by itself is not sufficient for inducing differentiation of IL-17 secreting T cells, IL-23 promotes proliferation and phenotypic maintenance of Th17 cells in helper T cells [40-44]. IL-23R is up-regulated during

activation and provides activated cells responsive to IL-23 and also expressed in memory cell subset [200]. The exact role of IL-23/IL-23R in Th17 differentiation is still controversial. In addition, the role of IL-23 in $CD8^+$ T cells has not been well documented. Our data suggest that environmental conditions in SLE result in the expression of IL-23R on IL-17 producing T cells and allow IL-23 to sustain and strengthen the IL-17 producing T cells phenotype [200]. Interestingly, the difference between the active and inactive group was not obvious in our study, suggesting that expression of IL-23R on T cells may not correlate with disease severity.

IL-17A is a pro-inflammatory cytokine that is produced by several cell types including $CD4^+$ T cells $CD8^+$ T cells [54, 55], γδ T cells [53], NK T cells [56, 57], NK cells [58], eosinophils [59], neutrophils [54] and monocytes [60]. Having observed that the higher frequency of $IL\text{-}23R^+$ T cells is SLE patients, we expected that IL-17 producing T cells would be higher in SLE patients. Unexpectedly, however, we found that $IL\text{-}17^+CD4^+$ T cells, the predominant source of IL-17A [51], in freshly isolated PBMC (day 0) from normal subjects were significantly higher than SLE patients (Figure 7A, left). Our results are inconsistent with the studies by Crispin *et al.* [201] that observed trend of increased in the percentage of $IL\text{-}17^+CD4^+$ T cells in Caucasian SLE patients when compared to those of the normal subjects, but did not reach statistical significance. This discrepancy might be explained by the complexity of SLE which is influenced by genetic background. A study by Mok *et al.* indicated that Asian patients with SLE show more severity in the progression of the disease, especially with renal involvement [8]. Seventeen of twenty-nine Thai SLE patients (58.6%) that were recruited for this study are patients with renal involvement. Infiltration of activated T cells are also found in tissues from affected organs such as the kidneys and skin in SLE [202-204]. Therefore, the low percentage of $IL\text{-}17^+CD4^+$ T cells in PBMCs from SLE patients might be due to infiltration of T cells from circulation to site of inflammation.

However, after *in vitro* stimulation with plate-bound anti-CD3 and CD-28 antibodies (day 3), we observed the increased trend in the percentage of IL-17$^+$CD4$^+$ T cells in SLE patients, when compared to that of normal subjects, but did not reach statistical significance (Figure 7A, right). These results are consistent with the studies by Crispin *et al.* [201] that observed a significantly higher percentage of IL-17$^+$CD4$^+$ T cells in PBMCs from SLE patients after long-term (> 5 days) *in vitro* stimulation with plate-bound anti-CD3 antibodies in the presence of monocytes (accessory cells) than in the normal subjects. Absence of accessory cells in our stimulating conditions may be one of the factors rendering the difference in our study did not reach statistically significance.

In addition, we also investigated the percentage of CD8$^+$IL-17$^+$ T cells in PBMCs from SLE patients and normal subjects. Even though the percentage of CD8$^+$IL-17$^+$ T cells were not different between SLE patients and normal subjects (Figure 8A, left), the percentage of CD8$^+$IL-17$^+$ T cells on day 3 in SLE patients were significantly higher than normal subjects (Figure 8A, right). Similar increase in CD8$^+$IL-17$^+$ T cells was observed in skin biopsies from psoriasis patients [184]. However, little is known about the role of CD8$^+$IL-17$^+$ T cells in normal immune response or in certain disease condition. Further studies are needed to clarify the role of CD8$^+$IL-17$^+$ T cells in SLE.

We next determined the fold increase in percentages of IL-17$^+$ T cells from day 0 to day 3 from individual sample. These studies revealed the fold increase in the percentage of IL-17$^+$CD4$^+$ T cells and IL-17$^+$CD8$^+$ T cells from all SLE patient groups (the inactive group, the active group, total SLE patients combining inactive and active groups) were significantly higher than those of the normal subjects (Figure 9A and 9B, respectively). Together with the tendency of increased IL-17 producing T cells in SLE patients from freshly isolated PBMCs, these observations indicated that SLE patients selectively increased the frequency of IL-17 producing T cells after *in vitro* stimulation and/or the IL-17 producing T cells outgrown other subsets of helper T cells.

As described above, IL-23 has been shown to aid the expansion and survival of IL-17 producing T cells. Thus, the expression of IL-23R on T cells is of interest, relevant to the numbers of IL-17–producing cells. Interestingly, we observed the significant positive correlation between percentages of $CD4^+IL\text{-}23R^+$, $CD8^+IL\text{-}23R^+$ T lymphocytes and $CD4^+IL\text{-}17^+$, $CD8^+IL\text{-}17^+$ T lymphocytes, respectively in SLE patients on day 3 (Figure 10). The significantly higher in the percentage of T lymphocytes expressing IL-23R and trend of increased in the percentage of T lymphocytes expressing IL-17A in PBMCs from SLE patients may play an important role in pathology of SLE.

When the level of IL-17A in sera and plasma were measured by ELISA (Figure 11 and 13, respectively), only two samples from 28 SLE patients were found positive at 5.12, 6.78 pg/ml and 5.75, 8.50 pg/ml, respectively, while the level in the rest of the samples was below detectable level (which was at 4 pg/ml). When IL-23 was measured in both sera and plasma by ELISA, all samples showed negative results (Figure 12 and 14, respectively). Our results are inconsistent with the studies by Wong *et al*. [71, 73] which reported that plasma IL-17A and IL-23 level were significantly elevated in 36 and 80 SLE patients, respectively. Moreover, they also reported that plasma IL-17A level correlated with the score of SLEDAI. However, there was no significant correlation of plasma IL-23 level and the score of SLEDAI. Kurasawa *et al*. [72] have shown that there was no difference in levels of serum IL-17A between SLE patients and the normal subjects. Therefore, it is still controversial whether the serum IL-17A and IL-23 in SLE patients is always higher than the controls. The discrepancy may be due to different patient background, drug treatment and other unknown factors.

When the expression of *IL-17A* was analyzed by realtime RT-PCR, the level of *IL-17A* mRNA expression in freshly isolated PBMCs (day 0) was undetectable in all subjects. Although our results are consistent with the studies by Kurasawa *et al* [72] showed that *IL-17A* mRNA, as determined by RT-PCR, was undetectable in freshly isolated PBMCs from all SLE patients and all normal subjects, they are inconsistent with

the studies by Yang *et al.* [199] that showed higher *IL-17A* mRNA expression levels in freshly isolated PBMC from SLE patients when compared with those from normal subjects. Moreover, our results in this part are inconsistent with our results from flow cytometry that could detect IL-17A expression in PBMCs. Currently; the cause of this discrepancy is not known. However, after *in vitro* stimulation with plate-bound anti-CD3 and anti-CD28 antibodies, the level of *IL-17A* mRNA expression in PBMCs at day 3 from the SLE patients were higher than those of the normal subjects, but did not reach statistical significance (Figure 15). Taken together, our results from flow cytometry, ELISA and realtime RT-PCR showed that IL-17 expression increased in SLE, which may indicate that the elevation of proinflammatory cytokine IL-17 is involved in SLE. It will be of great interest to use purified T cells for this experiment.

The orphan nuclear receptor RORγt (RORC in human) is the key transcription factor expressed in IL-17 producing T cells [42, 133]. Our results showed that the level of *RORC* mRNA expression in freshly isolated PBMCs (day 0) from SLE patients was significantly lower when compared with those of the normal subjects (Figure 16A), consistent with the lower *IL-17A* expression. Similarly, in PBMCs which were cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3), the level of *RORC* mRNA expression was significantly lower in SLE patients when compared with those of the normal subjects (Figure 16B). Our results are inconsistent with the studies by Yang *et al.* [199] that showed higher *RORC* mRNA expression in freshly isolated PBMC from SLE patients compared with those from normal subjects, but did not reach statistical significance. The discrepancy of this study might be explained by heterogeneous PBMC population which we did not determine the cellular origin of the mRNA in our experiment. The studies by Kwan *et al.* [205] showed the negative correlation between urinary *IL-17A* and *RORγ* mRNA expression and the score of SLEDAI. The investigator suggested that it is possible that there is retention of IL-17 producing T cells within the kidney in patients with active lupus nephritis, resulting in

reduction in urinary expression in the presence of high disease activity. Perhaps the discrepancy of our study and previous studies might be explained by the reasons described above.

Next, we analyzed the correlations of the results from flow cytometric analysis and the results from the semi-quantitative real-time RT-PCR analysis for *IL-17A* and *RORC*. We found that the percentages of $CD4^+$IL-$23R^+$ T cells and $CD4^+$IL-17^+ T cells at day 3 correlated well with *IL-17A* and *RORC* expression in SLE patients (Figure 17A, 17C, 18E and 18G, respectively). However, we did not found the same correlation in $CD8^+$ T cells in SLE patients. Moreover, we found that the percentage of IL-17^+CD4^+ T cells and IL-17^+CD8^+ T cells are higher in PBMC on day 3 from the active SLE patients and correlated with the score of SLEDAI-2K (Figure 19G, middle row and 19H, middle row, respectively). This data imply that IL-17 producing T cells may play a role in SLE disease progression.

Interestingly, it was recently reported that SLE patients have increased $CD4^-CD8^-$ double negative T cells which express IL-17A. Subsequent studies revealed that this subset of cells may derive from $CD8^+$ T cells [201]. Therefore, our results of increased $CD8^+$ IL$23R^+$ T cells may indicate that SLE patients have higher percentage if this subset which can develop to IL-17^+ DN T cells in periphery.

Our primary samples were derived from PBMCs which are composed of heterogenous circulating immune cells, including lymphocytes, monocytes and dendritic cells. Most of these cells can be the source of IL-17A and, less so of RORC. Thus, the results from mRNA expression should be clarified with the use of mRNA from purified T cells. Moreover, most SLE patients that were recruited in these studies were treated with immunosuppressive drug that suppress the immune system which may have an effect on IL-17 production in T cells. It will also be interesting to investigate this IL-17^+ T cells in lesions which are affected in SLE patients.

CHAPTER VII

CONCLUSION

The results obtained in this study can be summarized as follows:

1. The frequency of $CD4^+$ and $CD8^+$ T cells which expressed IL-23R in SLE patients was significantly higher than those in the normal subjects. Moreover, higher frequency of $CD4^+$IL-17^+ and significantly higher frequency of $CD8^+$IL-17^+ T cells in SLE patients were observed.

2. When IL-17A level was measured by ELISA, only two SLE patients were found to show detectable IL-17A. On the other hand, all samples showed undetectable when IL-23 level was measured by the same method.

3. The expression of *IL-17A* mRNA in PBMCs from patients was higher but did not reach statistically significant level. Difference in the level of *RORC* mRNA between patients and normal subjects was observed.

4. The percentages of $CD4^+$IL-$23R^+$ and $CD4^+$IL-17^+ T cells correlated well with *IL-17A* and *RORC* expression in SLE patients. Moreover, the percentages of IL-17^+CD4^+ and IL-17^+CD8^+ T cells are higher in PBMCs from the active SLE patients and correlated with the score of SLEDAI-2K.

Because IL-23 has been shown to play an important role in the phenotypic maintenance and proliferation of IL-17 producing T cells, and possibly in their differentiation (at least in human), therefore further investigation is needed to study the effect of exogenouse IL-23 on the production of IL-17 from peripheral blood mononuclear cells (PBMC) in ex vivo setting. Moreover, the culture supernatant of *ex vivo* culture should be subjected to subsequent ELISA IL-17 detection.

REFERENCES

[1] Foster MH. 2007. T cells and B cells in lupus nephritis. Semin Nephrol 27 (Jan 2007): 47-58.

[2] Lahita RG. 2004. Systemic Lupus Erythematosus. fourth ed. Vol. New York: Academic Press.

[3] Tutuncu ZN, Kalunian KC. 2006. The Definition ans Classification of Systemic Lupus Erythematosus. In Wallace DJ, Hahn BH (eds.), Dubois' Lupus Erythematosus seventh ed., p. 16. Philadelphia,PA: Lippincott Williams & Wilkins.

[4] Lau CS, Yin G, Mok MY. 2006. Ethnic and geographical differences in systemic lupus erythematosus: an overview. Lupus 15 2006): 715-719.

[5] Shapira Y, Weinberger A, Wysenbeek AJ. 1996. Lymphadenopathy in systemic lupus erythematosus. Prevalence and relation to disease manifestations. Clin Rheumatol 15 (Jul 1996): 335-338.

[6] Danchenko N, Satia JA, Anthony MS. 2006. Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. Lupus 15 2006): 308-318.

[7] Feng PH. 2007. Systemic lupus erythematosus: the face of Asia. Ann N Y Acad Sci 1108 (Jun 2007): 114-120.

[8] Mok CC, Lau CS. 2003. Lupus in Hong Kong Chinese. Lupus 12 2003): 717-722.

[9] Mok CC, Lau CS. 2003. Pathogenesis of systemic lupus erythematosus. J Clin Pathol 56 (Jul 2003): 481-490.

[10] Hoffman RW. 2004. T cells in the pathogenesis of systemic lupus erythematosus. Clin Immunol 113 (Oct 2004): 4-13.

[11] Rahman A, Isenberg DA. 2008. Systemic lupus erythematosus. N Engl J Med 358 (Feb 28 2008): 929-939.

[12] Shivakumar S, Tsokos GC, Datta SK. 1989. T cell receptor alpha/beta expressing double-negative (CD4-/CD8-) and CD4+ T helper cells in humans augment the production of pathogenic anti-DNA autoantibodies associated with lupus nephritis. J Immunol 143 (Jul 1 1989): 103-112.

[13] Datta SK, Patel H, Berry D. 1987. Induction of a cationic shift in IgG anti-DNA autoantibodies. Role of T helper cells with classical and novel phenotypes in three murine models of lupus nephritis. J Exp Med 165 (May 1 1987): 1252-1268.

[14] Zeng D, Dick M, Cheng L, Amano M, Dejbakhsh-Jones S, Huie P, et al. 1998. Subsets of transgenic T cells that recognize CD1 induce or prevent murine lupus: role of cytokines. J Exp Med 187 (Feb 16 1998): 525-536.

[15] Kaplan MJ, Beretta L, Yung RL, Richardson BC. 2000. LFA-1 overexpression and T cell autoreactivity: mechanisms. Immunol Invest 29 (Nov 2000): 427-442.

[16] Yi Y, McNerney M, Datta SK. 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 (Dec 1 2000): 6627-6634.

[17] Batuca JR, Ames PR, Amaral M, Favas C, Isenberg DA, Delgado Alves J. 2009. Anti-atherogenic and anti-inflammatory properties of high-density lipoprotein are affected by specific antibodies in systemic lupus erythematosus. Rheumatology (Oxford) 48 (Jan 2009): 26-31.

[18] Kovacs B, Vassilopoulos D, Vogelgesang SA, Tsokos GC. 1996. Defective CD3-mediated cell death in activated T cells from patients with systemic lupus erythematosus: role of decreased intracellular TNF-alpha. Clin Immunol Immunopathol 81 (Dec 1996): 293-302.

[19] Aringer M, Wintersberger W, Steiner CW, Kiener H, Presterl E, Jaeger U, 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 (Oct 1994): 1423-1430.

[20] Budagyan VM, Bulanova EG, Sharova NI, Nikonova MF, Stanislav ML, Yarylin AA. 1998. The resistance of activated T-cells from SLE patients to apoptosis induced by human thymic stromal cells. Immunol Lett 60 (Jan 1998): 1-5.

[21] Xu L, Zhang L, Yi Y, Kang HK, Datta SK. 2004. Human lupus T cells resist inactivation and escape death by upregulating COX-2. Nat Med 10 (Apr 2004): 411-415.

[22] Emlen W, Niebur J, Kadera R. 1994. Accelerated in vitro apoptosis of lymphocytes from patients with systemic lupus erythematosus. J Immunol 152 (Apr 1 1994): 3685-3692.

[23] Herrmann M, Voll RE, Zoller OM, Hagenhofer M, Ponner BB, Kalden JR. 1998. Impaired phagocytosis of apoptotic cell material by monocyte-derived macrophages from patients with systemic lupus erythematosus. Arthritis Rheum 41 (Jul 1998): 1241-1250.

[24] Crispin JC, Martinez A, Alcocer-Varela J. 2003. Quantification of regulatory T cells in patients with systemic lupus erythematosus. J Autoimmun 21 (Nov 2003): 273-276.

[25] Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, et al. 2005. Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol 175 (Dec 15 2005): 8392-8400.

[26] Lee JH, Wang LC, Lin YT, Yang YH, Lin DT, Chiang BL. 2006. Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus. Immunology 117 (Feb 2006): 280-286.

[27] Monk CR, Spachidou M, Rovis F, Leung E, Botto M, Lechler RI, 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 (Apr 2005): 1180-1184.

[28] Linker-Israeli M, Deans RJ, Wallace DJ, Prehn J, Ozeri-Chen T, Klinenberg JR. 1991. Elevated levels of endogenous IL-6 in systemic lupus erythematosus. A putative role in pathogenesis. J Immunol 147 (Jul 1 1991): 117-123.

[29] Houssiau FA, Lefebvre C, Vanden Berghe M, Lambert M, Devogelaer JP, Renauld JC. 1995. Serum interleukin 10 titers in systemic lupus erythematosus reflect disease activity. Lupus 4 (Oct 1995): 393-395.

[30] Klinman DM, Steinberg AD. 1995. Inquiry into murine and human lupus. Immunol Rev 144 (Apr 1995): 157-193.

[31] Tokano Y, Morimoto S, Kaneko H, Amano H, Nozawa K, Takasaki Y, et al. 1999. Levels of IL-12 in the sera of patients with systemic lupus erythematosus (SLE)--relation to Th1- and Th2-derived cytokines. Clin Exp Immunol 116 (Apr 1999): 169-173.

[32] Davas EM, Tsirogianni A, Kappou I, Karamitsos D, Economidou I, Dantis PC. 1999. Serum IL-6, TNFalpha, p55 srTNFalpha, p75srTNFalpha, srIL-2alpha levels and disease activity in systemic lupus erythematosus. Clin Rheumatol 18 1999): 17-22.

[33] al-Janadi M, al-Balla S, al-Dalaan A, Raziuddin S. 1993. Cytokine profile in systemic lupus erythematosus, rheumatoid arthritis, and other rheumatic diseases. J Clin Immunol 13 (Jan 1993): 58-67.

[34] Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, et al. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 201 (Jan 17 2005): 233-240.

[35] Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, et al. 2005. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 6 (Nov 2005): 1123-1132.

[36] Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 6 (Nov 2005): 1133-1141.

[37] Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441 (May 11 2006): 235-238.

[38] Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, et al. 2006. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature 441 (May 11 2006): 231-234.

[39] Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 24 (Feb 2006): 179-189.

[40] Chen Z, Tato CM, Muul L, Laurence A, O'Shea JJ. 2007. Distinct regulation of interleukin-17 in human T helper lymphocytes. Arthritis Rheum 56 (Sep 2007): 2936-2946.

[41] Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. 2007. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. Nat Immunol 8 (Sep 2007): 942-949.

[42] Wilson NJ, Boniface K, Chan JR, McKenzie BS, Blumenschein WM, Mattson JD, et al. 2007. Development, cytokine profile and function of human interleukin 17-producing helper T cells. Nat Immunol 8 (Sep 2007): 950-957.

[43] Manel N, Unutmaz D, Littman DR. 2008. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat Immunol 9 (Jun 2008): 641-649.

[44] Volpe E, Servant N, Zollinger R, Bogiatzi SI, Hupe P, Barillot E, et al. 2008. A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses. Nat Immunol 9 (Jun 2008): 650-657.

[45] Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, et al. 2008. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. Nature 454 (Jul 17 2008): 350-352.

[46] Cosmi L, De Palma R, Santarlasci V, Maggi L, Capone M, Frosali F, et al. 2008. Human interleukin 17-producing cells originate from a CD161+CD4+ T cell precursor. J Exp Med 205 (Aug 4 2008): 1903-1916.

[47] Santarlasci V, Maggi L, Capone M, Frosali F, Querci V, De Palma R, et al. 2009. TGF-beta indirectly favors the development of human Th17 cells by inhibiting Th1 cells. Eur J Immunol 39 (Jan 2009): 207-215.

[48] Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et al. 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. Nat Immunol 8 (Jun 2007): 639-646.

[49] Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. 2007. Phenotypic and functional features of human Th17 cells. J Exp Med 204 (Aug 6 2007): 1849-1861.

[50] Singh SP, Zhang HH, Foley JF, Hedrick MN, Farber JM. 2008. Human T cells that are able to produce IL-17 express the chemokine receptor CCR6. J Immunol 180 (Jan 1 2008): 214-221.

[51] Afzali B, Lombardi G, Lechler RI, Lord GM. 2007. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. Clin Exp Immunol 148 (Apr 2007): 32-46.

[52] Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL. 2003. Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 278 (Jan 17 2003): 1910-1914.

[53] Lockhart E, Green AM, Flynn JL. 2006. IL-17 production is dominated by gammadelta T cells rather than CD4 T cells during Mycobacterium tuberculosis infection. J Immunol 177 (Oct 1 2006): 4662-4669.

[54] Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A. 2003. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. J Immunol 170 (Feb 15 2003): 2106-2112.

[55] Shin HC, Benbernou N, Esnault S, Guenounou M. 1999. Expression of IL-17 in human memory CD45RO+ T lymphocytes and its regulation by protein kinase A pathway. Cytokine 11 (Apr 1999): 257-266.

[56] Michel ML, Keller AC, Paget C, Fujio M, Trottein F, Savage PB, et al. 2007. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. J Exp Med 204 (May 14 2007): 995-1001.

[57] Yoshiga Y, Goto D, Segawa S, Ohnishi Y, Matsumoto I, Ito S, et al. 2008. Invariant NKT cells produce IL-17 through IL-23-dependent and -independent pathways with potential modulation of Th17 response in collagen-induced arthritis. Int J Mol Med 22 (Sep 2008): 369-374.

[58] Cupedo T, Crellin NK, Papazian N, Rombouts EJ, Weijer K, Grogan JL, et al. 2009. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. Nat Immunol 10 (Jan 2009): 66-74.

[59] Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N, et al. 2001. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J Allergy Clin Immunol 108 (Sep 2001): 430-438.

[60] Zhou Q, Desta T, Fenton M, Graves DT, Amar S. 2005. Cytokine profiling of macrophages exposed to Porphyromonas gingivalis, its lipopolysaccharide, or its FimA protein. Infect Immun 73 (Feb 2005): 935-943.

[61] Zheng Y, Valdez PA, Danilenko DM, Hu Y, Sa SM, Gong Q, et al. 2008. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med 14 (Mar 2008): 282-289.

[62] Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, et al. 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. Nat Med 14 (Mar 2008): 275-281.

[63] Krueger GG, Langley RG, Leonardi C, Yeilding N, Guzzo C, Wang Y, et al. 2007. A human interleukin-12/23 monoclonal antibody for the treatment of psoriasis. N Engl J Med 356 (Feb 8 2007): 580-592.

[64] Hwang SY, Kim HY. 2005. Expression of IL-17 homologs and their receptors in the synovial cells of rheumatoid arthritis patients. Mol Cells 19 (Apr 30 2005): 180-184.

[65] Chabaud M, Durand JM, Buchs N, Fossiez F, Page G, Frappart L, et al. 1999. Human interleukin-17: A T cell-derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum 42 (May 1999): 963-970.

[66] Ziolkowska M, Koc A, Luszczykiewicz G, Ksiezopolska-Pietrzak K, Klimczak E, Chwalinska-Sadowska H, et al. 2000. High levels of IL-17 in rheumatoid arthritis patients: IL-15 triggers in vitro IL-17 production via cyclosporin A-sensitive mechanism. J Immunol 164 (Mar 1 2000): 2832-2838.

[67] Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, Cayrol R, Bernard M, et al. 2007. Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. Nat Med 13 (Oct 2007): 1173-1175.

[68] Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, et al. 2008. Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. Am J Pathol 172 (Jan 2008): 146-155.

[69] Nielsen OH, Kirman I, Rudiger N, Hendel J, Vainer B. 2003. Upregulation of interleukin-12 and -17 in active inflammatory bowel disease. Scand J Gastroenterol 38 (Feb 2003): 180-185.

[70] Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, et al. 2003. Increased expression of interleukin 17 in inflammatory bowel disease. Gut 52 (Jan 2003): 65-70.

[71] Wong CK, Ho CY, Li EK, Lam CW. 2000. Elevation of proinflammatory cytokine (IL-18, IL-17, IL-12) and Th2 cytokine (IL-4) concentrations in patients with systemic lupus erythematosus. Lupus 9 2000): 589-593.

[72] Kurasawa K, Hirose K, Sano H, Endo H, Shinkai H, Nawata Y, et al. 2000. Increased interleukin-17 production in patients with systemic sclerosis. Arthritis Rheum 43 (Nov 2000): 2455-2463.

[73] Wong CK, Lit LC, Tam LS, Li EK, Wong PT, Lam CW. 2008. Hyperproduction of IL-23 and IL-17 in patients with systemic lupus erythematosus: implications for Th17-mediated inflammation in auto-immunity. Clin Immunol 127 (Jun 2008): 385-393.

[74] Cohen AS, Reynolds WE, Franklin EC. 1971. Preliminary criteria for the classification of Systemic Lupus Erythematosus Bulletin on the rheumatic diseases 21 1971): 643-648.

[75] Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 25 (Nov 1982): 1271-1277.

[76] Hochberg MC. 1997. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 40 (Sep 1997): 1725.

[77] Griffiths B, Mosca M, Gordon C. 2005. Assessment of patients with systemic lupus erythematosus and the use of lupus disease activity indices. Best Pract Res Clin Rheumatol 19 (Oct 2005): 685-708.

[78] Gladman DD, Ibanez D, Urowitz MB. 2002. Systemic lupus erythematosus disease activity index 2000. J Rheumatol 29 (Feb 2002): 288-291.

[79] Lawrence RC, Helmick CG, Arnett FC, Deyo RA, Felson DT, Giannini EH, et al. 1998. Estimates of the prevalence of arthritis and selected musculoskeletal disorders in the United States. Arthritis Rheum 41 (May 1998): 778-799.

[80] Chakravarty EF, Bush TM, Manzi S, Clarke AE, Ward MM. 2007. Prevalence of adult systemic lupus erythematosus in California and Pennsylvania in 2000: estimates obtained using hospitalization data. Arthritis Rheum 56 (Jun 2007): 2092-2094.

[81] Pons-Estel GJ, Alarcon GS, Scofield L, Reinlib L, Cooper GS. 2009. Understanding the Epidemiology and Progression of Systemic Lupus Erythematosus. Semin Arthritis Rheum (Jan 9 2009).

[82] Cervera R, Khamashta MA, Font J, Sebastiani GD, Gil A, Lavilla P, et al. 1993. Systemic lupus erythematosus: clinical and immunologic patterns of disease expression in a cohort of 1,000 patients. The European Working Party on Systemic Lupus Erythematosus. Medicine (Baltimore) 72 (Mar 1993): 113-124.

[83] Pistiner M, Wallace DJ, Nessim S, Metzger AL, Klinenberg JR. 1991. Lupus erythematosus in the 1980s: a survey of 570 patients. Semin Arthritis Rheum 21 (Aug 1991): 55-64.

[84] Deesomchok U, Tumrasvin T. 1983. Clinical study of Thai patients with systemic lupus erythematosus. J Med Assoc Thai 66 (Nov 1983): 680-687.

[85] Kasitanon N, Louthrenoo W, Piyasirisilp S, Sukitawu W, Wichainun R. 2002. Neuropsychiatric manifestations in Thai patients with systemic lupus erythematosus. Asian Pac J Allergy Immunol 20 (Sep 2002): 179-185.

[86] Block SR, Winfield JB, Lockshin MD, D'Angelo WA, Christian CL. 1975. Studies of twins with systemic lupus erythematosus. A review of the literature and presentation of 12 additional sets. Am J Med 59 (Oct 1975): 533-552.

[87] Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, et al. 1992. A revised estimate of twin concordance in systemic lupus erythematosus. Arthritis Rheum 35 (Mar 1992): 311-318.

[88] Mills JA. 1994. Systemic lupus erythematosus. N Engl J Med 330 (Jun 30 1994): 1871-1879.

[89] Criswell LA. 2008. The genetic contribution to systemic lupus erythematosus. Bull NYU Hosp Jt Dis 66 2008): 176-183.

[90] Sigurdsson S, Nordmark G, Goring HH, Lindroos K, Wiman AC, Sturfelt G, et al. 2005. Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. Am J Hum Genet 76 (Mar 2005): 528-537.

[91] Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, Bauer JW, et al. 2006. A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet 38 (May 2006): 550-555.

[92] Graham RR, Kyogoku C, Sigurdsson S, Vlasova IA, Davies LR, Baechler EC, et al. 2007. Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus. Proc Natl Acad Sci U S A 104 (Apr 17 2007): 6758-6763.

[93] Lee YH, Rho YH, Choi SJ, Ji JD, Song GG, Nath SK, et al. 2007. The PTPN22 C1858T functional polymorphism and autoimmune diseases--a meta-analysis. Rheumatology (Oxford) 46 (Jan 2007): 49-56.

[94] Yang W, Ng P, Zhao M, Hirankarn N, Lau CS, Mok CC, et al. 2009. Population differences in SLE susceptibility genes: STAT4 and BLK, but not PXK, are associated with systemic lupus erythematosus in Hong Kong Chinese. Genes Immun 10 (Apr 2009): 219-226.

[95] Kim K, Sung YK, Kang CP, Choi CB, Kang C, Bae SC. 2009. A regulatory SNP at position -899 in CDKN1A is associated with systemic lupus erythematosus and lupus nephritis. Genes Immun 10 (Jul 2009): 482-486.

[96] Nath SK, Han S, Kim-Howard X, Kelly JA, Viswanathan P, Gilkeson GS, et al. 2008. A nonsynonymous functional variant in integrin-alpha(M) (encoded by ITGAM) is associated with systemic lupus erythematosus. Nat Genet 40 (Feb 2008): 152-154.

[97] Rhodes B, Vyse TJ. 2008. The genetics of SLE: an update in the light of genome-wide association studies. Rheumatology (Oxford) 47 (Nov 2008): 1603-1611.

[98] Vedove CD, Del Giglio M, Schena D, Girolomoni G. 2009. Drug-induced lupus erythematosus. Arch Dermatol Res 301 (Jan 2009): 99-105.

[99] Kuhn A, Bijl M. 2008. Pathogenesis of cutaneous lupus erythematosus. Lupus 17 2008): 389-393.

[100] Brown AC. 2000. Lupus erythematosus and nutrition: a review of the literature. J Ren Nutr 10 (Oct 2000): 170-183.

[101] Simard JF, Costenbader KH. 2007. What can epidemiology tell us about systemic lupus erythematosus? Int J Clin Pract 61 (Jul 2007): 1170-1180.

[102] Sarzi-Puttini P, Atzeni F, Iaccarino L, Doria A. 2005. Environment and systemic lupus erythematosus: an overview. Autoimmunity 38 (Nov 2005): 465-472.

[103] Cutolo M, Sulli A, Capellino S, Villaggio B, Montagna P, Seriolo B, et al. 2004. Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity. Lupus 13 2004): 635-638.

[104] Takeuchi C, Wentworth P. 2007. Essential Components and functions of the Immune system. In Meyers RA (ed.), Immunology: from cell biology to disease p. 5: Wiley-VCH.

[105] Bolland S, Ravetch JV. 2000. Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis. Immunity 13 (Aug 2000): 277-285.

[106] Floto RA, Clatworthy MR, Heilbronn KR, Rosner DR, MacAry PA, Rankin A, et al. 2005. Loss of function of a lupus-associated FcgammaRIIb polymorphism through exclusion from lipid rafts. Nat Med 11 (Oct 2005): 1056-1058.

[107] Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, et al. 1999. Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. J Exp Med 190 (Dec 6 1999): 1697-1710.

[108] Stohl W, Metyas S, Tan SM, Cheema GS, Oamar B, Xu D, et al. 2003. B lymphocyte stimulator overexpression in patients with systemic lupus erythematosus: longitudinal observations. Arthritis Rheum 48 (Dec 2003): 3475-3486.

[109] Grammer AC, Lipsky PE. 2003. B cell abnormalities in systemic lupus erythematosus. Arthritis Res Ther 5 Suppl 4 2003): S22-27.

[110] Renaudineau Y, Pers JO, Bendaoud B, Jamin C, Youinou P. 2004. Dysfunctional B cells in systemic lupus erythematosus. Autoimmun Rev 3 (Nov 2004): 516-523.

[111] Kalden JR, Winkler TH, Herrmann M, Krapf F. 1991. Pathogenesis of SLE: immunopathology in man. Rheumatol Int 11 1991): 95-100.

[112] Williams RC, Jr., Malone CC, Huffman GR, Silvestris F, Croker BP, Ayoub EM, 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 (Jun 1995): 1075-1085.

[113] Spatz L, Iliev A, Saenko V, Jones L, Irigoyen M, Manheimer-Lory A, et al. 1997. Studies on the structure, regulation, and pathogenic potential of anti-dsDNA antibodies. Methods 11 (Jan 1997): 70-78.

[114] Suzuki N, Mihara S, Sakane T. 1997. Development of pathogenic anti-DNA antibodies in patients with systemic lupus erythematosus. FASEB J 11 (Oct 1997): 1033-1038.

[115] Wofsy D, Seaman WE. 1987. Reversal of advanced murine lupus in NZB/NZW F1 mice by treatment with monoclonal antibody to L3T4. J Immunol 138 (May 15 1987): 3247-3253.

[116] Mihara M, Ohsugi Y, Saito K, Miyai T, Togashi M, Ono S, et al. 1988. Immunologic abnormality in NZB/NZW F1 mice. Thymus-independent occurrence of B cell abnormality and requirement for T cells in the development of autoimmune disease, as evidenced by an analysis of the athymic nude individuals. J Immunol 141 (Jul 1 1988): 85-90.

[117] Kariuki SN, Niewold TB. Genetic regulation of serum cytokines in systemic lupus erythematosus. Transl Res 155 (Mar: 109-117.

[118] Segal R, Bermas BL, Dayan M, Kalush F, Shearer GM, Mozes E. 1997. Kinetics of cytokine production in experimental systemic lupus erythematosus: involvement of T helper cell 1/T helper cell 2-type cytokines in disease. J Immunol 158 (Mar 15 1997): 3009-3016.

[119] Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136 (Apr 1 1986): 2348-2357.

[120] Del Prete GF, De Carli M, Mastromauro C, Biagiotti R, Macchia D, Falagiani P, et al. 1991. Purified protein derivative of Mycobacterium tuberculosis and excretory-secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production. J Clin Invest 88 (Jul 1991): 346-350.

[121] Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, et al. 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell 84 (Feb 9 1996): 431-442.

[122] Kaplan MH, Sun YL, Hoey T, Grusby MJ. 1996. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. Nature 382 (Jul 11 1996): 174-177.

[123] Kaplan MH, Schindler U, Smiley ST, Grusby MJ. 1996. Stat6 is required for mediating responses to IL-4 and for development of Th2 cells. Immunity 4 (Mar 1996): 313-319.

[124] Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100 (Mar 17 2000): 655-669.

[125] Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. 2006. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. Cell Res 16 (Jan 2006): 3-10.

[126] Reiner SL. 2001. Helper T cell differentiation, inside and out. Curr Opin Immunol 13 (Jun 2001): 351-355.

[127] Korn T, Bettelli E, Oukka M, Kuchroo VK. 2009. IL-17 and Th17 Cells. Annu Rev Immunol 27 2009): 485-517.

[128] Harrington LE, Mangan PR, Weaver CT. 2006. Expanding the effector CD4 T-cell repertoire: the Th17 lineage. Curr Opin Immunol 18 (Jun 2006): 349-356.

[129] Miller SA, Weinmann AS. 2009. Common themes emerge in the transcriptional control of T helper and developmental cell fate decisions regulated by the T-box, GATA and ROR families. Immunology 126 (Mar 2009): 306-315.

[130] Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, et al. 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature 448 (Jul 26 2007): 484-487.

[131] Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, et al. 2007. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature 448 (Jul 26 2007): 480-483.

[132] Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, Seymour B, et al. 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 421 (Feb 13 2003): 744-748.

[133] Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126 (Sep 22 2006): 1121-1133.

[134] Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, et al. 2007. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem 282 (Mar 30 2007): 9358-9363.

[135] Awasthi A, Kuchroo VK. 2009. Th17 cells: from precursors to players in inflammation and infection. Int Immunol 21 (May 2009): 489-498.

[136] Constantinescu CS, Wysocka M, Hilliard B, Ventura ES, Lavi E, Trinchieri G, et al. 1998. Antibodies against IL-12 prevent superantigen-induced and spontaneous relapses of experimental autoimmune encephalomyelitis. J Immunol 161 (Nov 1 1998): 5097-5104.

[137] Leonard JP, Waldburger KE, Goldman SJ. 1995. Prevention of experimental autoimmune encephalomyelitis by antibodies against interleukin 12. J Exp Med 181 (Jan 1 1995): 381-386.

[138] Segal BM, Dwyer BK, Shevach EM. 1998. An interleukin (IL)-10/IL-12 immunoregulatory circuit controls susceptibility to autoimmune disease. J Exp Med 187 (Feb 16 1998): 537-546.

[139] Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, et al. 2000. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13 (Nov 2000): 715-725.

[140] Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, et al. 2003. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. J Exp Med 198 (Dec 15 2003): 1951-1957.

[141] Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. 2006. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. J Immunol 177 (Jul 1 2006): 566-573.

[142] Nakae S, Nambu A, Sudo K, Iwakura Y. 2003. Suppression of immune induction of collagen-induced arthritis in IL-17-deficient mice. J Immunol 171 (Dec 1 2003): 6173-6177.

[143] Louten J, Boniface K, de Waal Malefyt R. 2009. Development and function of TH17 cells in health and disease. J Allergy Clin Immunol 123 (May 2009): 1004-1011.

[144] Evans HG, Suddason T, Jackson I, Taams LS, Lord GM. 2007. Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. Proc Natl Acad Sci U S A 104 (Oct 23 2007): 17034-17039.

[145] van Beelen AJ, Zelinkova Z, Taanman-Kueter EW, Muller FJ, Hommes DW, Zaat SA, et al. 2007. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. Immunity 27 (Oct 2007): 660-669.

[146] Burgler S, Ouaked N, Bassin C, Basinski TM, Mantel PY, Siegmund K, et al. 2009. Differentiation and functional analysis of human T(H)17 cells. J Allergy Clin Immunol 123 (Mar 2009): 588-595, 595 e581-587.

[147] Weaver CT, Hatton RD, Mangan PR, Harrington LE. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol 25 2007): 821-852.

[148] Bettelli E, Korn T, Oukka M, Kuchroo VK. 2008. Induction and effector functions of T(H)17 cells. Nature 453 (Jun 19 2008): 1051-1057.

[149] Zhou L, Ivanov, II, Spolski R, Min R, Shenderov K, Egawa T, et al. 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 8 (Sep 2007): 967-974.

[150] Batten M, Li J, Yi S, Kljavin NM, Danilenko DM, Lucas S, et al. 2006. Interleukin 27 limits autoimmune encephalomyelitis by suppressing the development of interleukin 17-producing T cells. Nat Immunol 7 (Sep 2006): 929-936.

[151] Stumhofer JS, Laurence A, Wilson EH, Huang E, Tato CM, Johnson LM, et al. 2006. Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system. Nat Immunol 7 (Sep 2006): 937-945.

[152] Rouvier E, Luciani MF, Mattei MG, Denizot F, Golstein P. 1993. CTLA-8, cloned from an activated T cell, bearing AU-rich messenger RNA instability sequences, and homologous to a herpesvirus saimiri gene. J Immunol 150 (Jun 15 1993): 5445-5456.

[153] Chen Z, O'Shea JJ. 2008. Regulation of IL-17 production in human lymphocytes. Cytokine 41 (Feb 2008): 71-78.

[154] Toy D, Kugler D, Wolfson M, Vanden Bos T, Gurgel J, Derry J, et al. 2006. Cutting edge: interleukin 17 signals through a heteromeric receptor complex. J Immunol 177 (Jul 1 2006): 36-39.

[155] Haudenschild D, Moseley T, Rose L, Reddi AH. 2002. Soluble and transmembrane isoforms of novel interleukin-17 receptor-like protein by RNA splicing and expression in prostate cancer. J Biol Chem 277 (Feb 8 2002): 4309-4316.

[156] Kuestner RE, Taft DW, Haran A, Brandt CS, Brender T, Lum K, et al. 2007. Identification of the IL-17 receptor related molecule IL-17RC as the receptor for IL-17F. J Immunol 179 (Oct 15 2007): 5462-5473.

[157] Liang SC, Long AJ, Bennett F, Whitters MJ, Karim R, Collins M, et al. 2007. An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. J Immunol 179 (Dec 1 2007): 7791-7799.

[158] Kawaguchi M, Adachi M, Oda N, Kokubu F, Huang SK. 2004. IL-17 cytokine family. J Allergy Clin Immunol 114 (Dec 2004): 1265-1273; quiz 1274.

[159] Dumoutier L, Louahed J, Renauld JC. 2000. Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9. J Immunol 164 (Feb 15 2000): 1814-1819.

[160] Fouser LA, Wright JF, Dunussi-Joannopoulos K, Collins M. 2008. Th17 cytokines and their emerging roles in inflammation and autoimmunity. Immunol Rev 226 (Dec 2008): 87-102.

[161] Lecart S, Morel F, Noraz N, Pene J, Garcia M, Boniface K, et al. 2002. IL-22, in contrast to IL-10, does not induce Ig production, due to absence of a functional IL-22 receptor on activated human B cells. Int Immunol 14 (Nov 2002): 1351-1356.

[162] Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, et al. 2007. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature 445 (Feb 8 2007): 648-651.

[163] Ouyang W, Kolls JK, Zheng Y. 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 28 (Apr 2008): 454-467.

[164] Boniface K, Bernard FX, Garcia M, Gurney AL, Lecron JC, Morel F. 2005. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. J Immunol 174 (Mar 15 2005): 3695-3702.

[165] Sa SM, Valdez PA, Wu J, Jung K, Zhong F, Hall L, et al. 2007. The effects of IL-20 subfamily cytokines on reconstituted human epidermis suggest potential roles in cutaneous innate defense and pathogenic adaptive immunity in psoriasis. J Immunol 178 (Feb 15 2007): 2229-2240.

[166] Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. 2004. IL-22 increases the innate immunity of tissues. Immunity 21 (Aug 2004): 241-254.

[167] Wolk K, Witte E, Wallace E, Docke WD, Kunz S, Asadullah K, et al. 2006. IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. Eur J Immunol 36 (May 2006): 1309-1323.

[168] Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, et al. 2006. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. Am J Physiol Gastrointest Liver Physiol 290 (Apr 2006): G827-838.

[169] Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, et al. 2008. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity 28 (Jan 2008): 29-39.

[170] Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, et al. 2008. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature 453 (May 1 2008): 106-109.

[171] Kimura A, Naka T, Nohara K, Fujii-Kuriyama Y, Kishimoto T. 2008. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. Proc Natl Acad Sci U S A 105 (Jul 15 2008): 9721-9726.

[172] Passerini L, Allan SE, Battaglia M, Di Nunzio S, Alstad AN, Levings MK, et al. 2008. STAT5-signaling cytokines regulate the expression of FOXP3 in CD4+CD25+ regulatory T cells and CD4+CD25- effector T cells. Int Immunol 20 (Mar 2008): 421-431.

[173] Hori S, Nomura T, Sakaguchi S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. Science 299 (Feb 14 2003): 1057-1061.

[174] Fontenot JD, Gavin MA, Rudensky AY. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 4 (Apr 2003): 330-336.

[175] Awasthi A, Riol-Blanco L, Jager A, Korn T, Pot C, Galileos G, et al. 2009. Cutting edge: IL-23 receptor gfp reporter mice reveal distinct populations of IL-17-producing cells. J Immunol 182 (May 15 2009): 5904-5908.

[176] Aujla SJ, Dubin PJ, Kolls JK. 2007. Th17 cells and mucosal host defense. Semin Immunol 19 (Dec 2007): 377-382.

[177] Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P, et al. 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med 194 (Aug 20 2001): 519-527.

[178] Huang W, Na L, Fidel PL, Schwarzenberger P. 2004. Requirement of interleukin-17A for systemic anti-Candida albicans host defense in mice. J Infect Dis 190 (Aug 1 2004): 624-631.

[179] Ishigame H, Kakuta S, Nagai T, Kadoki M, Nambu A, Komiyama Y, et al. 2009. Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. Immunity 30 (Jan 16 2009): 108-119.

[180] Tesmer LA, Lundy SK, Sarkar S, Fox DA. 2008. Th17 cells in human disease. Immunol Rev 223 (Jun 2008): 87-113.

[181] Chan JR, Blumenschein W, Murphy E, Diveu C, Wiekowski M, Abbondanzo S, et al. 2006. IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2-dependent mechanisms with implications for psoriasis pathogenesis. J Exp Med 203 (Nov 27 2006): 2577-2587.

[182] Kryczek I, Bruce AT, Gudjonsson JE, Johnston A, Aphale A, Vatan L, et al. 2008. Induction of IL-17+ T cell trafficking and development by IFN-gamma: mechanism and pathological relevance in psoriasis. J Immunol 181 (Oct 1 2008): 4733-4741.

[183] Zaba LC, Fuentes-Duculan J, Eungdamrong NJ, Abello MV, Novitskaya I, Pierson KC, et al. 2009. Psoriasis is characterized by accumulation of immunostimulatory and Th1/Th17 cell-polarizing myeloid dendritic cells. J Invest Dermatol 129 (Jan 2009): 79-88.

[184] Ortega C, Fernandez AS, Carrillo JM, Romero P, Molina IJ, Moreno JC, et al. 2009. IL-17-producing CD8+ T lymphocytes from psoriasis skin plaques are cytotoxic effector cells that secrete Th17-related cytokines. J Leukoc Biol 86 (Aug 2009): 435-443.

[185] Koenders MI, van den Berg WB. Translational mini-review series on Th17 cells: are T helper 17 cells really pathogenic in autoimmunity? Clin Exp Immunol 159 (Feb: 131-136.

[186] Ikeuchi H, Kuroiwa T, Hiramatsu N, Kaneko Y, Hiromura K, Ueki K, et al. 2005. Expression of interleukin-22 in rheumatoid arthritis: potential role as a proinflammatory cytokine. Arthritis Rheum 52 (Apr 2005): 1037-1046.

[187] Kim HR, Kim HS, Park MK, Cho ML, Lee SH, Kim HY. 2007. The clinical role of IL-23p19 in patients with rheumatoid arthritis. Scand J Rheumatol 36 (Jul-Aug 2007): 259-264.

[188] Maloy KJ. 2008. The Interleukin-23 / Interleukin-17 axis in intestinal inflammation. J Intern Med 263 (Jun 2008): 584-590.

[189] Monteleone I, Pallone F, Monteleone G. 2009. Interleukin-23 and Th17 cells in the control of gut inflammation. Mediators Inflamm 2009 2009): 297645.

[190] Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, et al. 2006. A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. Science 314 (Dec 1 2006): 1461-1463.

[191] Andoh A, Yasui H, Inatomi O, Zhang Z, Deguchi Y, Hata K, et al. 2005. Interleukin-17 augments tumor necrosis factor-alpha-induced granulocyte and granulocyte/macrophage colony-stimulating factor release from human colonic myofibroblasts. J Gastroenterol 40 (Aug 2005): 802-810.

[192] Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, et al. 2008. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. Nat Immunol 9 (Feb 2008): 166-175.

[193] Kang HK, Liu M, Datta SK. 2007. Low-dose peptide tolerance therapy of lupus generates plasmacytoid dendritic cells that cause expansion of autoantigen-specific regulatory T cells and contraction of inflammatory Th17 cells. J Immunol 178 (Jun 15 2007): 7849-7858.

[194] Wang D, John SA, Clements JL, Percy DH, Barton KP, Garrett-Sinha LA. 2005. Ets-1 deficiency leads to altered B cell differentiation, hyperresponsiveness to TLR9 and autoimmune disease. Int Immunol 17 (Sep 2005): 1179-1191.

[195] Bombardier C, Gladman DD, Urowitz MB, Caron D, Chang CH. 1992. Derivation of the SLEDAI. A disease activity index for lupus patients. The Committee on Prognosis Studies in SLE. Arthritis Rheum 35 (Jun 1992): 630-640.

[196] Vanaudenaerde BM, Dupont LJ, Wuyts WA, Verbeken EK, Meyts I, Bullens DM, et al. 2006. The role of interleukin-17 during acute rejection after lung transplantation. Eur Respir J 27 (Apr 2006): 779-787.

[197] Sodsai P, Hirankarn N, Avihingsanon Y, Palaga T. 2008. Defects in Notch1 upregulation upon activation of T Cells from patients with systemic lupus erythematosus are related to lupus disease activity. Lupus 17 (Jul 2008): 645-653.

[198] Dean GS, Tyrrell-Price J, Crawley E, Isenberg DA. 2000. Cytokines and systemic lupus erythematosus. Ann Rheum Dis 59 (Apr 2000): 243-251.

[199] Yang J, Chu Y, Yang X, Gao D, Zhu L, Wan L, et al. 2009. Th17 and natural Treg cell population dynamics in systemic lupus erythematosus. Arthritis Rheum 60 (May 2009): 1472-1483.

[200] Bettelli E, Oukka M, Kuchroo VK. 2007. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol 8 (Apr 2007): 345-350.

[201] Crispin JC, Oukka M, Bayliss G, Cohen RA, Van Beek CA, Stillman IE, et al. 2008. Expanded double negative T cells in patients with systemic lupus erythematosus produce IL-17 and infiltrate the kidneys. J Immunol 181 (Dec 15 2008): 8761-8766.

[202] Adalid-Peralta L, Mathian A, Tran T, Delbos L, Durand-Gasselin I, Berrebi D, et al. 2008. Leukocytes and the kidney contribute to interstitial inflammation in lupus nephritis. Kidney Int 73 (Jan 2008): 172-180.

[203] Enghard P, Humrich JY, Rudolph B, Rosenberger S, Biesen R, Kuhn A, et al. 2009. CXCR3+CD4+ T cells are enriched in inflamed kidneys and urine and provide a new biomarker for acute nephritis flares in systemic lupus erythematosus patients. Arthritis Rheum 60 (Jan 2009): 199-206.

[204] Robak E, Niewiadomska H, Robak T, Bartkowiak J, Blonski JZ, Wozniacka A, et al. 2001. Lymphocyctes Tgammadelta in clinically normal skin and peripheral blood of patients with systemic lupus erythematosus and their correlation with disease activity. Mediators Inflamm 10 (Aug 2001): 179-189.

[205] Kwan BC, Tam LS, Lai KB, Lai FM, Li EK, Wang G, et al. 2009. The gene expression of type 17 T-helper cell-related cytokines in the urinary sediment of patients with systemic lupus erythematosus. Rheumatology (Oxford) 48 (Dec 2009): 1491-1497.

APPENDICES

APPENDIX A

REAGENTS FOR CELLS CULTURE

1. Roswell Park Memorial Institute (RPMI)-1640 medium

RPMI-1640	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 (RPMI-1640 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. 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.

5. Ficoll-Hypaque solution

store in dark at 4°C.

6. 1% Trypan blue

Trypan blue	0.3	g
distilled water	30	ml

APPENDIX B

REAGENTS FOR FLOW CYTOMETRY

1. 2.5 mg/ml Phorbol-12-myristate-13-acetate (PMA)

Phorbol-12-myristate-13-acetate (PMA)	1	mg
Dimethyl sulfoxide (DMSO)	400	µl

store at -80°C.

2. 2.5 mg/ml Ionomycin

Ionomycin	1	mg
Dimethyl sulfoxide (DMSO)	400	µl

store at -80°C.

3. 5 mg/ml Brefeldin A

Brefeldin A	1	mg
Dimethyl sulfoxide (DMSO)	200	µl

store at -20°C.

4. 4% Sodium azide (NaN_3) solution

Sodium azide (NaN_3)	0.4	g
1X PBS	10	ml

store in dark at 4°C.

5. **10% saponin solution**

1X PBS	1	ml
Saponin	0.1	g

store in dark at 4°C.

6. **Staining buffer**

1X PBS	49.25	ml
4% Sodium azide (NaN_3) solution	250	µl
Fetal bovine serum (FBS)	500	µl

store in dark at 4°C.

7. **Permeabilization buffer**

1X PBS	2.91	ml
10% saponin solution	30	µl
4% Sodium azide (NaN_3) solution	30	µl
Fetal bovine serum (FBS)	30	µl

store in dark at 4°C.

8. **4% Paraformaldehyde**

Paraformaldehyde	4	g
1X PBS	100	ml

9. **1% Paraformaldehyde**

4% Paraformaldehyde	5	ml
1X PBS	15	ml

APPENDIX C

REAGENTS FOR ELISA

1. **Coating Buffer**

ELISA Coating Buffer powder	1	pack

Adjust volume up to 1 liter (L) with distilled water and sterile with filter 0.2 µm

2. **Capture Antibody**

Purified anti-human IL-17A	48	µl
Coating Buffer	12	ml

3. **Wash Buffer**

1X PBS	1000	ml
Tween-20	500	µl

4. **1X Assay diluent**

5X Assay diluent	10	ml
Distilled water	40	ml

5. **Standard solution**

1 µg/ml Recombinant human IL-17A	5	µl
1X Assay diluent	10	ml

6. **Detection Antibody**

Biotin-conjugate anti-human IL-17A	48	µl
1X Assay diluent	12	ml

7. Enzyme solution

Avidin-HRP solution	48	µl
1X Assay diluent	12	ml

8. Substrate solution

1X TMB solution	20	ml

9. Stop Solution

1M H_3PO_4	30	ml

BIOGRAPHY

NAME	Miss Hathaipat Phuwipirom
DATE OF BIRTH	9th February 1984
PLACE OF BIRTH	Pattani, Thailand
INSTITUTION ATTENDED	Kasetsart University,
	2002-2005
	Bachelor of Sciences (Microbiology)