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โครงการวิจัยเรื่อง
(ภาษาไทย) ระดับของ IL-17 ในผู้ป่วยเอสแอลอีชาวไทย
(ภาษาอังกฤษ) Level of IL-17 in Thai SLE Patients

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กิตติกรรมประกาศ

โครงการวิจัยนี้ดำเนินการโดยได้รับการสนับสนุนจากทุนอุดหนุนการวิจัยจากเงินอุดหนุนทั่วไปจากรัฐบาล ประจำปีงบประมาณ 2552 คณะผู้วิจัยขอขอบคุณ รองศาสตราจารย์นายแพทย์ยิ่งยศ อวิหิงสานนท์ และแพทย์หญิงปูชนียะดา วิเชียรธรรม ที่ได้ให้ความอนุเคราะห์ในการเก็บตัวอย่างจากผู้ป่วย คุณสุปราณี บูรณประดิษฐ์กุล ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัยและคุณนพดล สะอาดเอี่ยม คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ที่ช่วยในการวิเคราะห์ตัวอย่างโดยเครื่องโฟลไซโตมิเตอร์ สุดท้ายนี้ คณะผู้วิจัยขอขอบคุณอาสามัครทุกท่านที่กรุณาเข้าร่วมในโครงการวิจัยนี้

บทคัดย่อ

โรคเอสแอลอี (Systemic Lupus Erythematosus; SLE) จัดเป็นโรคออโต้อิมมูนชนิดหนึ่ง ซึ่งมีผลกระทบในหลายระบบของร่างกาย ซึ่งในปัจจุบันสาเหตุของโรคยังไม่ทราบแน่ชัด แต่หนึ่งในสาเหตุที่คาดว่ามีบทบาทสำคัญต่อการเกิดโรค คือ ความผิดปกติของเครือข่ายไซโตไคน์และเซลล์ลิมโฟซัยท์ โดยได้มีรายงานก่อนหน้านี้ว่ามีการเพิ่มสูงขึ้นของไซโตไคน์ในผู้ป่วยเอสแอลอี เช่น IL-1β IL-6 IL-23 เป็นต้น ในงานวิจัยนี้จึงมีจุดประสงค์ที่จะศึกษาระดับของไซโตไคน์ IL-17 ซึ่งเป็นไซโตไคน์ที่ผลิตจากเซลล์หลายชนิดรวมถึงทีลิมโฟซัยท์ด้วย CD4+ ทีลิมโฟซัยท์ชนิดที่ผลิต IL-17 เป็นหลักเรียกว่า Th17 ซึ่งได้มีรายงานมาก่อนหน้านี้ว่า Th17 อยู่ภายใต้การควบคุมของไซโตไคน์ IL-1β/IL-6 และ IL-23 อีกทั้ง IL-23 ยังมีบทบาทสำคัญในการรักษาฟีโนไทป์ของ Th17 อีกด้วย โดยในงานวิจัยนี้ได้ทำการทดลองในตัวอย่างจากผู้ป่วยเอสแอลอีจำนวน 29 ราย จากโรงพยาบาลจุฬาลงกรณ์ โดยแยกตามระดับความรุนแรงของโรค SLEDAI Score เป็นผู้ป่วยในระยะ active จำนวน 13 รายและระยะ inactive จำนวน 16 ราย และกลุ่มควบคุมปกติจำนวน 10 ราย โดยวิเคราะห์การแสดงออกของ IL-23R ใน PBMC พบว่าในผู้ป่วยเอสแอลอีมีเปอร์เซนต์ของ $CD4^+IL\text{-}23R^+$ และ $CD8^+IL\text{-}23R^+$ ทีลิมโฟซัยท์ที่สูงกลุ่มควบคุมอย่างมีนัยสำคัญ ทั้งใน PBMC ที่ไม่ได้รับการกระตุ้น (day 0) (เปอร์เซนต์ของ $CD4^+IL\text{-}23R^+$; $p = 0.0025$, $p = 0.0201$ และ $p = 0.0021$ ในกลุ่ม inactive, active และ total SLE เมื่อเปรียบเทียบกับกลุ่มควบคุม ตามลำดับ) (เปอร์เซนต์ของ $CD8^+IL\text{-}23R^+$; $p = 0.0021$, $p = 0.0032$ และ $p = 0.0008$ ในกลุ่ม inactive, active และ total SLE เมื่อเปรียบเทียบกับกลุ่มควบคุม ตามลำดับ) และ PMBC ที่กระตุ้น *ex vivo* โดยแอนติบอดีต่อ CD3 และ CD28 (day 3) (เปอร์เซนต์ของ $CD4^+IL\text{-}23R^+$; $p = 0.0057$, $p = 0.0011$ และ $p = 0.0007$ ในกลุ่ม inactive, active และ total SLE เมื่อเปรียบเทียบกับกลุ่มควบคุม ตามลำดับ) (เปอร์เซนต์ของ $CD8^+IL\text{-}23R^+$; $p = 0.0041$, $p = 0.0101$ และ$p = 0.0019$ ในกลุ่ม inactive, active และ total SLE เมื่อเปรียบเทียบกับกลุ่มควบคุม ตามลำดับ) เมื่อวิเคราะห์เปอร์เซนต์ของทีลิมโฟซัยท์ที่สามารถสังเคราะห์ IL-17 พบว่าผู้ป่วยเอสแอลอีมีเปอร์เซนต์ของ $CD4^+IL\text{-}17^+$ ที่ต่ำกว่ากลุ่มควบคุมอย่างมีนัยสำคัญเฉพาะที่ day 0 ($p = 0.0219$ และ $p = 0.0197$ ในกลุ่ม inactive และ total SLE เมื่อเปรียบเทียบกับกลุ่มควบคุม ตามลำดับ) และมี $CD8^+IL\text{-}17^+$ สูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญที่ day 3 ($p = 0.0007$, $p = 0.0120$ และ$p = 0.0007$ ในกลุ่ม inactive, active และ total SLE เมื่อเปรียบเทียบกับกลุ่มควบคุม ตามลำดับ) เมื่อเปรียบเทียบระดับของ IL-17 ในซีรัมและพลาสมาของผู้ป่วยกับกลุ่มควบคุมโดยวิธี ELISA พบว่ามีผู้ป่วยจำนวน 2 รายที่สามารถตรวจพบ IL-17 ได้ในระดับ 5.12-6.78 pg/mL แต่พบว่าตัวอย่างส่วนใหญ่ไม่สามารถตรวจพบ IL-17 ได้ เมื่อตรวจหา IL-23 ในซีรัมโดย ELISA ก็ไม่สามารถตรวจพบได้ จึงได้วิเคราะห์การแสดงออกของยีน *IL-17A* โดยวิธี Realtime RT-PCR พบว่าใน PBMC จากผู้ป่วยมีแนวโน้มการเพิ่มขึ้นของ *IL-17A* เมื่อเปรียบเทียบกับกลุ่มควบคุมแต่ยังไม่ถึงระดับที่มีนัยสำคัญ เมื่อนำผลที่ได้ทั้งหมดมาหาความสัมพันธ์พบว่าเปอร์เซนต์ของทีลิมโฟซัยท์ในผู้ป่วยที่มีฟีโนไทป์ $CD4^+IL\text{-}23R^+$, $CD8^+IL\text{-}23R^+$ มีความสัมพันธ์ในทางบวกกับเปอร์เซนต์ของเซลล์ที่มีฟีโนไทป์ $CD4^+IL\text{-}17^+$, $CD8^+IL\text{-}17^+$ ตามลำดับอย่างมีนัยสำคัญ อีกทั้ง เปอร์เซนต์ของเซลล์ที่มีฟีโนไทป $CD4^+IL\text{-}23R^+$ ที่ day 3 มีความสัมพันธ์ในทางบวกกับระดับการแสดงออกของ *IL-17A* ในการเปรียบเทียบระหว่างกลุ่มผู้ป่วย active และ inactive นั้น ในตัวชี้วัดทั้งหมดของงานวิจัยนี้ ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติ ดังนั้น ผลที่ได้จากงานวิจัยนี้บ่งชี้ว่าทีลิมโฟซัยท์ในผู้ป่วยเอสแอลอีมีการแสดงออกของ IL-23R และ IL-17 มากกว่าในกลุ่มควบคุม ซึ่งอาจจะมีบทบาทสำคัญต่อการเกิดพยาธิสภาพของโรคเอสแอลอี

ABSTRACT

Systemic Lupus Erythematosus (SLE) is an autoimmune disorder which affects various systems. Currently, the etiology of this disease has not been fully elucidated. One of potential causes which may play an important role is the defects in cytokine network and the functions of T lymphocytes. Previously, it was reported that SLE patients showed elevated level of various cytokines such as IL-1β IL-6 IL-23. The aim of this study was to investigate the level of cytokine IL-17 which could be produced by various cell types including T lymphocytes. $CD4^+$ T lymphocytes mainly producing IL-17 form a distinct lineage called Th17. Differentiation of Th17 is under the influence IL-1β/IL-6 and IL-23. Furthermore, IL-23 also plays an important role in maintaining the phenotype of Th17. In this study, 29 SLE patients at King Chulalongkorn Memorial Hospital were recruited and were divided based on SLEDAI score which is an indicator of the severity of disease. There were 13 patients in active stages and 16 patients in inactive stages and 10 normal subjects were used as control group. First, the frequency of T lymphocytes expressing IL-23R in PBMC was analyzed. The frequency of $CD4^+$IL-$23R^+$ and $CD8^+$IL-$23R^+$ T lymphocytes in PBMC from SLE patients were found to be significantly higher than those of controls, both in freshly isolated PBMC (day 0) ($p = 0.0025$, $p = 0.0201$ and $p = 0.0021$ for $CD4^+$ and $p = 0.0021$, $p = 0.0032$ and $p = 0.0008$ for $CD8^+$ for the inactive, the active and the total SLE groups in comparison with normal subjects, respectively) and PBMC receiving *ex vivo* stimulation by anti-CD3 and CD28 antibodies (day 3) ($p = 0.0057$, $p = 0.0011$ and $p = 0.0007$ for $CD4^+$ and $p = 0.0041$, $p = 0.0101$ and $p = 0.0019$ for $CD8^+$ for the inactive, the active and the total SLE groups in comparison with normal subjects, respectively). When the frequency of T cells which could produce IL-17 was measured, PBMC from SLE patients showed significantly lower percentages of $CD4^+$IL-17^+ T lymphocytes than those from the control group in samples from day 0 ($p = 0.0219$ and $p = 0.0197$ for the inactive and the total SLE in comparison with normal subjects, respectively) but the trend reversed on day 3 when SLE patients exhibited a tendency to have higher percentage of $CD4^+$IL-17^+ T cells. In contrast, the frequency of $CD8^+$IL-17^+ T cells in SLE patients was significantly higher on day 3 ($p = 0.0007$, $p = 0.0120$ and $p = 0.0007$ for the inactive, the active and the total SLE in comparison with normal subjects, respectively), while there was no difference on day 0. When the level of IL-17 in serum and plasma were measured by ELISA, only two samples were found to show detectable IL-17 at 5.12-6.78 pg/ml, while the level in the rest of the samples was below detectable. When IL-23 was measured in sera by ELISA, all samples showed negative results. Therefore, the expression of *IL-17A* was analyzed by Realtime RT-PCR and it was found that PBMC from patients showed increased, but not statistically significant, level of *IL-17A*, when compared with the controls. Analysis of all results obtained in this study, the correlation was found to be significant between percentages of $CD4^+$IL-$23R^+$, $CD8^+$IL-$23R^+$ T lymphocytes and $CD4^+$IL-17^+, $CD8^+$IL-17^+ T lymphocytes in SLE patients. Furthermore, the percentages of $CD4^+$IL-$23R^+$ T cells at day 3 were found to have a correlation with *IL-17A* expression in SLE patients. When the active patients were compared against the inactive patients for all indicators investigated in this study, no statistically significant difference was detected. 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.

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ELISA: Enzyme-linked immunosorbent assay
Interleukin 17: IL-17
Peripheral blood mononuclear cells: PBMCs
SLE: Systemic Lupus Erythematosus
Th17: T helper 17
Threshold cycle (C_T)

Introduction

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 [1]. 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 [1].

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 [3]. The preliminary criteria were revised and updated to include new immunologic knowledge and improve disease classification in 1982 [4]. 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)(Table 1). All these indices have been validated and have excellent reliability, validity and responsiveness to change [5].

SLE has a worldwide distribution, with differences in prevalence related to race, geography and sex [6,7]. The reported prevalence of SLE in the general population is approximately 20-150 per 100,000 [8-10]. SLE primarily affects women in the child-bearing years, and is estimated to be 9–12 times more common in women than in men [11]. The disease is more frequent in blacks/African Americans, Hispanics and Indians/Native Americans than in whites/Caucasians [6,7]. The prevalence of SLE in Asia is estimated to be 50 to 100 per 100,000 [12]. 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 [13]. The frequencies of renal disease were 50% in Chinese patients and 31-39% in Caucasian patients [14-16]. 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 [17]. Moreover, another manifestation such as neuropsychiatry was also found with higher frequency in Thai patients, compared to Chinese patients [18].

Table 1 Scoring for SLE Disease Activity Index (SLEDAI) 2000 (SLEDAI-2K) [19]

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, disorganized or catatonic behavior. Exclude uremia 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	$< 100 \times 10^9$ platelets/l, exclude drug causes
1	Leucopenia	$< 3 \times 10^9$ 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.

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 [14].

1) 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 [14,20-22]. 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 [23]. Other genes which contain risk variants for SLE are *IRF5* [24-26], *PTPN22* [27], *STAT4*, *BLK* [28], *CDKN1A* [29], *ITGAM* [30], *TNFSF4* and *BANK1* [31]. Some of the susceptibility genes may be population specific.

2) 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 [32]. 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 [33]. 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 [34,35]. 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 [36]. 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 [14,36]. 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 [14,37].

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 [38].

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 [39].

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 [40]. 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 [41]. 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 [42]. Moreover, elevated BAFF serum levels have been found in human SLE and this rising correlates with serum IgG and autoantibody levels [43]. Altered signals of B cells such as CD80/CD86, CD19 and CD22 also might be involved in autoimmunity [44,45]. Additionally, somatic hypermutation of immunoglobulin variable regions, abnormality of receptor editing, antigen-drived autoantibody production and defect of idiotypic networks could be associated with SLE development [46-49].

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 [50]. In addition, athymic mice do not develop SLE [51]. 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 [52] . 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 [53]. 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 [52].

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 [54-56]. 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 [57]. Overexpression of CD40L leading to prolong co-stimulation that sustains autoantibody producing B cells [58]. 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 [59].

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 [60]. SLE T cells resist anergy and apoptosis by upregulating *cox-2* and *bcl-2* expression, both of which encoding anti-apoptotic proteins [61-63]. Increased apoptosis, however, has been reported in SLE [64]. 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 [65]. 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 [66-68], whereas some investigators reported that suppressive functions of Treg cells were poor during active SLE [69]. Therefore, it is still controversial whether Treg plays a role in SLE.

Subsets of T Helper Cells

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 [70,71]. 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 [72,73]. Similarly, STAT-6 activation is necessary for Th2 development [74]. The lineage-specifying transcription factors for Th1 and Th2 are T box family protein expressed in T cells (T-bet) [75] and GATA-binding protein 3 (GATA-3) [76], 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 [77-80].

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 Th 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 [81-87] and the key transcription factors that are involved in its generation have been elucidated [88,89], firmly establishing Th17 cells as an independent T helper cell lineage in human and mice.

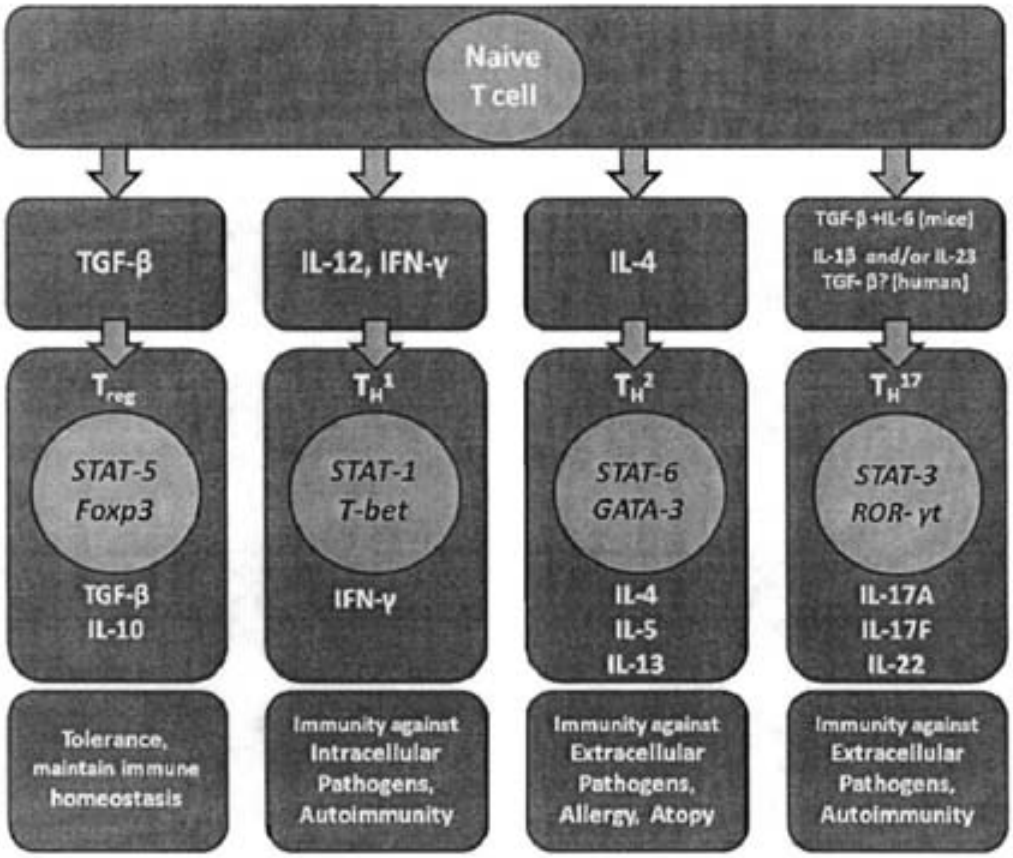


Figure 1 Subsets of Effector T Helper Cells
Antigen and specific cytokine signals induce the differentiation of naive T cells into various subsets of T helper cells (Th1, Th2 and Th17). While these subsets produce specific patterns of cytokines that induce immunity, regulatory T cells (Treg), naturally 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.

T Helper 17 (Th17)

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 [90-92]. This association, however, came into question with the discovery a new IL-12 family member, IL-23 [93]. 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 and colleagues revisited the development of EAE and CIA using mice deficient in IL-12 (*Il12p35*$^{-/-}$), IL-23 (*Il23p19*$^{-/-}$), or both (*Il12p40*$^{-/-}$) [86]. 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 and colleagues analyzed the cytokine production profiles of effector $CD4^+$T cells in CIA [94]. 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 [95,96]. 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 [97] 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 [87]. 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).

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 [87,98,99]. These cells contribute to host defense against extracellular pathogen, mainly at mucosal surface [83,100,101]. Their activities, however, are also pivotal in the development of autoimmune diseases under pathologic conditions [102].

After the identification of differentiation factors of mouse Th17 cells, 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 [103-106]. 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 [103,104,107]. 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 [108-110]. Volpe and colleagues [109] 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 and colleagues [108] 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 and colleagues [110] proposed that the combination of TGF-β and IL-21 activated the development of Th17 cells that produced IL-17. Manel and colleagues[108], however, did not detect production of IL-17 under the similar conditions. Finally, the recent

work of Cosmi and colleagues [111] 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 [112]. 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 [113]. Nevertheless, TGF-β was essential for expression of the key transcription factor *RORC2*, the closest human relative of murine RORγt [112]. Molecular mechanisms leading to differentiation and maturation of murine and human Th17 cells are shown in Figure 2.

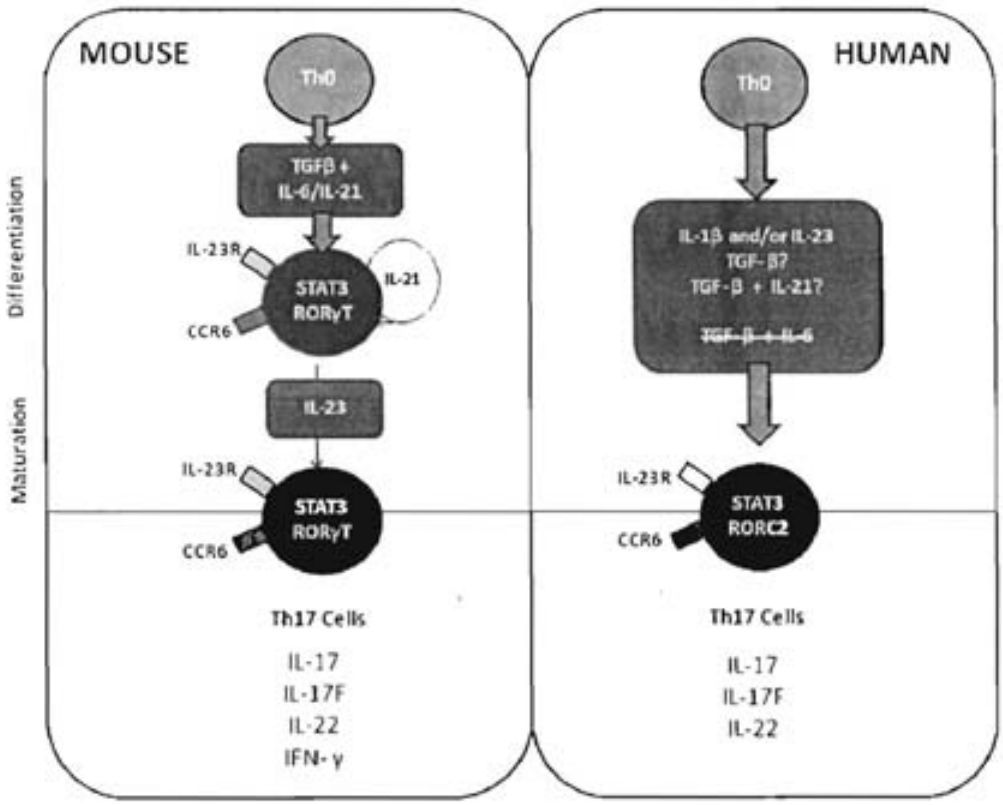


Figure 2 Molecular Mechanisms of Differentiation and Maturation of Murine and Human Th17 Cells

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.

The Signature Cytokine of Th17 Cells

Interleukin (IL)-17 or IL-17A

IL-17A was originally cloned and described by Rouvier and colleagues and named CTLA8 [114]. 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) [115]. 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 [116] and bind to the same receptors (IL-17RA and IL-17RC) [117]. IL-17RA is expressed broadly and mediates its effects through a number of immune and non-immune cells (particularly endothelial and epithelial cells) [97]. 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 [118,119]. 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. γδ T cells [120], $CD8^+$ memory T cells [121,122], NK T cells [123,124], NK cells [125], eosinophils [126], neutrophils [121] and monocytes [127] can also be a source of IL-17A. Nevertheless, the predominant source of IL-17A remains the $CD4^+$ T cell population [128].

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 [129]. 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 [130]. 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 [129]. 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 [119], 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) [131]. 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 [132]. Thus, immune cells are not target cells of IL-22 [133,134]. On the other hand, IL-22 elicits strong responses from many epithelial cells [135]. Data from many studies implicate the potential roles of IL-22 in host defense, inflammation and tissue repair [136-138]. 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 [137,139]. Furthermore, IL-22 also stimulates proliferation, abnormal differentiation, and migration of various epithelial cells *in vitro* [136,137,140]. Collectively, these data strongly support a role of IL-22 in host defense and epithelial-barrier function.

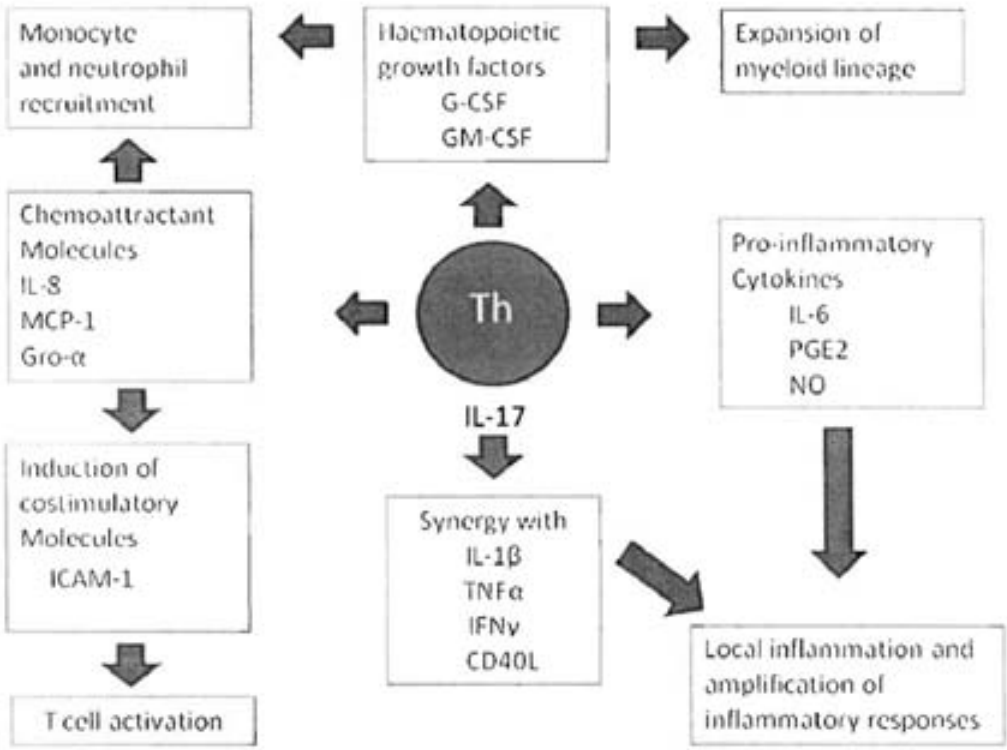


Figure 3 The Biological Function of IL-17A
IL-17A activates production of IL-6, nitric oxide and prostaglandin E_2 (PGE_2), while synergy with other inflammatory cytokines such as IL-1β, TNF-α, IFN-γ and CD40 ligand (by increasing surface levels of CD40) leads to progression and amplification of local 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. IL-17A also 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.

In addition to a unique cytokine expression profile, the T helper cell lineages are defined by lineage-specific transcription factors. 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 (RORC2 in human) is the key transcription factor that regulates the differentiation of Th17 lineage [88,107]. 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 [88]. 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* [141].

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 [142]. AhR deficiency was shown to impair, but not prevent, the generation of IL-17 producing T cells [142,143]. 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 [142].

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 [89].

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 [144]. 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 [145-147].

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

[148]. IL-17 promotes neutrophils and other myeloid cells recruitment at the site of infection by up-regulates 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* [149], and similarly, mice deficient in IL-17RA are seriously susceptible to *Candida albicans* [150].

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* [101] and *Citrobacter rodentium* infection [100], determined that IL-23–dependent production of IL-22 is essential for full protection of the host. Furthermore, IL-17F also plays a non-redundant role in protection against *C. rodentium* [151]. 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 2.

Table 2 Evidence for a Role of Th17 Cells in Immune-Mediated Diseases [152]

• 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 [107,139,153-155]. 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 [107]. 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 [156]. Interestingly, Ortega and colleagues 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 [157].

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 [158]. 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 [159-161]. Like psoriasis, there is also increased IL-22 and IL-23 in the synovium of RA patients [162,163].

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 [152], 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 and colleagues 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 [164]. 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 and colleagues 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 [165].

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 [166,167]. 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 [168]. Additionally, high IL-17 level is found in sera and colonic biopsies of Crohn's disease patients [169,170]. IL-22 is also overexpressed by colonic $CD4^+$ T cells in patients with IBD compared to healthy controls [171].

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 (Table 6) and patients with lupus.

Table 3 IL-17 in Murine Models of SLE

Murine Models	Experimental Evidence and Outcomes	References
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	[172]
SNF1	Increased numbers of IL-17$^+$ cells	[173]
Ets-1 knock-out	Enhanced differentiation of naive T cells into Th17 cells	[174]

In human SLE, many studies have reported potential roles for T-cell-derived cytokines in human SLE pathogenesis, but only recently have data involving IL-17 in SLE become available. Initial studies by Wong and colleagues in 2000, reported that the levels of IL-17 as measured by 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 [175]. In that same year, Kurasawa and colleagues 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 [176]. Eight years after these initial reports, in 2008, Wong and colleagues 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 [177].

Taken together, there are conflicting evidences in both human and mouse models on the role of IL-17 and its producing cells (Th17) in SLE progression.

Because SLE is known to be heterogeneous, it is likely that different mechanisms of pathogenesis occur in different cohorts of patients. Thus, the exact role of IL-17 and Th17 cells in SLE autoimmune disease need further investigation. This study aimed at investigating the IL-17 level, the frequency of IL-17 producing T cells in Thai SLE patients from King Chulalongkorn Memorial Hospital and Bhumibol Adulyadej Hospital. The results obtained from this study was the first to be conducted in SLE Thai patients and may lead to a better understanding of the the role IL-17 plays in this disease.

Materials and Methods

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, and disease activity was evaluated by the SLE disease activity index (SLEDAI) 2000 score (Table 1). Active SLE disease was defined as a SLEDAI-2K score ≥ 6 and inactive SLE disease was defined as a SLEDAI-2K score < 6 [178]. 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 4. 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 counted in hemacytometer chamber and adjusted to 1×10^6 cells/ml for the experiments described below. Four milliliters of venous peripheral blood from each patients and normal subjects were collected in serum collection tubes (BD Pharmingen Corp). 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, CA, USA) (both at 1 μg/ml), in RPMI-1640 medium (GIBCO, Germany) supplemented with

10% heat-inactivated fetal bovine serum (GIBCO), 100 U/mL penicillin (GIBCO), 100 μg/ml streptomycin (GIBCO) and non-essential amino acids (GIBCO) for 72 hours at 37°C in a 5% CO_2 environment (day 3). After incubation, PBMCs were harvested 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™) with 25 μg/ml PMA (Merck, Germany) and 10 μg/ml ionomycin (Merck) in different wells for PBMCs from each SLE patients and normal subjects, in the presence of 10 μg/ml brefeldin A (Merck). 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. PBMCs were then centrifuged at 1500 rpm for 10 minutes at 4°C. PBMCs were collected and subjected to surface and intracellular cytokine staining with antibodies as listed above.

Surface and Intracellular Cytokine Staining

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). The conjugated antibodies 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. Cells were fixed in 300 μl of fixation buffer (4% paraformaldehyde (Sigma-Aldrich) 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 milliliter of permeabilization buffer (PBS supplemented with 0.04% sodium azide (Sigma-Aldrich), 0.1% bovine serum albumin (GIBCO) and 0.1 %saponin)) was added to the cells and they were incubated for 10 minutes at room temperature in dark. 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. Cells were washed with 1 ml of permeabilization buffer by centrifugation at 1500 rpm for 10 minutes at 4°C, and 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 FACSCalibur 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, 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 was developed for detection and quantification of *IL-17A* and *RORC* transcripts using *β-actin* housekeeping transcripts as internal control. Primers were indicated in Table 5. 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, Indianapolis, 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 = CT (target) -ΔC_T (reference)

$\Delta\Delta C_T$ = ΔC_T (normalized target) -ΔC_T (normalized calibrator)

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 the plate was then sealed and incubated 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, San Diego, 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 4 Characteristics of SLE Patients Included in This Study

No.	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

Table 5 List of Primers Used for Analysis of the *IL-17A*, *RORC* and *β-actin*

Primers	Sequence (5'→3')	Product Size
IL-17A [179]		95 bp
- Forward	AATCTCCACCGCAATGAGGA	
- Reverse	ACGTTCCCATCAGCGTTGA	
RORC (This study)		
115 bp		
- Forward	CCGAGATGCTGTCAAGTTC	
- Reverse	CTTGACCACTGGTTCCTGTT	
β-actin [180]		380 bp
- Forward	ACCAACTGGGACGACATGGAGAA	
- Reverse	GTGGTGGTGAAGCTGTAGCC	

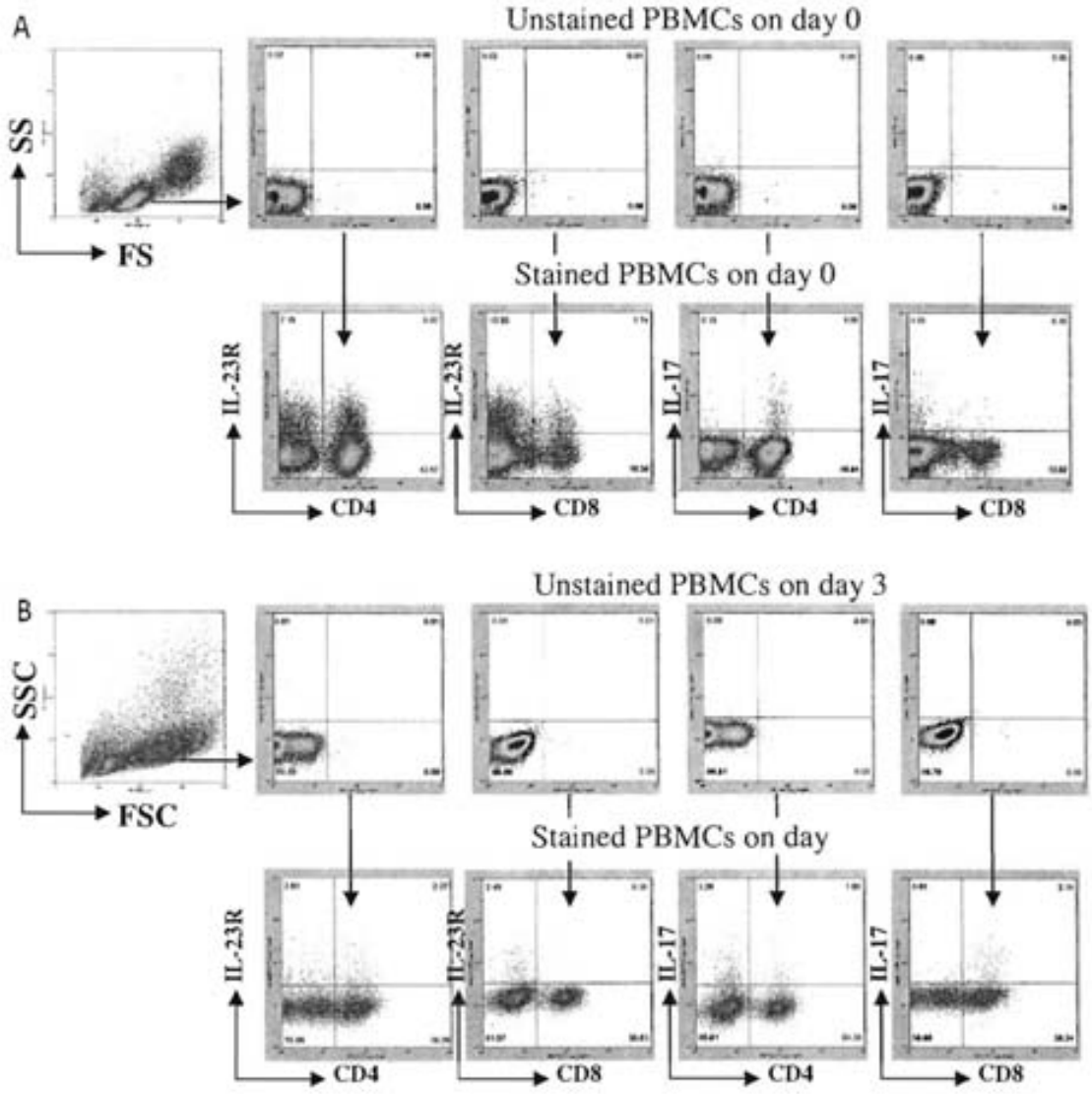


Figure 4 Representative FACS Plots and Gating Strategy Used in This Study
PBMCs were gated on small lymphocytes on the basis of their profiles of forward and side scatters. The gated populations were analyzed in CD4 vs. IL-23R, CD8 vs. IL-23R, CD4 vs. IL-17 and CD8 vs. IL-17 scatter plot. Values of negative 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.

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 6. 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 7.

Table 6 Characteristics of Individual Subjects Recruited for Flow Cytometric Analysis, Serum Cytokine Detection by ELISA and Semi-Quantitative Real-time RT-PCR Analysis

Sample No.	Stage	Age (yr)	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	Day 0 *β-actin* Ct	Day 3 *β-actin* Ct
Normal 1	normal	28	F	-	-	-	-	✓	✓	✓	23.19	20.45
Normal 2	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
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

Table 6 (Continued)

Sample No.	Stage	Age (yr)	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
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
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

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. The samples where highlighted in grey were excluded from further analysis.

Table 7 Characteristics of Individual Subjects Recruited for Plasma Cytokine Detection by ELISA

Sample No.	Stage	Age (yr)	Sex	Treatments	Doses (mg/day)	SLEDAI-2K Scores	Clinical Features	Sample No.	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

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 to play a role 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 freshly isolated PBMCs (day 0), and in PBMCs cultured in the presence of plate-bound anti-CD3 and anti-CD28 antibodies (day 3). 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 (CD4$^+$ and CD8$^+$) may be one of the features of SLE patients.

Since IL-17 has been shown to play an important role in many autoimmune and inflammatory diseases, we next analyzed the production of IL-17 from T cells by intracellular cytokine staining. When we studied freshly isolated cells (day 0), the percentages of IL-17$^+$CD4$^+$ T cells in normal subjects (Figure 7A: left panel) 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 7A: left panel) 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 9A; right panel). 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 panel). 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.

Interestingly, we observed a correlation 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 9A and 9C,

respectively). Moreover, a significant correlation 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).

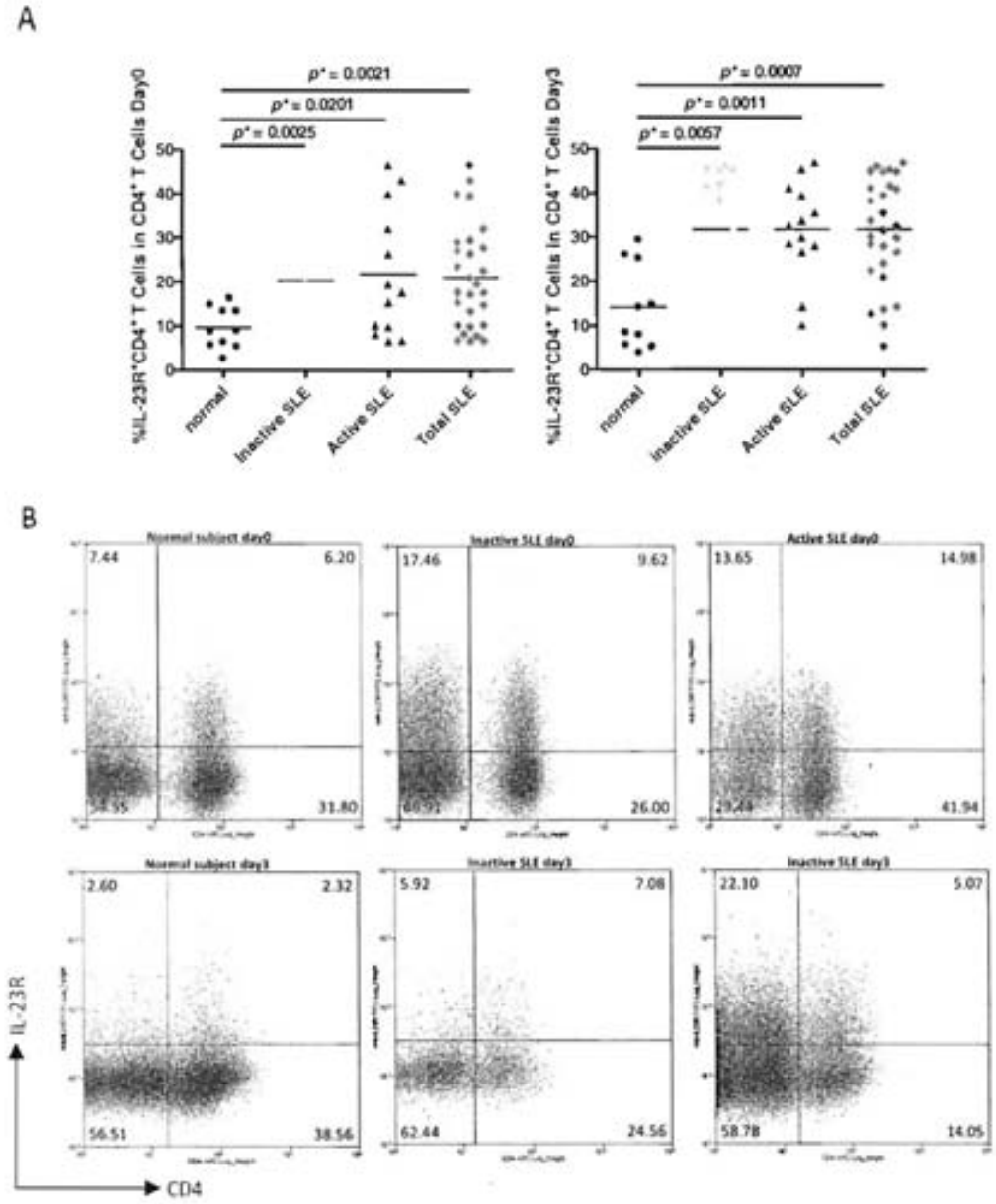


Figure 5 The Percentages of IL-23R$^+$CD4$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects

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 panel) and day 3 (A: right panel). 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) are shown.

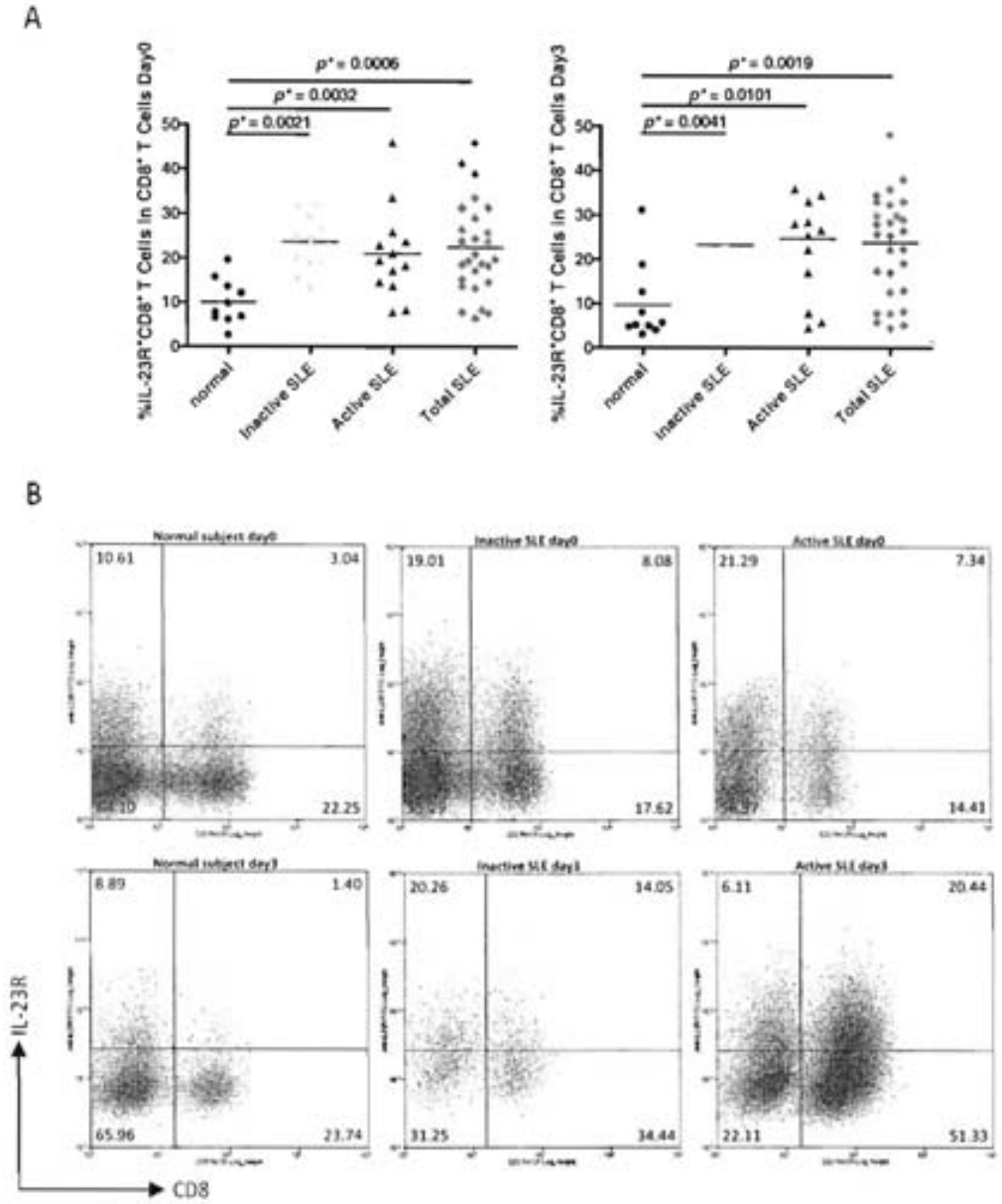


Figure 6 The Percentages of IL-23R$^+$CD8$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects

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 panel) and day 3 (A: right panel). 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) are shown.

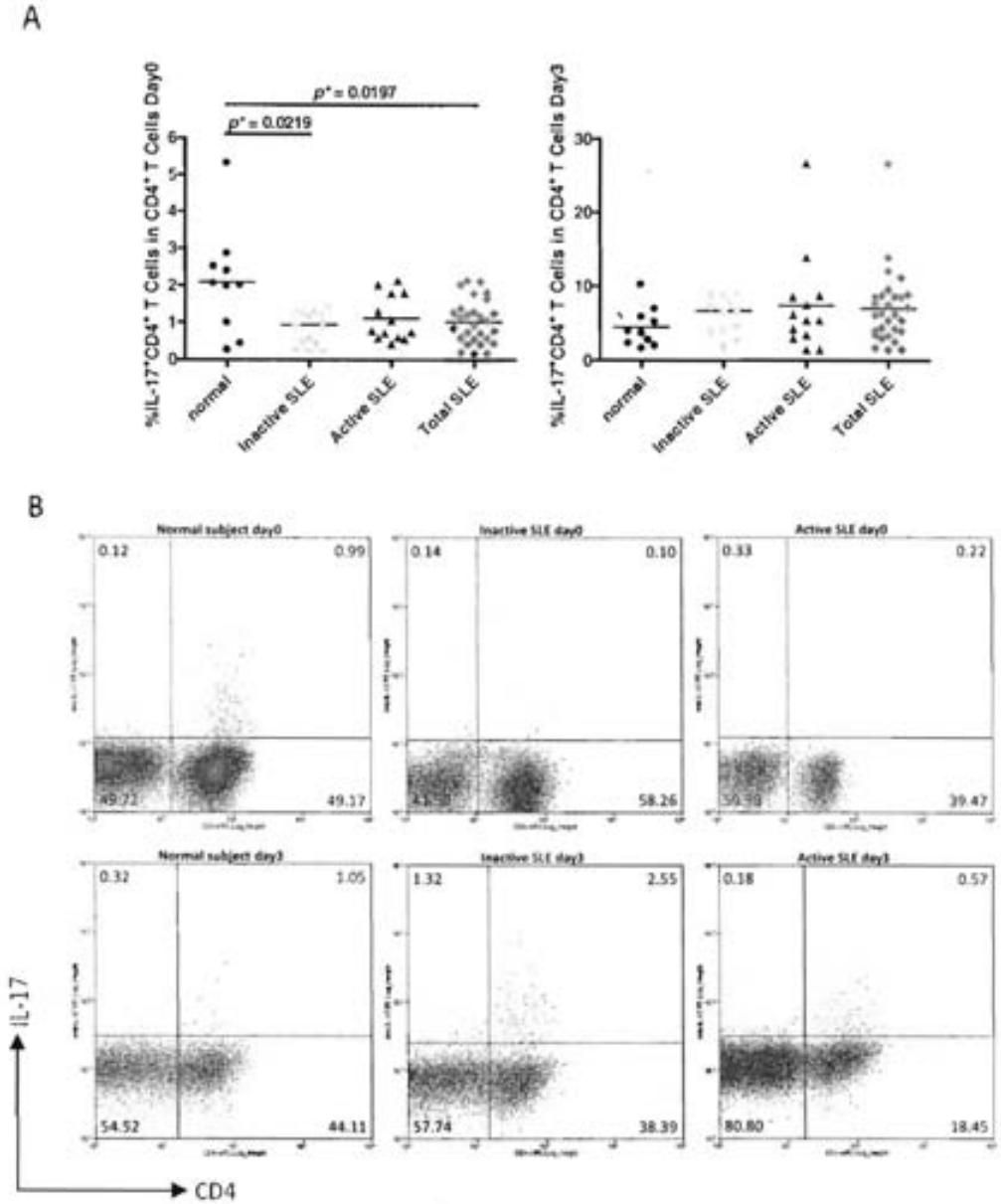


Figure 7 The Percentages of IL-17$^+$CD4$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects

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 panel) and day 3 (A: right panel). 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) are shown.

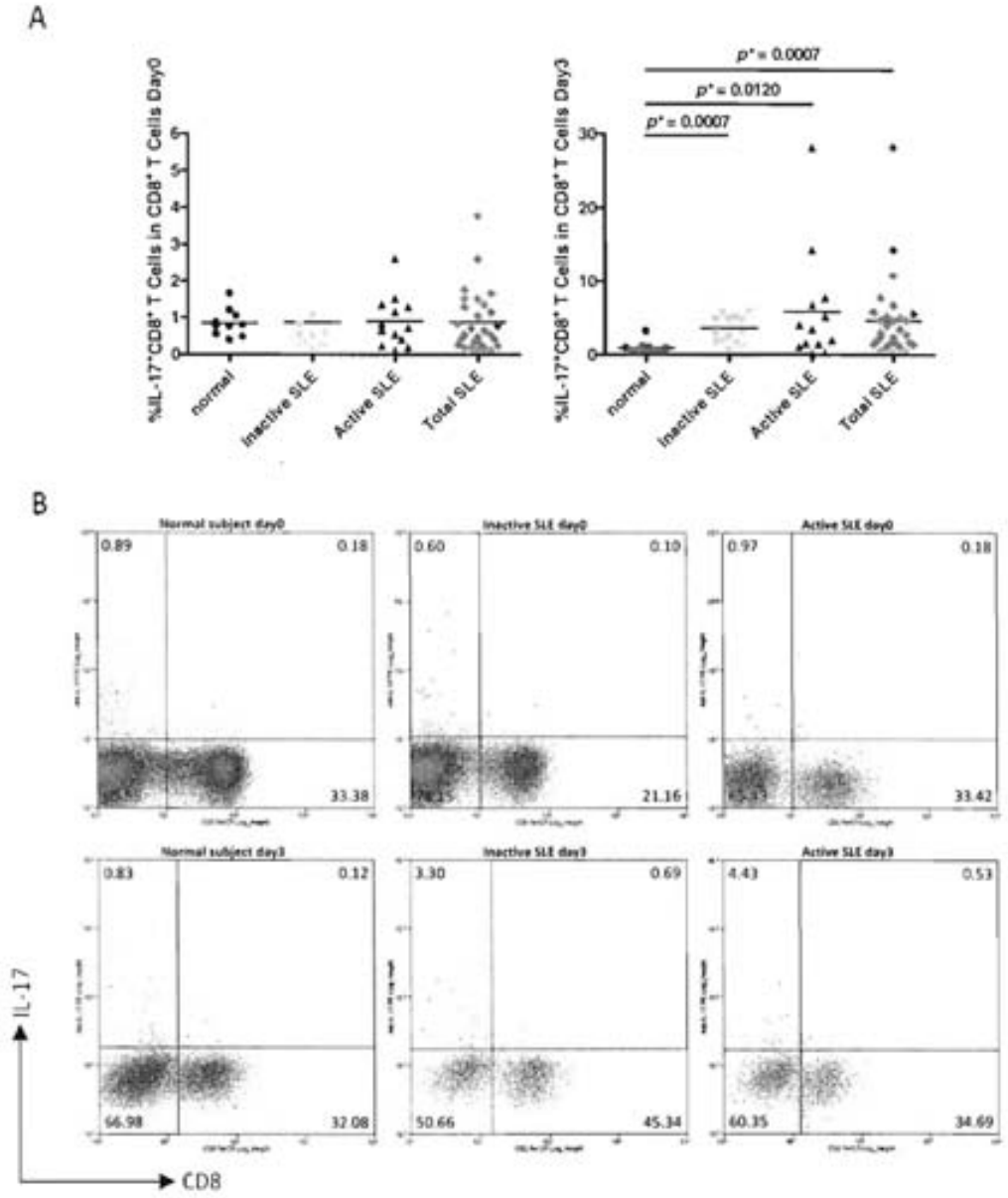


Figure 8 The Percentages of IL-17$^+$CD8$^+$ T Cells in PBMCs from SLE Patients and Normal Subjects

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 panel) and day 3 (A: right panel). 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) are shown.

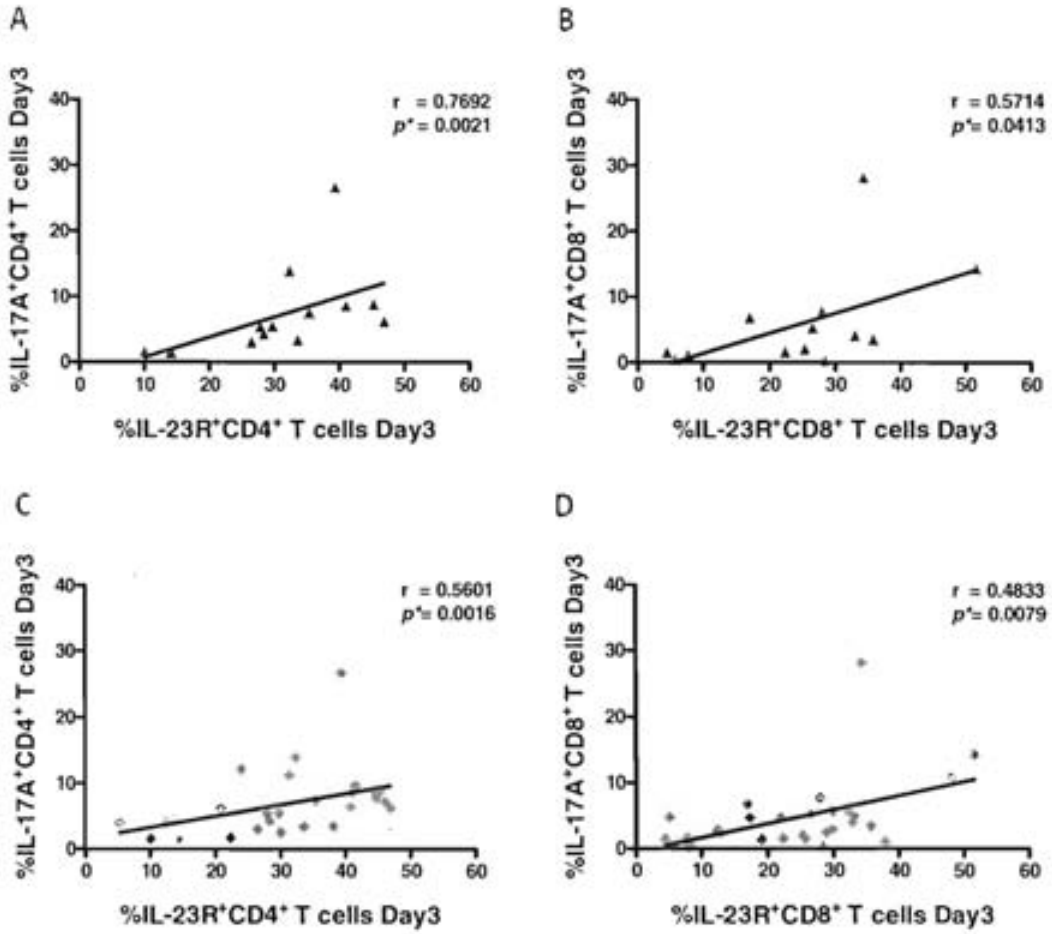


Figure 9 Correlations Between the Percentages of IL-23R$^+$ T Cells and the Percentages of IL-17$^+$ T Cells

(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

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 than those from healthy controls. 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 the rest of SLE patients (Figure 10). 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 11). Since previous studies [175,177] 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 8). 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 the rest of SLE patients (Figure 12). For IL-23 detection in plasma, all samples yielded undetectable results (Figure 13).

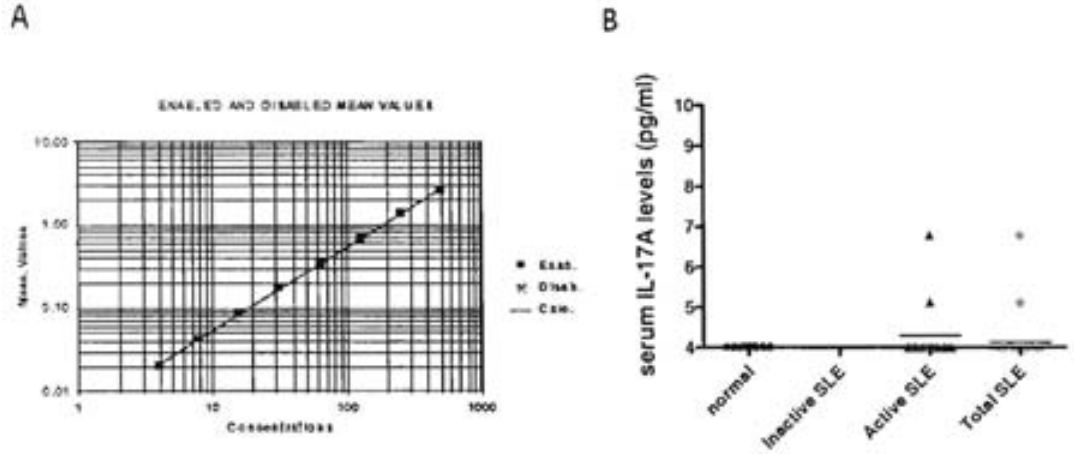
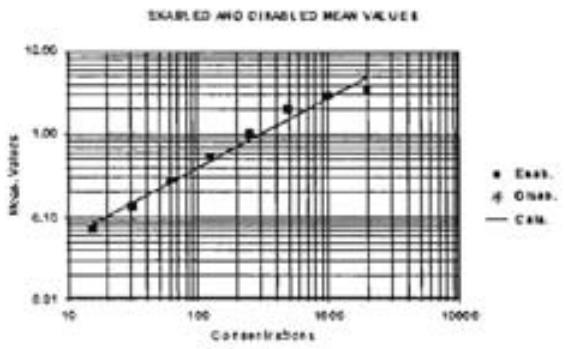


Figure 10 Detection of Serum IL-17A Levels in SLE Patients and Normal Subjects by ELISA

(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 samples from active SLE patients (5.12 and 6.78 pg/ml, respectively).

A



B

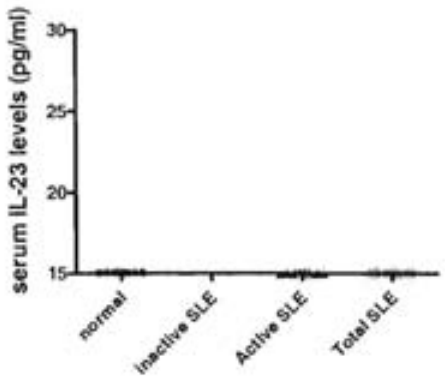


Figure 11 Detection of Serum IL-23 levels in SLE Patients and Normal Subjects by ELISA

(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.

Table 8 Individual Characteristics and Summary of the Results of Samples Recruited for Plasma Cytokine Detection by ELISA

Sample No.	Stage	Treatments	Doses (mg/day)	SLEDAI-2K scores	Clinical Features	Plasma Cytokine (pg/ml)		No. Samples	Stage	Treatments	Doses (mg/day)	SLEDAI-2K scores	Clinical Features	Plasma Cytokine (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	-	8.50	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

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.

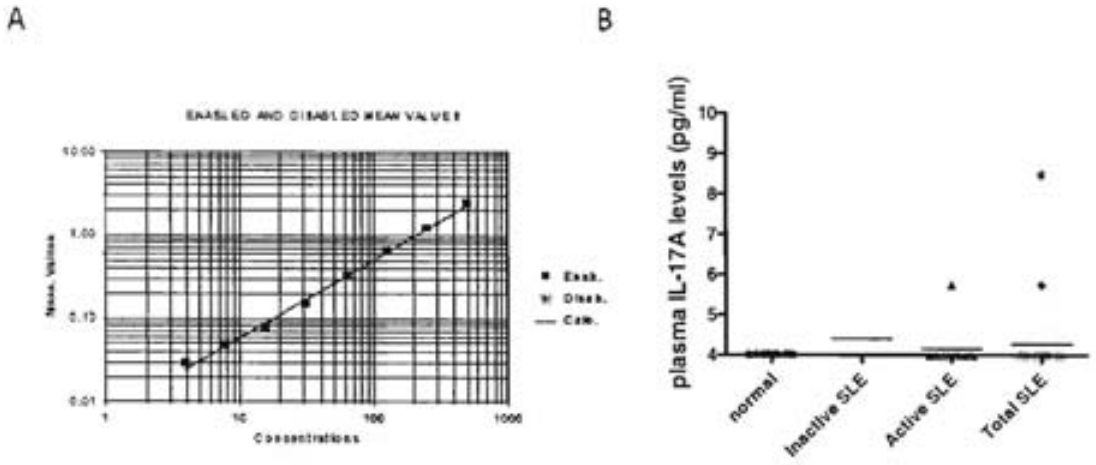


Figure 12 Detection of Plasma IL-17A Levels in SLE Patients and Normal Subjects by ELISA

(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 sample from active and one sample from inactive SLE patient (5.75 and 8.50, respectively).

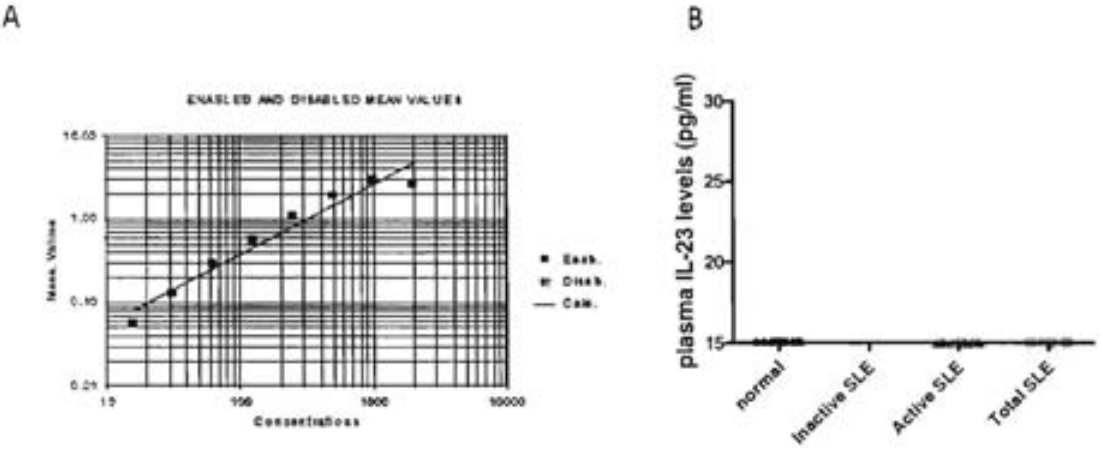


Figure13 Detection of Plasma IL-23 Levels in SLE Patients and Normal Subjects by ELISA

(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 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 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 (the threshold cycle (C_t) values for *IL-17A* 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 14).

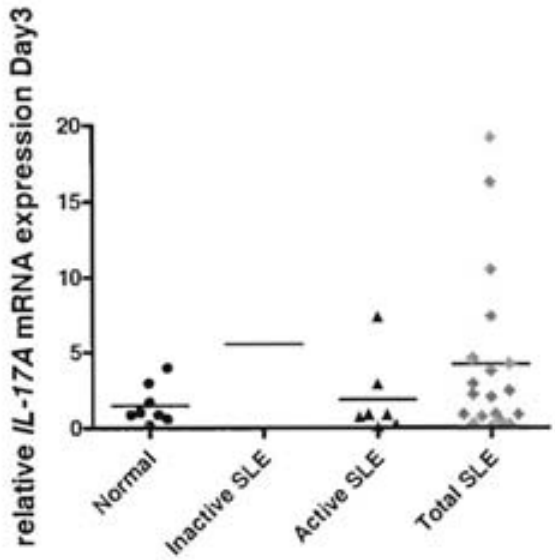


Figure 14 Semi-Quantitative Real-time RT-PCR Analysis for *IL-17A* in Stimulated PBMCs at Day 3 from SLE Patients and Normal Subjects

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 normalized to β-actin. 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 *RORC* mRNA, in both freshly isolated PBMCs (day 0) and 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. 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 15. 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 14A). 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 15B). The level of *RORC* mRNA expression at day 3 in PBMCs from the inactive SLE group was lower when compared with those of the normal subjects, but did not reach statistical significance (p>0.05) (Figure 15B).

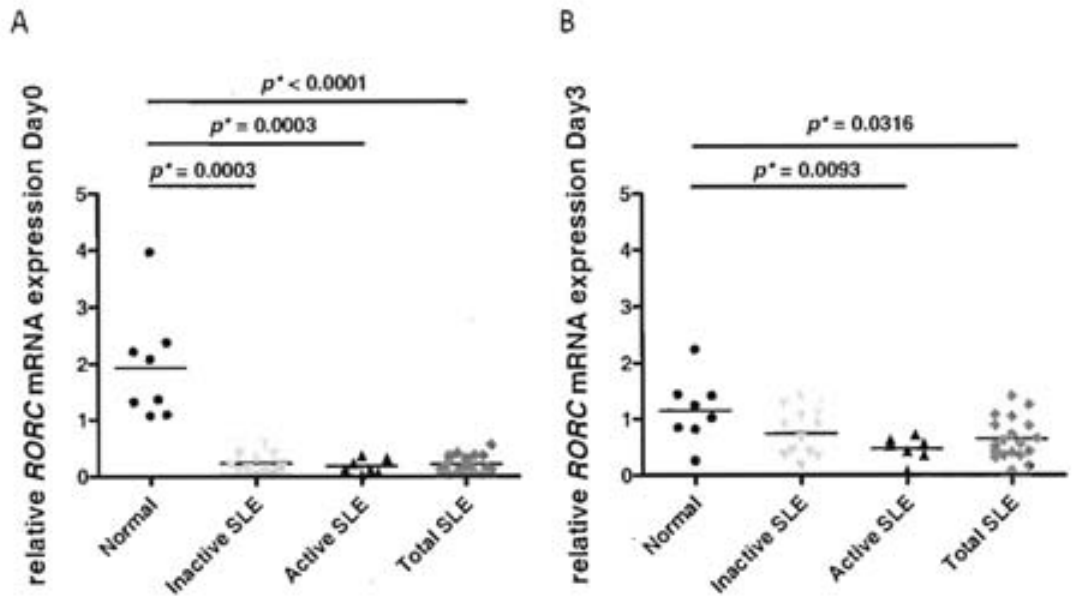


Figure 15 Semi-Quantitative Real-time RT-PCR Analysis for *RORC* Expression in PBMCs at Day 0 and Day 3 from SLE Patients and Normal Subjects

(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 the 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 9.

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 16). 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 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) (Figure16 A; ▲ = the active SLE patients and ◆ = the total SLE patients, respectively) Moreover, we found a significant 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 16C; ●= the normal subjects, = the inactive SLE patients and ◆ = the total SLE patients, respectively).

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 16. We did not find any correlation between any two pairs of the data set from all subjects in PBMCs on day 0 (Figure 16; A-D). A significant correlation, 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 17E; ▲ = the active SLE patients and ◆ = the total SLE patients, respectively). A significant 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 16G; ▼ = the inactive SLE patients, ▲ = the active SLE patients and ◆ = the total SLE patients, respectively).

Table 9 Summary of the Results of the Subjects Recruited for Flow Cytometric Analysis, Serum Cytokine Detection by ELISA and Semi-Quantitative Real-time RT-PCR Analysis

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
Normal 1	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
Normal 2	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
Normal 3	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
Normal 4	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
Normal 5	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
Normal 6	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
Normal 7	normal	14.90	15.63	0.45	0.48	25.20	18.76	3.69	0.88	UD	UD	ND		
Normal 8	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
Normal 9	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		
SLE 1	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
SLE 2	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
SLE 3	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
SLE 4	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
SLE 5	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
SLE 6	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 9 (Continued)

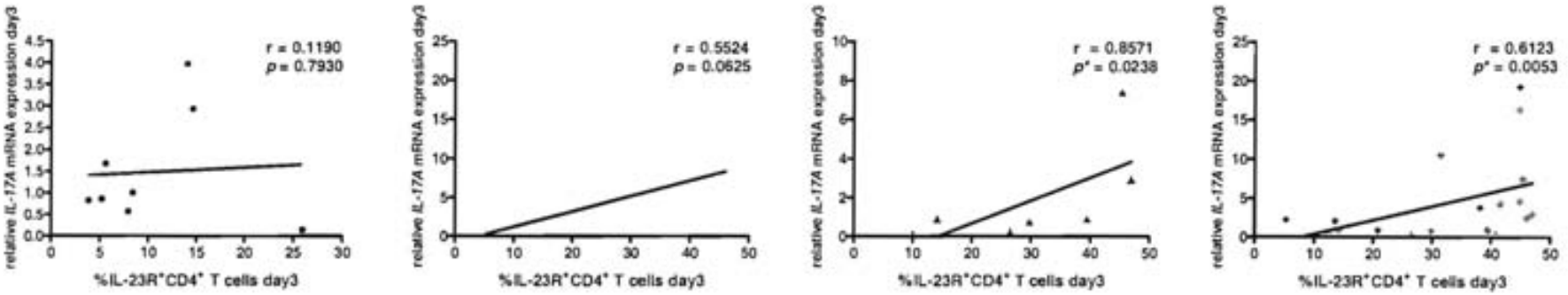
No. Samples	Stage	Flow Cytometric Analysis (% T cells)								Serum Cytokine Levels Detection (pg/ml)		Quantitative Real-time RT-PCR Analysis		
		Day 0				Day 3						IL-17A	RORC	
		IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-17A	IL-23	Day3	Day0	Day3
SLE 7	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
SLE 8	inactive	39.46	39.00	0.58	0.55	22.38	8.23	1.71	0.49	UD	UD	ND		
SLE 9	inactive	17.16	18.62	0.92	0.26	30.00	19.14	2.54	1.42	UD	UD	ND		
SLE 10	inactive	14.73	13.27	0.18	0.24	45.20	29.91	8.59	2.93	UD	UD	ND		
SLE 11	inactive	10.18	19.86	1.17	1.05	24.02	22.10	12.06	4.69	UD	UD	ND		
SLE 12	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
SLE 13	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
SLE 14	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
SLE 15	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
SLE 16	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
SLE 17	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
SLE 18	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
SLE 19	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
SLE 20	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
SLE 21	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
SLE 22	active	46.59	25.89	0.78	0.23	33.61	26.56	3.36	5.17	UD	UD	ND		
SLE 23	active	6.77	8.25	1.78	1.52	32.40	51.64	13.88	14.29	UD	UD	ND		
SLE 24	active	8.14	14.63	0.55	1.36	27.92	7.75	5.35	1.09	6.78	UD	ND		

Table 9 (Continued)

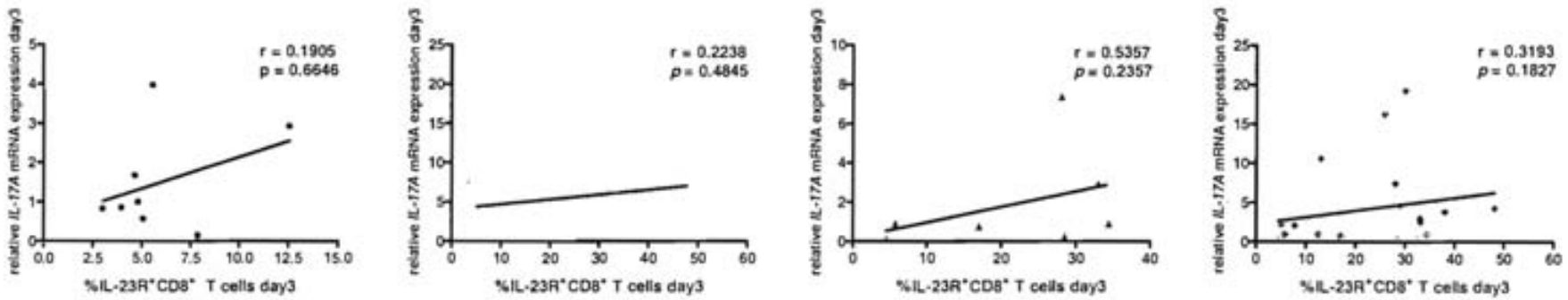
No. Samples	Stage	Flow Cytometric Analysis (% T cells)								Serum Cytokine Levels Detection (pg/ml)		Quantitative Real-time RT-PCR Analysis		
		Day 0				Day 3						IL-17A	RORC	
		IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-23R$^+$CD4$^+$	IL-23R$^+$CD8$^+$	IL-17$^+$CD4$^+$	IL-17$^+$CD8$^+$	IL-17A	IL-23	Day3	Day0	Day3
SLE 25	active	9.89	19.49	0.43	0.19	41.05	22.37	8.58	1.50	UD	UD	ND		
SLE 26	active	26.32	33.75	0.60	1.15	28.38	25.38	4.26	1.98	UD	UD	ND		
SLE 27	active	17.50	22.94	1.29	0.66	35.39	35.82	7.49	3.41	5.12	UD	ND		
SLE 28	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
SLE 29	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

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
%IL-23R+CD4+ T cells day3
r = 0.1190
p = 0.7930
r = 0.5524
p = 0.0625
r = 0.8571
p* = 0.0238
r = 0.6123
p* = 0.0053

B


relative IL-17A mRNA expression day3
%IL-23R+CD8+ T cells day3
r = 0.1905
p = 0.6646
r = 0.2238
p = 0.4845
r = 0.5357
p = 0.2357
r = 0.3193
p = 0.1827

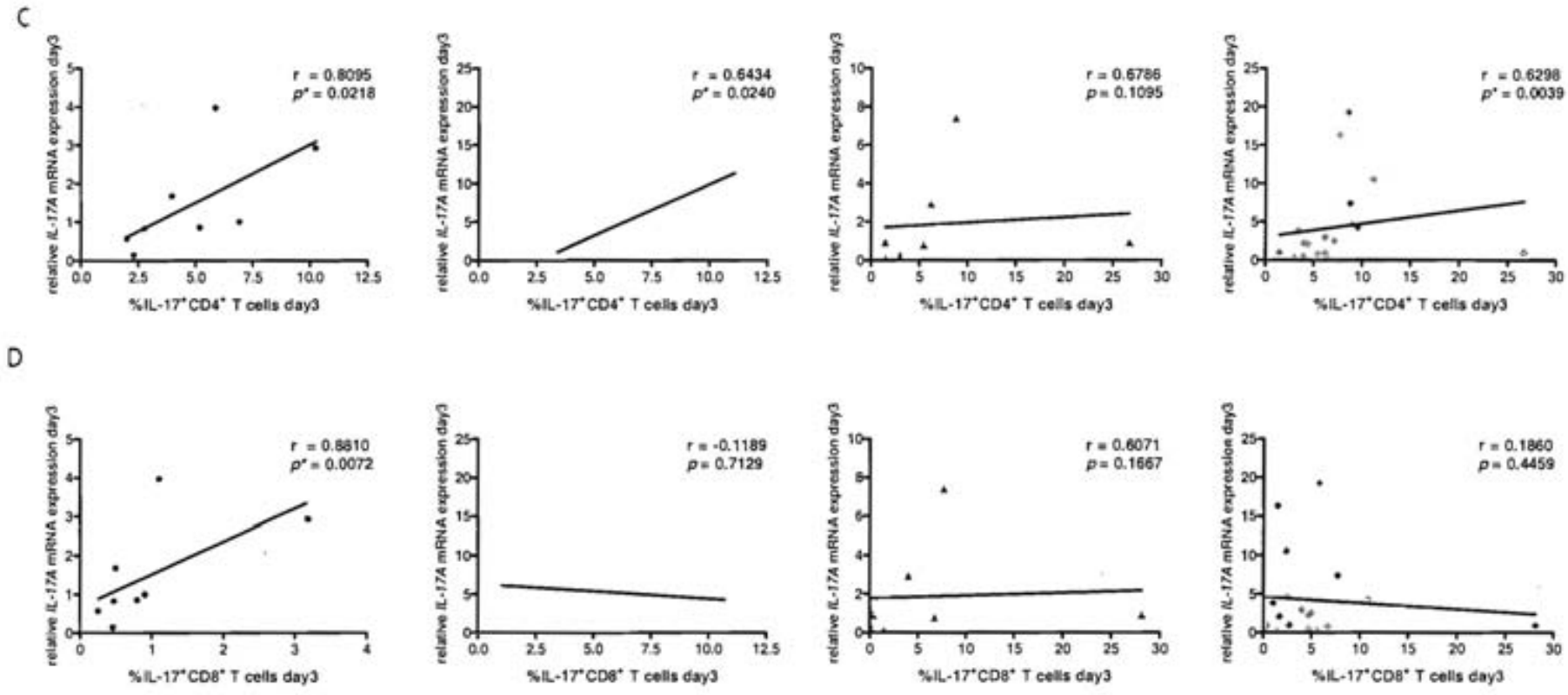
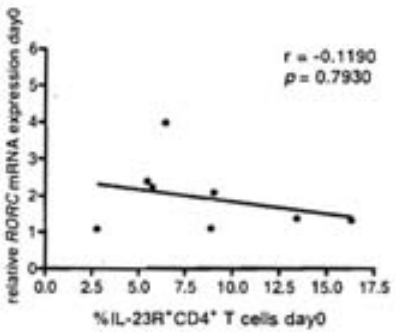
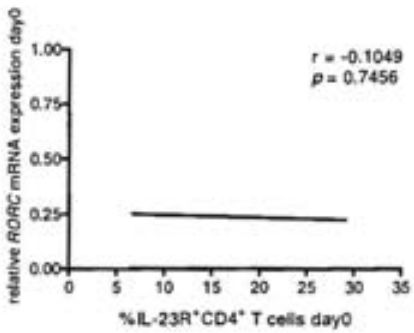


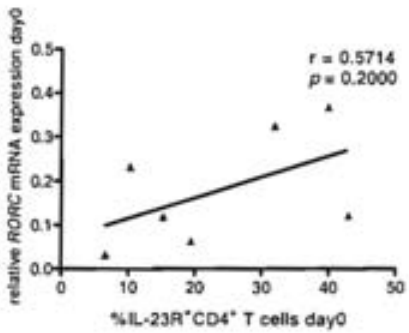
Figure 16 Correlation Between Relative *IL-17A* Expression and the Results from Flow Cytometric Analysis

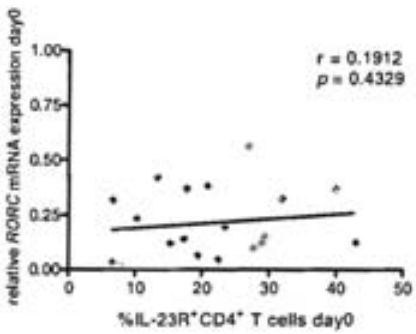
The relative *IL-17A* mRNA expression in PBMC at day 3 was plotted against the followings: (A) %IL-23R$^+$CD4$^+$T cells at day 3, (B) %IL-23R$^+$CD8$^+$T cells at day 3, (C) %IL-17$^+$CD4$^+$T cells at day 3 and (D) %IL-17$^+$CD8$^+$T cells at day 3. Spearman's correlation test was used to analyze these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

A

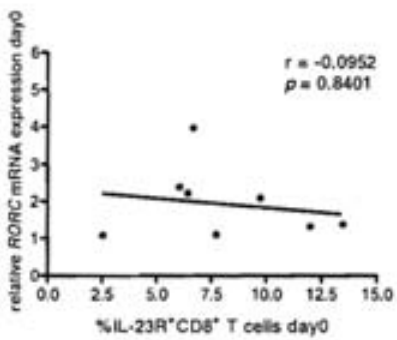

r = -0.1190
p = 0.7930
relative RORC mRNA expression day0
%IL-23R+CD4+ T cells day0

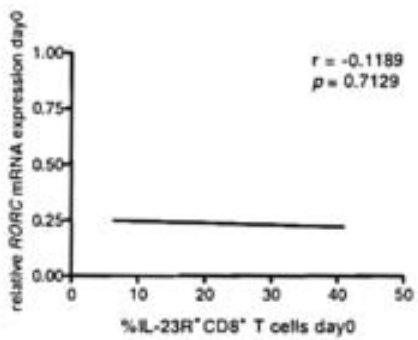

r = -0.1049
p = 0.7456
relative RORC mRNA expression day0
%IL-23R+CD4+ T cells day0


r = 0.5714
p = 0.2000
relative RORC mRNA expression day0
%IL-23R+CD4+ T cells day0


r = 0.1912
p = 0.4329
relative RORC mRNA expression day0
%IL-23R+CD4+ T cells day0

B


r = -0.0952
p = 0.8401
relative RORC mRNA expression day0
%IL-23R+CD8+ T cells day0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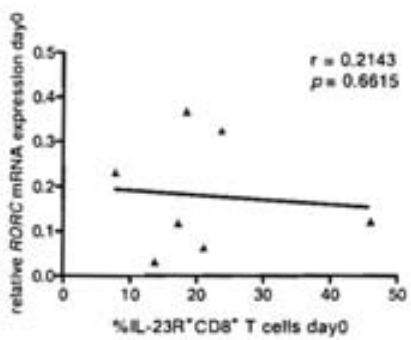
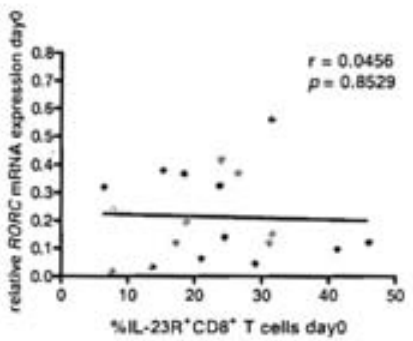
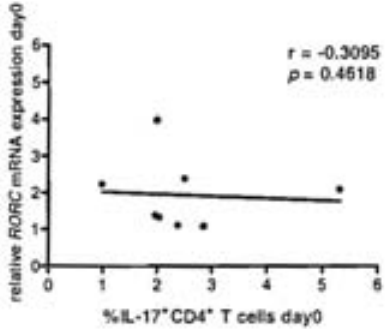
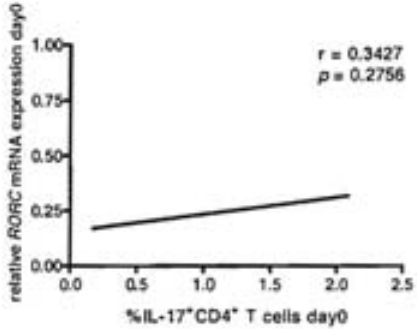
p = 0.7129
relative RORC mRNA expression day0
%IL-23R+CD8+ T cells day0

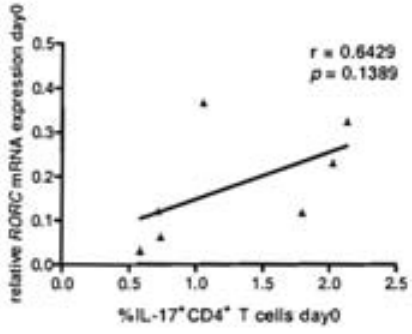

r = 0.2143
p = 0.6615
relative RORC mRNA expression day0
%IL-23R+CD8+ T cells day0

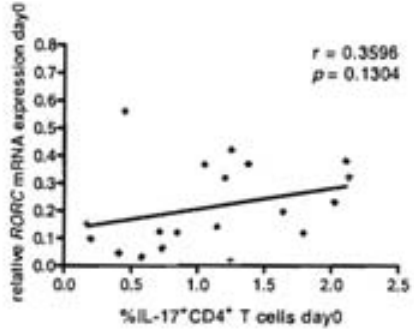

r = 0.0456
p = 0.8529
relative RORC mRNA expression day0
%IL-23R+CD8+ T cells day0

C

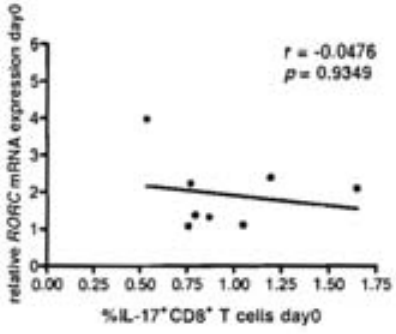

relative RORC mRNA expression day0
r = -0.3095
p = 0.4618
%IL-17+CD4+ T cells day0

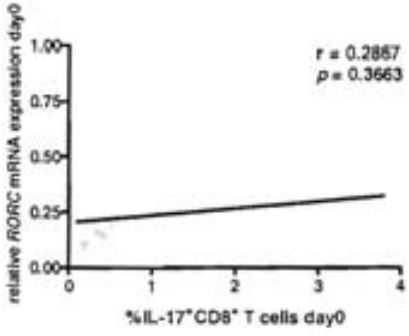

relative RORC mRNA expression day0
r = 0.3427
p = 0.2756
%IL-17+CD4+ T cells day0


relative RORC mRNA expression day0
r = 0.6429
p = 0.1389
%IL-17+CD4+ T cells day0


relative RORC mRNA expression day0
r = 0.3596
p = 0.1304
%IL-17+CD4+ T cells day0

D


relative RORC mRNA expression day0
r = -0.0476
p = 0.9349
%IL-17+CD8+ T cells day0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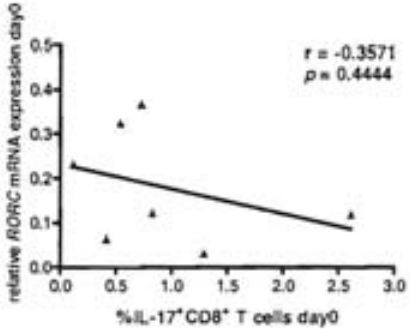
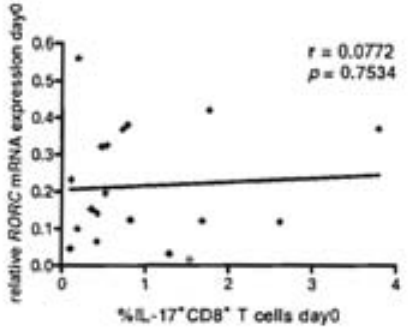
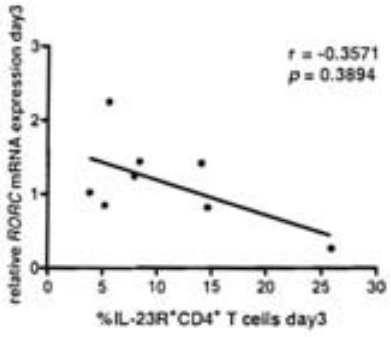
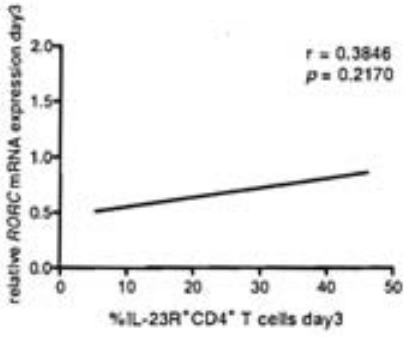
r = 0.2867
p = 0.3663
%IL-17+CD8+ T cells day0


relative RORC mRNA expression day0
r = -0.3571
p = 0.4444
%IL-17+CD8+ T cells day0


relative RORC mRNA expression day0
r = 0.0772
p = 0.7534
%IL-17+CD8+ T cells day0

E


relative RORC mRNA expression day3
r = -0.3571
p = 0.3894
%IL-23R+CD4+ T cells day3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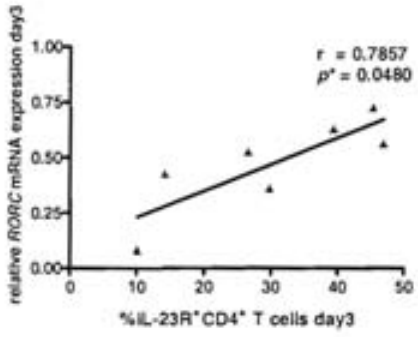
p = 0.2170
%IL-23R+CD4+ T cells day3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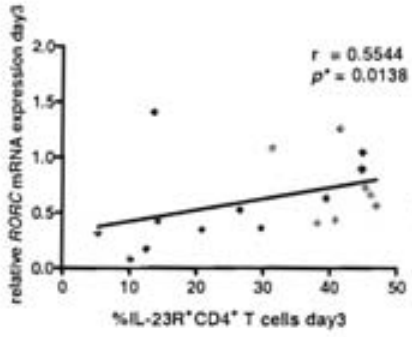
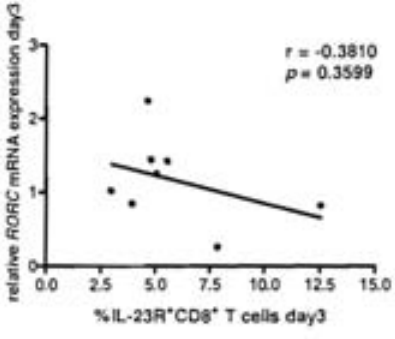
p* = 0.0480
%IL-23R+CD4+ T cells day3


relative RORC mRNA expression day3
r = 0.5544
p* = 0.0138
%IL-23R+CD4+ T cells day3

F


relative RORC mRNA expression day3
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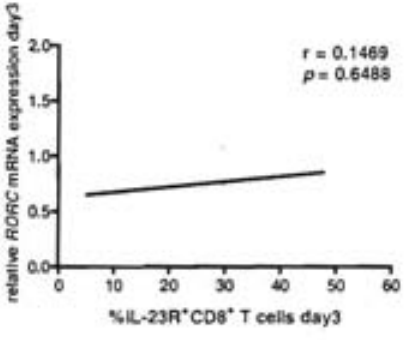
p = 0.3599
%IL-23R+CD8+ T cells day3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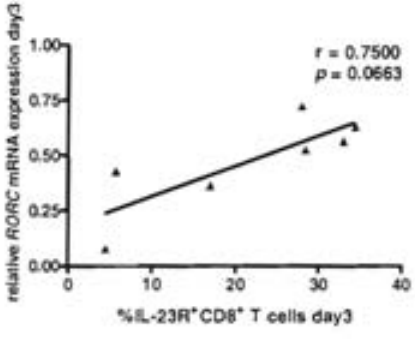
p = 0.6488
%IL-23R+CD8+ T cells day3


relative RORC mRNA expression day3
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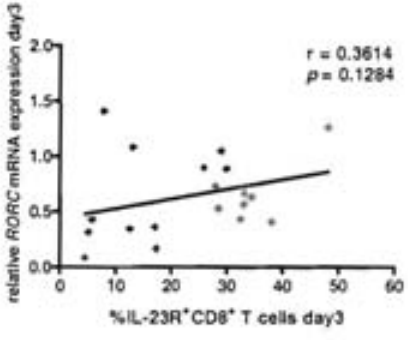
p = 0.0663
%IL-23R+CD8+ T cells day3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r = 0.3614
p = 0.1284
%IL-23R+CD8+ T cells day3

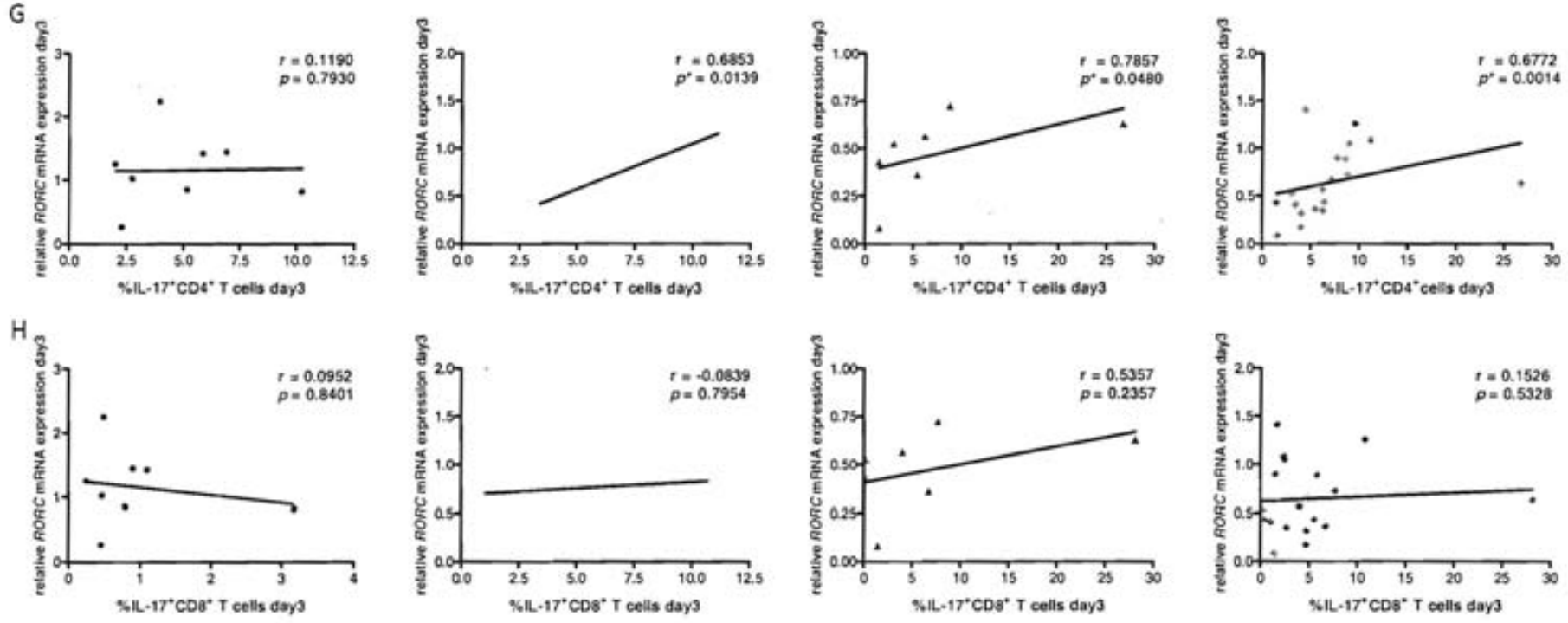


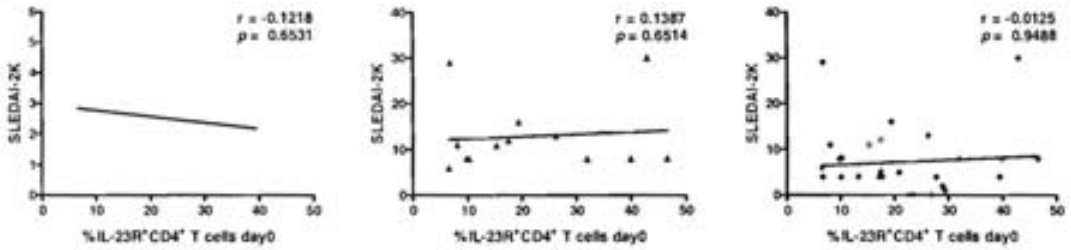
Figure 17 Correlation Between Relative *RORC* Expression and the Results from Flow Cytometric Analysis

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)

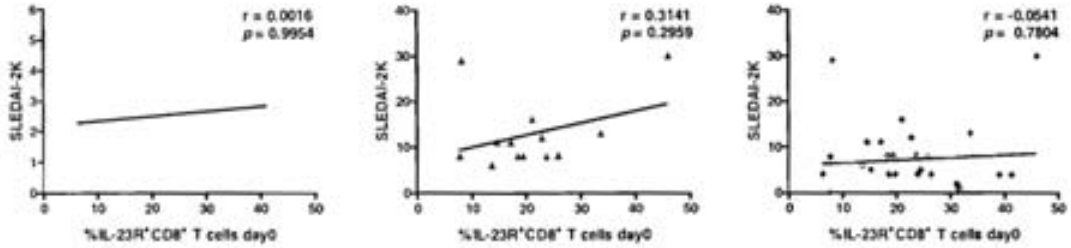
7. Correlation with the SLEDAI-2K Scores

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. 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 18G, middle row and 18H, middle row, respectively). We, however, 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 18; A-H; ▼ = the inactive SLE patients and ◆ = the total SLE patients). We observed a correlation 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 18F, 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 19).

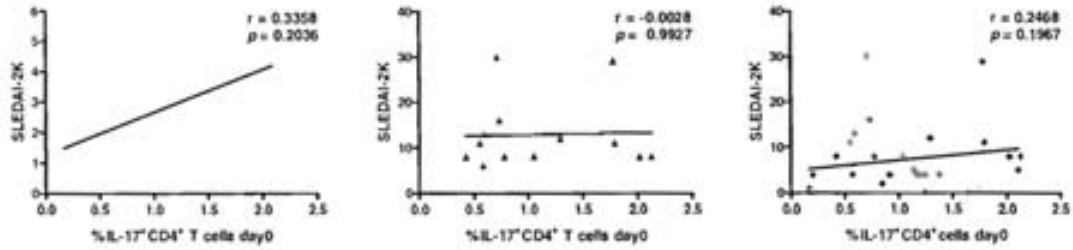
A


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

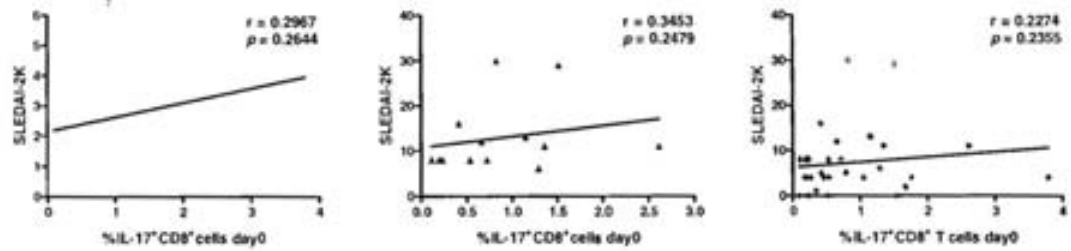
B


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

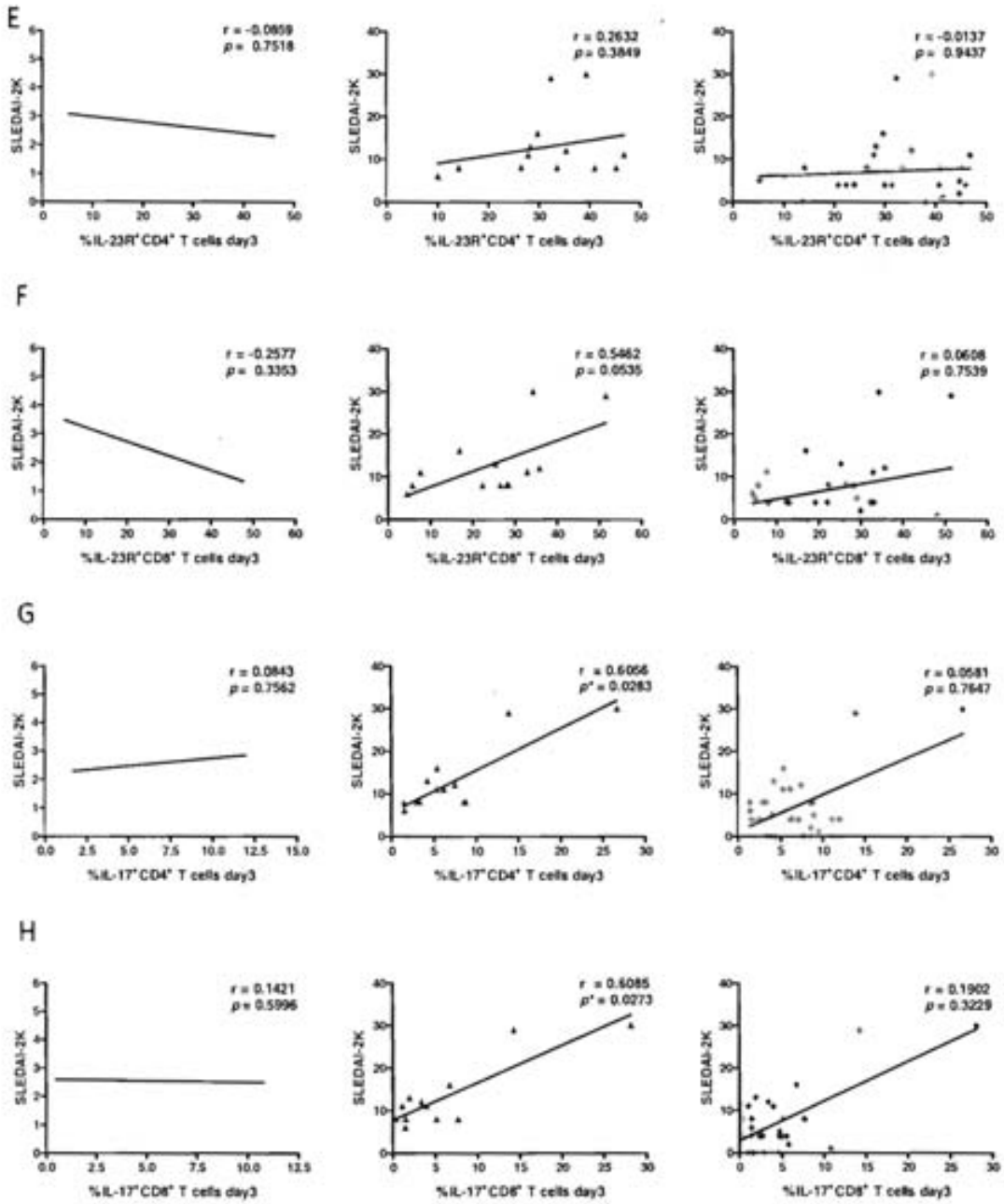


Figure 18 Correlation Analyses Between the Scores of SLEDAI-2K and the Percentages of IL-23R$^+$CD4$^+$ T Cells, IL-23R$^+$CD8$^+$ T Cells, IL-17$^+$CD4$^+$ T Cells and IL-17$^+$CD8$^+$ T Cells in PBMCs from 29 SLE Patients

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).

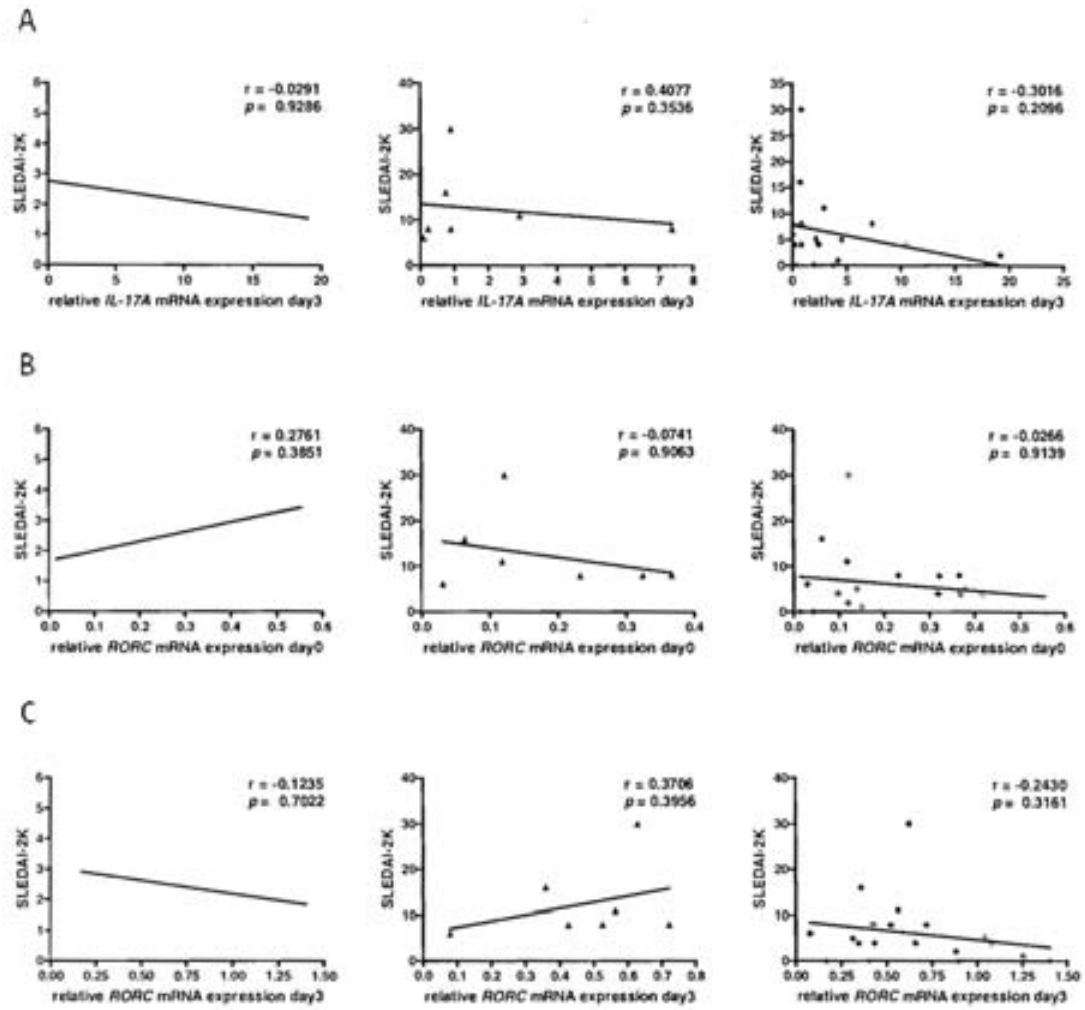


Figure 19 Correlation Analyses Between the Scores of SLEDAI-2K and Relative mRNA Expression in PBMCs from 19 SLE Patients

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.

8. Effects of IL-23 on IL-17 Producing T Cells

From the results obtained above, we speculated that T cells (both CD4+ and CD8+) T cells with higher IL-23R in SLE would respond to cytokine IL-23 better than those of the normal subjects. We tested this hypothesis by stimulating PBMCs from SLE patients and normal subjects as previously described in the presence or absence of exogenous recombinant IL-23 for 1 and 3 days. Cells were then analyzed for IL-17 and

IL-23R expression by flow cytometric analysis. Consistently, PBMCs from both groups showed a decreased IL-23R+ T cells (data not shown). This is probably due to the binding of the recombinant IL-23 to the receptor interfered with antibody binding to the receptors or IL-23R was removed from the cell surface when it is engaged by the ligand. When IL-17+ T cells were examined, there was no detectable increase in this population with or without addition of IL-23 (data not shown). Therefore, we could not conclude that higher numbers of IL-23R+ T cells found in PBMC of SLE patients play any role in producing IL-17 when IL-23 is present.

Discussions

In this study, we undertook an association analysis of IL-17/IL-23 axis with SLE in Thai patients for the first time. The patients were originally recruited from the King Chulalongkorn Memorial Hospital, but because of the limitation in numbers of cases, additional patients were recruited from Bhumibol Adulyadej Hospital. We found a significant increased in IL-23R$^+$ T cells, both in CD4$^+$ and CD8$^+$ subpopulation in SLE patients, and this trend was seen in both freshly isolated PBMC and *ex vivo* activated PBMC. Although it has been reported in several studies that serum IL-23 in SLE patients is higher than the healthy control [177], this is the first report on increased frequency of IL-23R$^+$ T cells in SLE patients. Since IL-23 is one of the key cytokines which regulate differentiation and proliferation of Th17, the results obtained in this study suggest that SLE patients may harbor subpopulation of pro-inflammatory T cells which are posed to be activated. In fact, the larger differences between healthy controls and SLE patients were often observed with *ex vivo* activated T lymphocytes. These observations imply that T cells from SLE patients with IL-17$^+$ and IL-23R$^+$ are selectively outgrown other subsets of T cell after stimulation through TCR/CD28. Interestingly, the difference between active and inactive groups was not detected for IL-23R$^+$ T cells, indicating that this increasing T cells subpopulation and the disease activity are not related. An increased in IL-17 producing T cells was also observed in our study. This trend was observed in only freshly isolated PBMC for CD4$^+$ T cells, whereas the trend appeared after stimulation in CD8$^+$ T cell population. This may indicate that CD4$^+$ T cells in PBMC of SLE patients do not have advantages for expansion in culture condition we used. Furthermore, CD8$^+$ T cells producing IL-17 are selectively expanded in SLE patients. Currently, most studies focus on the role of IL-17 producing CD4+ T cells but characterization of CD8+ T cells which can produce IL-17 is lacking. Recently, it was reported that CD4CD8 double negative αβ TCR T cells which produce IL-17 increased in SLE patients and this population derived from CD8$^+$ T cells [181,182]. Therefore, this reported observation together with our results may point to the importance of CD8$^+$ T cells which can produce IL-17 in SLE. The characteristics, differentiation, phenotypes of this subpopulation have not yet been elucidated.

Based on our observation at the cellular level, we expected to see an increased serum IL-17 and IL-23 in SLE patients. Using commercial ELISA test kit which sufficient sensitivity, we could only detect serum IL-17 in 2 out of 29 patients while none was detected in control subjects. For IL-23 detection, no samples yielded any positive results, suggesting that the sera we used contained extremely low amount of both cytokines. Since it has been reported that plasma from SLE patients in Chinese patients contained higher IL-17 and IL-23 [177], we also tried plasma sample and could not detect both cytokines. This is probably due to several confounding factors such as different groups of patients used in our studies and others, the treatment our patients received. In

addition, IL-17 and IL-23 have found in the kidney biopsy from lupus nephritis patients, suggesting the local production of these cytokines where T cells infiltrate the tissues which results in tissue pathology [181,183].

In an effort to try to pinpoint whether a conventional CD4+ Th17 increased in SLE, we detected expression of *RORC* mRNA in PBMC by semi-quantitative RT-PCR. RORC is a human homolog of murine RORγt which has been shown to be a master regulator of Th17 differentiation [88]. Unexpectedly, the expression of this gene was higher in freshly isolated PBMC from the control groups and this trend persisted even after *ex vivo* stimulation. This result may indicate that *RORC* is expressed constitutively in PBMC and its expression may not be a good indicator whether Th17 is expanded or not. Another possibility is that mRNA of *RORC* has been shown to exist in different splicing isoforms. Although our primer sets used in this study could detect at least 2 splicing isoforms, we cannot rule out the possibility of other isoforms which may correlate with Th17.

One of the confounding factors which may interfere with our result interpretation is that whole PBMCs were used in our study. There are various cell types which can produce IL-17 in PBMC. Therefore, analysis at single cell level such as ICS was helpful in determing the exact population which is relevant. The experiments which used whole PBMC such as ELISA and RT-PCR, thus, may be complicated by other cell types in the PBMC. Using purified population, thus, may help clarify the results obtained in this study.

Conclusions and Suggestions

We reported here that Thai SLE patients, irrespective of the disease status, showed increased IL-23R$^+$CD4$^+$ and IL-23R$^+$CD8$^+$ T cells. In addition, increased frequency of CD8$^+$ T cells producing IL-17 were observed in our patients. The results obtained here need further investigation into the significance of this trend. In particular, the biological functions of IL-23R$^+$ T cells and the identity of CD8$^+$ T cells which can produce IL-17.

Using purified subset of cells will be needed for clarification some of the differences observed in our study and others. In addition, more SLE subjects receiving lower doses of drugs may be needed to investigate the serum cytokine production.

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