The Relation between Intra-Renal Gene Expression and Histological Pattern of Lupus Nephritis

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Statement

The work presented in this thesis has not been submitted in whole or in part for any other higher degree at any University or institute of learning. Unless otherwise stated, the work presented here is my own.

The histological assessment of this project was done by Professor Fernand Lai; the clinical data was retrieved from the database kept by Professor CC Szeto.

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ABSTRACT

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by aberrant cytokines milieu. Although almost all organs in the body could be involved, lupus nephritis remains the leading cause of morbidity and mortality in SLE. The precise pathogenic mechanism of lupus nephritis is not completely understood. However, cytokine aberration is a cardinal phenomenon of lupus nephritis. It is important to realize that cytokines may not only be involved in the initial triggering of aberrant immune regulation, but also participate in the perpetuation of local inflammatory processes that ultimately lead to tissue destruction. Unfortunately, most published studies on lupus nephritis focus on the cytokine profile of peripheral blood in SLE patients, and the results are often inconsistent. Since specific organ or tissue involvement in SLE probably involve local cytokine aberrations that do not appear in the systemic circulation, study of the immunological aberration should logically focus at specific sites of lupus involvement. To complicate the issue, there are several different histological patterns of lupus nephritis, and, in each of them, the aberrant immunological response is probably different between the glomerular and tubulointerstitial compartment of the renal parenchyma. In this series of work, we studied the gene expression of a panel of inflammatory mediators in the glomerulus and tubulointerstitium, and examined their relation with the histological pattern of lupus

nephritis.

In the first step, we studied the renal biopsy specimen of 42 patients with active lupus nephritis. By laser microdissection, glomerulus and tubulointerstitium of the biopsy specimen were separated, and the gene expression of a panel of pre-defined inflammatory mediators was compared to those of 10 controls. We found that there was an increase in expression of TWEAK, Fn14, NGAL and TLR9, and a decrease in CXCR3 expression in both glomerulus and tubulointerstitium of lupus nephritis. Notably, glomerular CXCR3 expression correlates with the degree of proteinuria, while tubulointerstitial CXCR3 expression correlates with renal function. In addition, tubulointerstitial expression of NGAL correlated with proteinuria, renal function, and therapeutic response. Our findings suggested that TWEAK/Fn14 and IP-10/CXCR3 axis probably contribute to the pathogenesis of lupus nephritis.

Next, we explored the relation between intra-renal gene expression and histological pattern of lupus nephritis. We studied the change in the histological pattern and the progression of histological scarring in 156 SLE patients who had repeat renal biopsies (a total of 412 biopsy specimens were reviewed). We found that a change in the histological class of lupus nephritis is common during lupus disease flare. More

importantly, the histological pattern could not be predicted by baseline clinical, biochemical, or pathological parameters. Our result indicates that when there is lupus flare with renal involvement, repeat renal biopsy is often necessary to guide the treatment.

We then quantified and compared the glomerular and tubulointerstitial expression of TWEAK/Fn14, IP-10/CXCR3, NGAL and TLR9 in patients with paired renal biopsy. We found that there was a significant change in gene expression upon renal biopsy with progressive renal scarring. In short, the tubulo-interstitial IP-10 expression consistently decreased when the histology changed to membranous nephritis in a 2nd biopsy. Besides, tubulo-interstitial TLR9 expression trends to increase when change to membranous while decrease when change from membranous. On the contrary, tubulo-interstitial TWEAK expression trends to decreased when change to membranous while increased when change from membranous. Our findings suggest that TWEAK/Fn14, IP-10/CXCR3 axis, NGAL, TLR9 appeared to have a consistent change in expression in parallel with the alteration in histological pattern of lupus nephritis and may contribute to the pathogenesis of lupus nephritis.

Finally, we assessed intra-renal micro-RNA expression in patients with lupus nephritis.

We found that intra-renal expression of miR-638, miR-198, and miR-146a are differentially expressed between patients with lupus nephritis and normal controls. Furthermore, the degree of change correlated with disease severity. The results suggested that these micro-RNA species may play a role in the pathogenesis of lupus nephritis. Our data also suggest a regulatory role of miR146a, miR155, and miR663 in the expression of inflammatory genes such as TLR9, CXCR3, and Fn14.

Taking together, this series of work indicates that the TWEAK/Fn14 and IP-10/CXCR3 axis, as well as TLR9 and NGAL may participate in the pathogenesis and progression of lupus nephritis. Our data seem to suggest that the TWEAK/Fn14 axis and IP10 are important in the determination of specific histological class of lupus nephritis, CXCR3 is involved in governing the severity of disease, while NGAL is related to therapeutic response irrespective to histological class. In addition, a panel of micro-RNA seems to be involved in the pathogenesis of lupus nephritis, possibly via their effect on the transcription of TWEAK/Fn14 and IP10/CXCR3 axis.

中文摘要

系統性紅斑狼瘡（SLE）是一種多系統自身免疫性疾病，其特徵為異常的細胞因子環境。雖然幾乎所有身體器官都可能受損，狼瘡性腎炎仍就是 SLE 發病率和死亡率的主要原因。雖然狼瘡性腎炎的確切致病機制仍未完全了解，但細胞因子異常是狼瘡性腎炎的基本現象。重要的是我們要認識到細胞因子可能不僅參與了最初的觸發免疫調節異常，也參與了局部長期存在的炎症過程并最終導致組織破壞。不幸的是，大多數已發表的關於狼瘡性腎炎的研究都只關注與 SLE 患者外周血中的細胞因子模式，其結果往往是不一致的。由於特定的受累器官或組織可能只涉及局部細胞因子的異常而不會表現在全身循環，我們應把免疫學異常的研究集中在特定的狼瘡累及部位。更為複雜的問題是狼瘡性腎炎有多種不同的組織學形態，並對每種類型而言，異常的免疫反應可能因腎實質里不同的腎小球及腎小管間質而不同。在這一系列工作中，我們研究了一組炎症介質在腎小球和腎小管間質的基因表達，並探討了它們與狼瘡性腎炎組織學模式之間的關係。

第一步，我們研究了 42 例活動性狼瘡性腎炎患者的腎穿刺活檢標本。通過激光顯微切割，分離活檢標本的腎小球和腎小管間質，測定一組預定的炎症介質的基因表達，并與 10 例正常對照進行比較。我們發現在狼瘡性腎炎組腎小球和腎小管間質中，TWEAK，Fn14，NGAL 和 TLR9 表達增加而 CXCR3 表達減少。值得注意的是，腎小球 CXCR3 表達與蛋白尿程度相關，而腎小管間質 CXCR3 表達與腎

功能相關。此外，腎小管間質 NGAL 表達與蛋白尿，腎功能和治療反應相關。我們的研究結果表明，TWEAK/Fn14 和 IP-10/CXCR3 軸可能參與了狼瘡性腎炎的發病機制。

接下來，我們探討了狼瘡性腎炎腎內基因表達和組織學模式之間的關係。我們通過研究 156 例接受重複腎活組織檢查的 SLE 患者（共 412 活檢標本）中的組織學模式和組織學疤痕進展變化，發現在狼瘡性腎炎發作期出現組織學變化是常見的。更重要的是，組織學模式無法通過臨床基線，生化或病理參數預測。我們的結果表明當有狼瘡發作與腎臟受累時，通常需要進行重複腎活檢來指導治療。

然後，我們量化和比較了配對腎活檢患者的腎小球和腎小管間質表達 TWEAK/Fn14，IP-10/CXCR3，NGAL 和 TLR9 的情況。我們發現在腎疤痕進行性發展過程之中，腎臟活檢組織基因表達有一個顯著的變化。如果第二次活檢時組織學類型轉變為膜性腎炎，那麼腎小管間質 IP - 10 的表達持續下降。此外，當轉變為膜性腎炎時腎小管間質 TLR9 表達上升，而由膜性腎炎轉變為其他類型腎炎時表達下降。同此相反，轉變為膜性腎炎時腎小管間質 TWEAK 表達反而下降，而由膜性腎炎轉變為其他類型腎炎時表達卻上升。我們的研究結果表明，TWEAK/Fn14，IP-10/CXCR3 軸，NGAL，TLR9 似乎與組織學模式變化相匹配，并參與了狼瘡性腎炎的發病機制。

最後，我們評估了狼瘡性腎炎患者的腎內 microRNA 表達。我們發現，腎炎患者 miR - 638， miR - 198，和 miR - 146a 的腎內表達明顯區別于正常對照組。此外，變化程度與疾病的嚴重程度相關。結果表明這些 microRNA 在狼瘡性腎炎的發病機制中可能發揮作用。我們的數據也表明 miR146a，miR155，miR663 對炎症基因，如 TLR9，CXCR3 和 FN14 的表達有調節作用。

綜上所述，這一系列工作表明 TWEAK/Fn14 和 IP-10/CXCR3 軸，以及 TLR9 和 NGAL 可能參與狼瘡性腎炎的發病機制及進展。我們的數據似乎表明 TWEAK/Fn14 軸和 IP10 對狼瘡性腎炎特定組織學類型有重要意義，CXCR3 參與了對疾病嚴重性的控制，而 NGAL 不論組織學類如何都同治療反應有關。此外，一組 microRNA 似乎也參與了狼瘡性腎炎的發病機制，其可能通過作用于 TWEAK/Fn14 和 IP10/CXCR3 軸的轉錄而發揮影響。

ABBREVIATIONS

Ab	antibody
ACR	The American College of Rheumatology
ACEI	angiotensin converting enzyme inhibitor;
α-GalCer	α-galactosylceramide
AI	activity index
AKI	acute kidney injury
ANA	anti-nuclear antibody
APCs	antigen presenting cells
ARB	angiotensin receptor blocker
BCR	B cell receptor
BILAG	British Isles Lupus Assessment Group
BLK	B lymphoid tyrosine kinase
cDNA	complementary deoxyribonucleic acid
CI	chronicity index
CKD	chronic kidney disease
CNS	central nervous system
CR	complete remission
C	complement
DCs	dendritic cells
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
DTT	dithiothreitol
EBV	Epstein-Barr virus
FoxP3	forkhead box P3

GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFR	glomerular filtration rate
HERVs	human endogenous retroviruses
HLA	human leukocyte antigen
HRES-1	HTLV-1-related endogenous sequence
IP-10	Interferon-γ-inducible protein 10
LE	lupus erythematosus
IFN-γ	interferon-gamma
IL	interleukin
LN	lupus nephritis
ISN/RPS	the International Society of Nephrology / Renal Pathology Society
ITGA	integrin alpha
ITP	immune thrombocytopenic purpura
LMD	laser-assisted microdissection
Lyp	lymphoidspecific phosphatase
miRNA	micro-RNA
mRNA	messenger ribonucleic acid
MDRD	Modification of Diet in Renal Disease
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NGAL	neutrophil gelatinase associated lipocalin
NK/T	natural killer / T cells.
NR	no response
NZB/W	New Zealand Black/New Zealand White F1
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction

PR	partial remission
PTPN22	protein tyrosine phosphatase non-receptor type 2
PDCD1	programmed cell death 1 gene
RT-QPCR	real time quantitative polymerase chain reaction
RBC	red blood cell
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT	reverse transcription
SCID	severe combined immunodeficient mice
SCr	serum creatinine concentration
SD	Standard deviation
SLAM	Systemic Lupus Activity Measures
SLE	systemic lupus erythematosus
SLEDAI	systemic lupus erythematosus disease activity index
SLICC	The Systemic Lupus International Collaborating Clinics
ssDNA	single-stranded DNA
STAT	signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	transforming growth factor-beta
Th	T-helper cells
TLR9	toll-like receptor-9
TNF	tumour necrosis factor
Treg	T regulatory cells
Tr1	T-regulatory 1 cells
Ts	T-suppressor cells
TWEAK	Tumor necrosis factor (TNF)-like weak inducer of apoptosis

UV	ultraviolet
WBC	white blood cell
WHO	World Health Organization

CHAPTER 1 – BACKGROUND REVIEW

1.1 Overview

Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease that has a highly variable clinical presentation. The common characteristic phenomenon of SLE is the production of autoantibodies to various ubiquitous cellular antigens, which include nucleic acids, molecules involved in the transcription and translation of nucleic acids, and components of cell membranes. The organ system involvement of SLE is highly diverse. Although muscoskeletal and dermatological manifestations are most frequent, there is often involvement of the kidney, heart, lung, and central nervous system (CNS). Because of the highly variable clinical feature, the diagnosis of SLE is based on a set of clinical criteria originally put up by the American Rheumatic Association in 1971 [1], which was subsequently updated in 1982 and then 1997 [2]. Despite the advances in modern medical care and immunosuppressive therapy, SLE remains a difficult disease to treat. Cardiovascular disease, infection, and lupus disease activity *per se* remain common causes of morbidity and mortality in SLE patients[3].

1.2 Historical Perspective

The history of SLE could be divided into three periods. In the classical period before the late 19th century, the first description of cutaneous manifestation of SLE was made. The term lupus (Latin for wolf) was first used by the thirteenth century physician Rogerius, who used the word to describe erosive facial lesions that were reminiscent of a wolf's bite [4,5]. The first published illustrations of lupus erythematosus were included in the *Atlas of Skin Diseases* by Ferdinand von Hebra, published in 1856.

In 1872, Moriz Kaposi, a student and son-in-law of the Austrian dermatologist Ferdinand von Hebra, first described the systemic manifestations of SLE. Kaposi actually distinguished two forms of lupus, which are what we now know as SLE and discoid lupus. The systemic form, i.e. SLE, was further described in detail by William Osler[6] and Josef Jadassohn [7].

In 1948, Malcolm Hargraves and coworkers described the appearance of lupus erythematosus cells (so called "LE cells") [8], which marked the modern period in the history of SLE. Initially, LE cells were found in the bone marrow of patients with acute disseminated lupus erythematosus, representing the first *in vivo* immunological test

dedicated for the diagnosis of SLE. Since then, the development of tests for laboratory autoantibodies together with the discovery of corticosteroid and cytotoxic agents for the treatment of severe SLE dramatically revolutionized the diagnosis and management of this devastating disease [9-11].

1.3 Epidemiology

Systemic lupus erythematosus (SLE) is predominantly a disease of women. Overall speaking, the female-to-male ratio is about 7:1, but the ratio is as high as 11:1 during childbearing years [12]. In fact, over 80 per cent of cases occur in women during their childbearing years, but SLE spares neither the neonatal nor geriatric populations. Paediatric-onset SLE (pSLE) represents as many as 10 to 20% of all SLE cases [13].

The overall prevalence of SLE is 14.6 to 50.8 per 100,000 population [14], with an incidence rate of 1.8 to 7.6 new cases per 100,000 population per year in United States, 12.5 to 39 per 100,000 in Europe, 30 to 50 per 100,000 in Asia [15], 40 to 70 per 100,000 in China, and 50 to 100 per 100,000 in Hong Kong [16,17]. The incidence of SLE has increased approximately 3- to 7-fold in USA from 1950 to 1992 [18]. The reason of this increasing incidence remains obscure.

Evidence suggests that geography and racial background may influence the prevalence and severity of SLE. Surveys in western countries based on hospitalized patients showed a considerably greater incidence in black [19] and Asian populations [20] as compared to the white population. In addition, the disease is 2 to 4 times more frequent,

and more severe, amongst non-white populations around the world. SLE also tends to be more severe in men, in paediatric cases, as well as those with late-onset disease [21].

1.4 Definitions of SLE

The diagnostic criteria of SLE were published by the American College of Rheumatology (ACR) in 1971. These criteria were revised in 1982 [1] and then 1997 [2]. The latest criteria are summarized in Table 1-1. In short, the diagnosis of SLE could be made when 4 of the 11 criteria are fulfilled.

The first four criteria cover the skin manifestations of SLE; these include photosensitivity, mouth ulcers, butterfly rashes and discoid lesions. The next four criteria are those of specific internal organ involvement, which include the serosal surface (pleura, pericardium, and peritoneum), joints, kidneys, and the central nervous system. The last three criteria are laboratory features, including abnormal blood counts, antinuclear antibody (ANA), and a panel of other auto-antibodies.

Table 1-1 1997 Update of the 1982 American College of Rheumatology Revised Criteria for Classification of Systemic Lupus Erythematosus

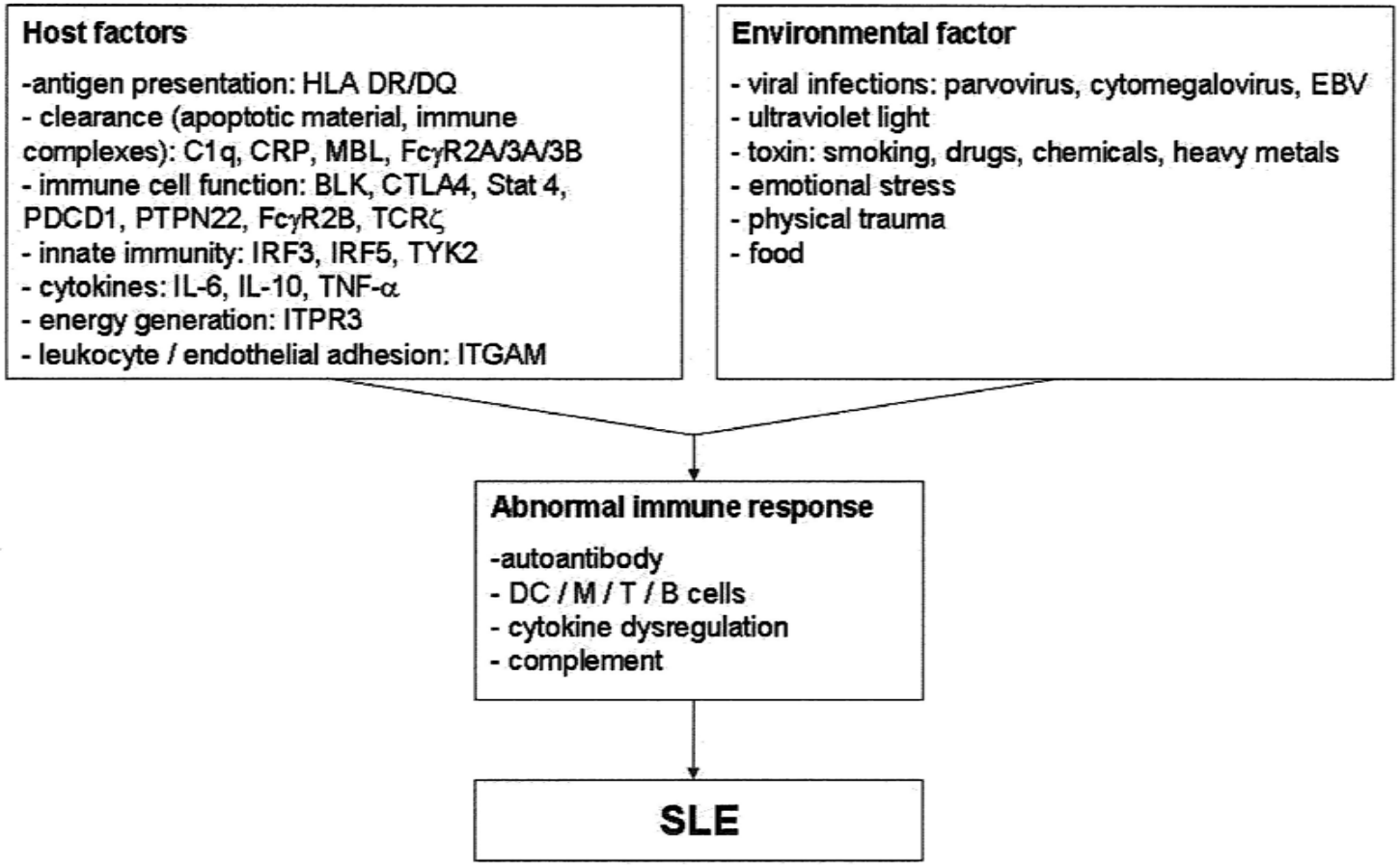
Criterion	Definition
1. Malar Rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin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

It should be noted that although widely regarded as the primary diagnostic tool of SLE, a positive ANA does not occur in all SLE patients. On the other hand, a number of other manifestations commonly found in SLE patients are not included in the ACR criteria because they are not statistically specific for SLE, and therefore could not be used for the purpose of diagnosis. Notable examples are Raynaud's phenomenon, alopecia, splenomegaly, and fundal cytoid bodies. Similarly, some other manifestations, such as pulmonary hemorrhage, restrictive lung disease, and liver function abnormalities, are occasionally found in SLE patients but not included in the criteria. The original purpose of establishing diagnostic criteria for SLE was to differentiate it from other rheumatic diseases, for the calculation of prevalence and incidence, as well as for defining subjects for clinical trials. Patients can have SLE even though their disease manifestations do not fulfill the criteria.

1.5 Pathogenesis

The pathogenesis of SLE remains incompletely understood. Nonetheless, it involves both host and environmental factors; abnormalities of both the innate and adaptive immune systems are present. The proposed pathogenic factors implicated in human SLE are summarized in Figure 1-1. In essence, under physiological condition, autoimmunity is prevented by a number of central and peripheral tolerance mechanisms. The initial trigger of SLE is likely an environmental stimulus, which somehow acts on a susceptible individual. Once the critical threshold of tolerance is breached, the immune system begins to mount abnormal responses to various tissues and organ systems.

Figure 1-1. Proposed pathogenetic factors implicated in human SLE.



1.5.1 Genetic factors

Genetic predisposition plays a crucial role in the susceptibility to SLE, both in animal models and human studies [22]. To begin with, there is a higher concordance rate of SLE in monozygotic twins than dizygotic ones (25% and 3%, respectively). There is an increased frequency of serological and immunological abnormalities in first-degree relatives of patients with SLE as compared to healthy controls. In addition, SLE occurs more frequently in certain ethnic groups, a difference that persists in spite of immigration to other countries.

A number of genes had been found to be associated with an excess risk for SLE. Traditionally, the major histocompatibility complex (MHC) on chromosome 6 represents the most important loci for the suseptibility genes of autoimmune diseases. Besides the MHC, chromosome 6 also encodes components of the complement pathway (C2, C4) as well as tumour necrosis factor (TNF)-α and TNF-β. All these genes have been reported to be associated to SLE [23].

In addition, chromosome 1 contains some of the loci most consistently identified in SLE. Several variants of Fcγ receptors, notably FCGR2A and FCGR3A, may result in

defective clearance of immune complex in the circulation, resulting in their deposition in internal organs such as in kidney and blood vessel [24]. Other susceptible genes include the protein tyrosine phosphatase non-receptor type 2 (PTPN22) on chromosome 1 [25], and programmed cell death 1 gene (PDCD1) [26] and the signal transducer and activator of transcription 4 (STAT4) [27] on chromosome 2. Notably, PTPN22 encodes a lymphoidspecific phosphatase (Lyp) that inhibits T cell receptor (TCR) signalling through Csk kinase. This target is now considered to be the strongest common genetic risk factor for human autoimmunity besides the MHC. More recently, Hom et al [28] reported two new genetic loci, which are a promoter-region allele associated with reduced expression of the B lymphoid tyrosine kinase (BLK) and increased expression of C8orf13 on chromosome 8, and variants in the integrin alpha M (ITGAM) and integrin alpha X (ITGAX) region on chromosome 16.

Other than genetic loci that have protein translational products, recent evidence indicates that micro-RNA (miRNA) plays an important role in the pathogenesis of many human diseases. Micro-RNAs (miRNAs) are short noncoding RNA molecules that inhibit gene expression through incomplete base pairing with the 3'-untranslated region of target mRNAs. The role of several micro-RNA species (for example, miR-125a, miR-371-5P, miR-423-5P, miR-638, miR-1224-3P, and miR-663) in the

post-transcriptional regulation of the immune system has been described, and their role in the pathogenesis of SLE is under active investigation [29,30].

1.5.2 Environmental factors

In epidemiological studies, exposure to ultraviolet (UV) light is the best described environmental factor related to the pathogenesis of SLE [31]. UV radiation has been shown to trigger and exacerbate the photosensitive lupus rash. There is also evidence that UV light may trigger an alteration in the structure of DNA, leading to the production of autoantibodies [32,33]. In addition, UV light has also been shown to induce apoptosis in human keratinocytes, resulting in the exposure of nuclear and cytoplasmic autoantigens onto the cell surface [34,35].

A common but yet to be proved hypothesis is that SLE is triggered by infectious agents, particularly viruses [36]. Notably, Epstein-Barr virus (EBV) has long been considered to be a potentially pathogenic virus, partly because of its well-known property of B cell activation [37]. Furthermore, a significant increase in the prevalence of EBV infection has been found in paediatric patients with SLE as compared to the control population [38]. Another potentially pathogenic species is human endogenous retroviruses

(HERVs). It has been suggested that the molecular mimicry between HTLV-1-related endogenous sequence (HRES-1) and the snRNP complex may initiate autoantibody formation [39]. Nonetheless, there is as yet no convincing evidence that any virus is directly pathogenic. Although the issue is frequently revisited, the role of virus in the pathogenesis of SLE continues to be controversial.

Other environmental factors have also been proposed to be involved in the development of SLE [40]. For instance, chemical and drug villains, such as aromatic amines and their derivatives (notably hydralazine and procainamide), are known to cause drug-induced lupus, especially in patients who are slow acetylators [40,41]. However, most of the ordinary SLE cases are unlikely to be directly related to the exposure of environmental toxins. Exposure to other environmental agents, including respirable crystalline silica, solvents, pesticides, and hair dye use, has also been implicated as the trigger of SLE [42]. In addition, physical trauma and emotional stress have been postulated to affect the clinical expression of SLE [43,44].

1.5.3 Autoantibody

A cardinal serological feature of SLE is the presence of circulating autoantibodies

against an entire panel of nuclear, cytoplasmic, and membrane antigens. Specifically, many of the auto-antibodies target at intracellular nucleoprotein particles. For example, 98% of SLE patients having antinuclear antibodies (ANA), while 70% have antibodies against double-stranded DNA (anti-dsDNA). Although antibodies to both native double-stranded DNA and denatured single-stranded DNA (ssDNA) could be present in SLE patients, but the former are more strongly associated with the development of lupus nephritis. A high titre of anti-dsDNA is virtually pathognomonic of SLE, and this antibody has been eluted from the kidney and skin samples of SLE patients. In addition to anti-DNA antibodies, many other autoantibodies have been implicated in the pathogenesis of SLE, with some linked to particular clinical manifestations of the disease [22]. The spectrum of autoantibodies in SLE is summarized in Table 1-2.

Table 1-2 Autoantibodies associated with clinical manifestations of SLE

Location	Antibody	Antigen / epitope	Prevalence (%)	Clinical association
Intracellular	DNA	dsDNA	40–90	Renal and cardiovascular/pulmonary disease
	Histones	H1, 2A, 2B, H3 & H4	30–80	Drug induced lupus (>95% positive)
	Sm	B/B′ & D	50–70 (Afro-Carribeans) 10–20 (Caucasians)	SLE specific (Afro-Carribeans associated with DR2 and nephritis)
	U1 RNP	68 kDa RNP	20–30	Mild disease (associated with DR4 and reduced risk of nephritis)
	Ro	60 and 52 kDa protein	25–40	Sjögren's syndrome, photosensitivity, and neonatal lupus syndrome
	La	47 kDa protein	10–15	Sjögren's syndrome and neonatal lupus syndrome
	Heatshock proteins	Hsp 90 (Hsp 70)	50 (40 mainly IgM)	Cardiovascular/pulmonary disease
	Ribosome P proteins	P0, P1, and P2	15–35	Link to lupus psychosis is controversial
Cell membrane	Cardiolipin	Phospholipids and DNA	20–40	Recurrent abortion and thrombosis
	Neuronal	Neuronal antigen	70–90 (+CNS) 10 (−CNS)	In serum and central nervous system
	Lymphocyte	HLA/CD4/CD8 markers	~74 (IgM) ~47 (IgG)	80% are cytotoxic (T cells affected more than B cells)
	RBC and platelets	Non-Rhesus related	<10	Haemolytic anaemia and ITP, respectively
Extracellular	Complement	C1q	~56	Rising titres indicative of proliferative glomerular nephritis
	Rheumatoid factor	Fc region of IgG	~25	Usually IgM (may be linked to erosive disease)

dsDNA, double-stranded DNA; RNP, ribonucleoprotein; ITP, immune thrombocytopenic purpura; RBC, red blood cells.

It is often believed that there is a pathogenic role of anti-dsDNA because of the close correlation between lupus disease activity and the serum levels of anti-dsDNA antibodies in many patients. Furthermore, anti-dsDNA could be eluded from the kidney of patients with lupus nephritis, while these antibodies are hardly ever found in relatives of SLE patients [45]. However, it has been shown in both murine models and human studies that only a subset of circulating anti-DNA antibodies deposit in the kidney and has pathogenic properties, and only 30% to 50% of SLE patients with these antibodies eventually develop lupus nephritis [46]. At present, it is not clear which properties distinguish pathogenic from non-pathogenic anti-dsDNA antibodies, although high-affinity anti-dsDNA antibodies of the IgG isotype, which have narrow cross reactivity with antigens other than naked DNA, are often believed to be pathogenic. It has also been suggested that DNA binding is enhanced by the presence of cationic amino acids, probably arising by somatic hypermutation in the complementarity determining regions. More recently, anti-DNA antibodies which can also bind nucleosomes are considered particularly pathogenic. This multiple reactivity of anti-DNA antibodies may be the result of variable antigen binding sites or the presence of shared epitopes on different antigens.

1.5.4. Involvement of immune cells

SLE is commonly regarded as an antibody mediated condition. Accumulating evidence, however, indicates that multiple functional defects are present amongst various cell types of the immune system, including T and B lymphocytes, dendritic cells (DCs), natural killer (NK) cells, and other antigen presenting cells (APCs). In SLE patients, peripheral lymphocyte count is frequently abnormal; lymphopenia with reduction in circulating T lymphocyte and concomitant increase in hyperactive B lymphocytes is the characteristic finding.

1.5.4.1 T lymphocytes

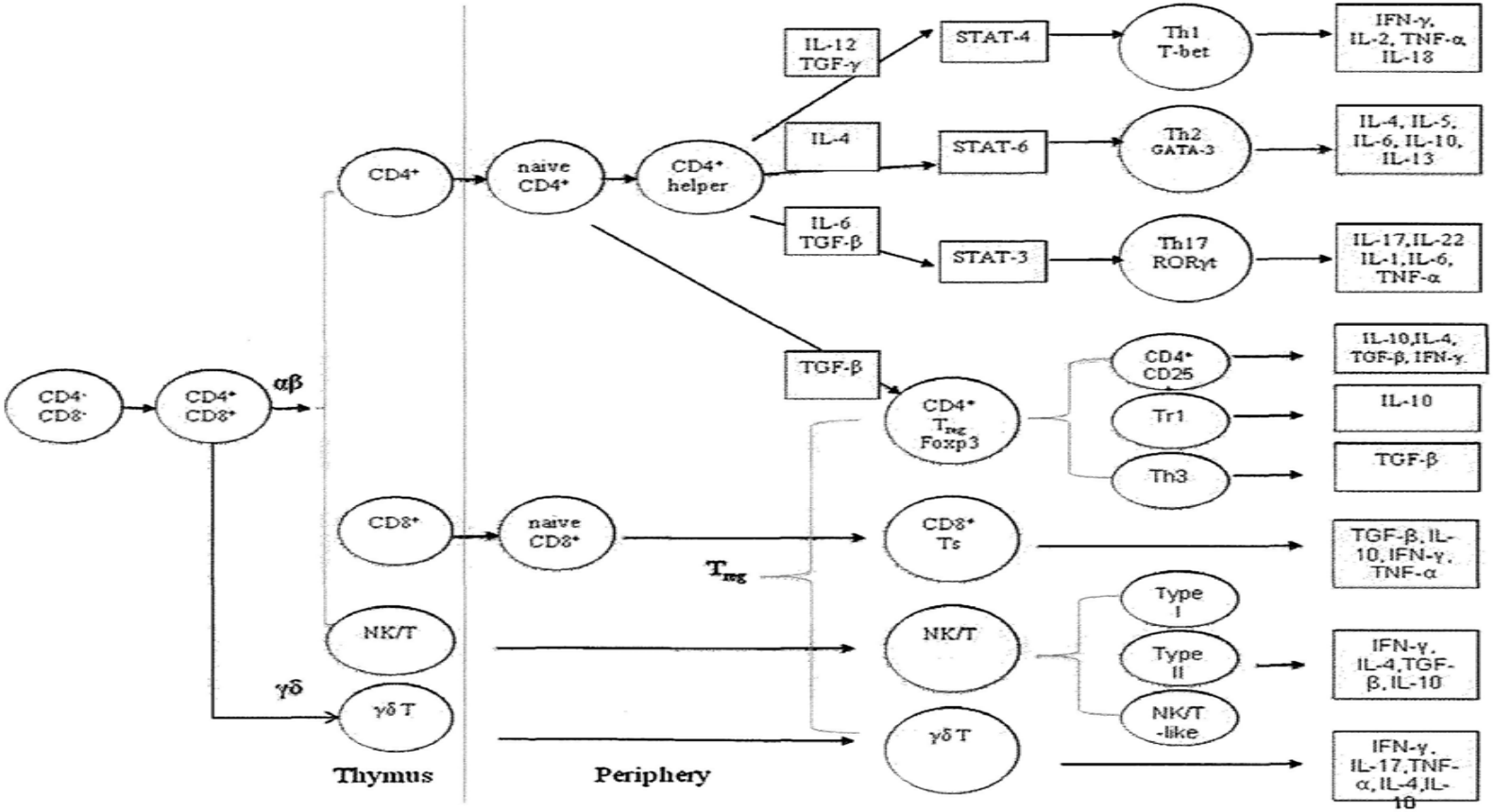
Intense T cells infiltration in the kidney is frequently observed in SLE patients [47]. T cells may contribute to the pathogenesis of lupus nephritis at multiple stages, such as providing help to B lymphocytes for the secretion of nephritogenic autoantibodies, and regulating the activity of other effector cells (for example, macrophages and NK cells) [48]. It has been reported that T cells in SLE patient are defective in the normal effector and regulatory functions [48,49]. Loss of self-tolerance that prevents the expansion of

autoreactive T-helper (Th) cells may also contribute to the development of SLE. Defects in the regulatory T (Treg) cells may result in skewing of the immune response from tolerance to autoreactivity.

Overview of T cell subset differentiation

Although T cells originated from haematopoietic stem cells in the bone marrow, they mature in the thymus and gain their full function in peripheral lymphoid tissues (Figure 1-2) [48,50-53]. In the thymus, CD4-CD8- (double negative, DN) T cells differentiate into CD4+CD8+ (double positive, DP) T cells. They then become committed to express either αβ or γδ T cell receptor chains, and further develop into single positive CD4+ , CD8+ [54,55] , NK/T, and γδ T cell subsets [50].

Figure 1-2 T cell subsets differentiation.



Th: T-helper cells. T_{reg}: regulatory T cells. Tr1: T-regulatory 1 cells. $CD8^+$ Ts: $CD8^+$ suppressor cells. NK/T: natural killer / T cells. STAT: signal transduction and activator of transcription. FoxP3: forkhead box P3. IL: interleukin. IFN: interferon. TNF: tumor necrosis factor. TGF: transforming growth factor.

In periphery, T cells are differentiated into effector subsets upon antigen stimulation. Naive CD4+ T cells are often called T-helper (Th) cells; they differentiated into effector cells that are characterized by their cytokine production profiles and immune-regulatory functions. A few subsets of Th cell could be discerned. For example, interleukin-12 (IL-12) promotes skewing towards type 1 T helper (Th1) cell commitment by activating signal transduction and activator of transcription (STAT)-4, which results in the expression of T-bet, the master regulator of Th1 development. Type 2 T helper (Th2) cell is initiated by T cell receptor (TCR) signaling acting in concert with IL-4, which up-regulates the expression of GATA3, the master regulator of Th2 differentiation, via STAT-6 [56]. Th1 cells are characterized by the expression of T-bet and production of interferon-γ (IFN-γ), IL-2, tumor necrosis factor-α (TNF-α), and IL-18 [57], whereas Th2 cells are characterized by the expression of GATA-3, and synthesis of IL-4, IL-5, IL-6, IL-10, and IL-13.

Besides the traditional Th1 and Th2, other T cell subsets are recently being identified. Amongst them, regulatory T (Treg) cells and type 17 T helper (Th17) are involved in many physiological and pathological processes. Both of them require the presence of transforming growth factor-β (TGF-β) for development, but the presence of IL-6 preferentially skews the response towards a Th17 phenotype. It is generally assumed,

though not completed proved beyond doubt, that there is a reciprocal relationship between Treg and Th17 cells, and that IL-6 plays a pivotal role in dictating whether the immune response is dominated by Th17 cells (usually believed to be pathogenic) or Treg cells (often regarded as protective) [58]. The master regulator of the Th17 differentiation program is the orphan nuclear receptor RORγt [59], while that for Treg is forkhead box P3 (FoxP3). It has been suggested that the differentiation into either Th17 or Treg cells depends on the cytokine-regulated balance of RORγt and FoxP3. TGF-β at low concentrations promote Th17 cell differentiation, while high concentrations of TGF-β favor Treg development [60]. The pro-inflammatory cytokine IL-17 is produced by Th17 cells [61], while Treg are characterized by the expression of FoxP3, IL-10 and TGF-β. Activation of different T cell subsets mentioned above will result in the migration of the cells via chemokine receptors and subsequent production of the indicated cytokines [62,63].

Functional T cell abnormalities in SLE

Accumulating evidence indicates that T cells in SLE patients have several phenotypic and functional abnormalities, which probably contribute to the pathogenesis of the disease. For example, lupus T cells showed decreased DNA methylation, low expression of TCR/CD 3 zeta chain, resulting in deficient activation-induced cell death [48,64]. On

the other hand, lupus T cells exhibited exaggerated intracellular calcium responses, increased intracellular phosphorylation and up-regulated expression of several costimulatory molecules [48,65,66]. Lupus T cells also have a lowered activation threshold, mitochondrial hyperpolarization and cytoplasmic alkalinization [49,63].

CD4-CD8- T cells

DN T cells represent a major component of thymocytes and a small proportion of peripheral blood T cells. DN T cells are expanded in the peripheral blood of patients with SLE and had been shown to produce IL-4 [67] , IL-17 and IFN-γ [68]. Besides, they induced anti-DNA autoantibody production as efficiently as CD4+ T cells [69]. Furthermore, DN T cells that produce IL-17 are found in kidney biopsies of patients with lupus nephritis, suggesting a direct pathogenic role in kidney damage [68].

T helper (Th) cells

According to the local cytokine milieu, CD4+ helper T cells are activated, proliferated and differentiated into different effector subsets as described above [70]. Generally speaking, activated autoreactive Th cells provide B cells help, so that the latter differentiate and produce a variety of pathogenic autoantibodies. Both autoantibody and the direct cell or cytokine-mediated effects of inflammatory Th cells promote tissue

injury characteristic of lupus [71].

Th1 and Th2 cells

Th1 and Th2 cells are characterized by distinct profiles of cytokine production. It is usually believed that Th1 cells promote cell-mediated immunity, while Th2 cells have functions in humoral immune responses and antibody induction [72]. Moreover, Th1 and Th2 cells counter-regulate each other. The balance of Th1/Th2 is essential for the normal human immunity. For example, several nutrients and hormones, including plant sterols or sterolins, melatonin, probiotics, progesterone, and the minerals of selenium and zinc, influence the Th1/Th2 balance [73]. In the progress of LN, antibody and immune complex stimulate cytokines release, resulting in the imbalance of Th1/Th2 subsets, which may directly lead to glomerulonephritis [74]. For example, a previous study found that SLE patients with nephritis had higher Th1 cytokines than non-nephritis patients [75]. Moreover, adjusting the Th1/Th2 balance has been shown to have therapeutic effects in various models of autoimmunity [76,77].

However, reports on the Th1/Th2 imbalance in LN remain conflicting. Some reports described the role of Th2 cytokines in the pathogenesis of LN [78-80], while others emphasized the importance of Th1 cytokines [81-84]. Notably, Lit et al [85] found that

the ratio of T-bet / GATA-3 gene expression is more informative than the level of either alone, and an elevated ratio correlated with lupus disease activity. Our group also showed previously that patients with active LN have increased T-bet and depressed GATA-3 expression in the urinary sediment and kidney tissue, indicating a predominant Th1 type of activation [86]. In addition, we have also shown that a high urinary T-bet expression was an independent predictor of lupus flare [87].

The inconsistency in published data is probably the result of different experimental approaches. However, it is clear that both Th1 and Th2 cells play prominent roles in the development of LN.

Furthermore, some studies also suggest a relationship between histological classification of LN and Th1/Th2 balance. Masutani K et al [88] found that Th1 cells is responsible for the proliferative change of diffuse lupus nephritis. In contrast, Th2 cytokines are more related to membranous lupus nephritis [89,90].

Th17 cells

A number of recent studies showed that Th17 cells represents a distinct pro-inflammatory Th-cell lineage [56]. Th17, an IL-23-dependent IL-17 producing

CD4+effector T cell subset, is important in host defense at mucosal surfaces and believed to be involved in pathogenesis of several autoimmune diseases. IL-17 is a pleiotropic proinflammatory cytokine that enhances T cell priming and stimulates epithelial, endothelial and fibroblastic cells to produce multiple proinflammatory mediators, including IL-1, IL-6, TNF-α, and several chemokines [91]. Elevated serum IL-17 levels have been reported in SLE patients, plasma IL-17, IL-23 and the number of Th17 cells were significantly elevated in SLE patients than health control subjects [92]. IL-17 can induce overproduction of autoantibody and overexpression IL-6 by peripheral blood mononuclear cells of patients with lupus nephritis [93]. In addition, elevated IL-17 concentrations correlated positively with SLE Disease Activity Index (SLEDAI) scores [92] [94]. All these findings indicate the important roles for IL-17 and Th17 cells in SLE.

Regulatory T cells

A variety of regulatory T (Treg) cells is defined by their role in the maintenance of peripheral tolerance. These regulatory T cells can be divided into CD4+ regulatory T cells, CD8+ suppressors (Ts), NK/T cells, and γδ T cells according to their cell surface phenotype and cytokine production [52,53]. All of them could suppress T or B cells proliferation in vitro. CD4+ regulatory T cells and CD8+ suppressors cells suppress

anti-DNA antibody production and delays the onset of nephritis in BWF1 lupus prone mice [95]. Notably, both CD4+CD25+ and CD8+CD25+ Treg cells exert strong suppressive activity on Th1, but to a much lower extend on Th2 cells, presumably because the latter could respond to growth factors other than IL-2 [96].

Although the actual mechanism is complicated, Treg act mainly via two pathways: either direct contact or cytokine secretion. Tolerance, however, is also induced by intrinsic mechanisms. For example, T- or B-cell receptor editing rendered anergic and apoptotic signals after contact with self-antigens [97].

CD4+ regulatory T cells

There are three major subsets of CD4+ regulatory T cells, including CD4+CD25+T cells, T-regulatory 1 (Tr1) cells, and Th3 cells [53]. CD4+CD25+ T cells belong to naturally occurring Treg cells, which arise in the thymus and results in the suppression of CD4+ T cells, CD8+T cells, monocytes, B cells, and dentritic cells in the early immune response via either cell-to-cell contact mechanisms [98] or perforin / granzyme pathway in the periphery to prevent autoimmune reactions [99]. The transcriptional repressor FoxP3 seems to be the most important biomarker of natural Treg, and was shown (at least in mice) to be specially expressed by this T cell subset. Both Th3 and Tr1 Treg cells are

induced Treg cells, which appear during disease or after tolerance induction as a consequence of activation of mature T cells [100]. The activation is antigen-restricted and they suppress T and B lymphocytes which participate in ongoing autoimmune responses.

CD4+CD25+T cells

The CD4+CD25+ Treg cells are currently the best defined and characterized regulatory T cells [101]. They are the crucial components of a complex machinery which is responsible for sustaining peripheral tolerance toward self-structures [102]. The CD4+CD25+ Treg cells are also involved in the regulation of T cell homeostasis via IL-10 and TGF-β [103,104].

The results in the published studies on the phenotype and function of CD4+CD25+Treg in patients with SLE are controversial. It is found that CD4+CD25+ Treg are numerically decreased or functionally defective during active SLE [105,106]. However, in another study, the suppressive function of CD4+CD25+ cells is only defective in one third of the patients, and there is no apparent abnormalities in the levels of different regulatory T cell subsets in the peripheral blood of SLE patients [107]. Miyara et al [108] suggested that the increased sensitivity of the Treg cells in SLE patients to Fas-mediated

apoptosis may account for the reduced number of Treg cells in SLE. In contrast, Yates et al [109] reported no association between defects in CD4+ CD25hi regulatory T cell function or a reduction in their frequency and the disease activity of LN patients. Another study also indicated that the increased FoxP3 mRNA level at early stage of B cell-depletion persisted in anti-CD20 antibody-treated SLE patients with clinically active remission of nephritis [110]. The differences of these reports may be due to the different stages of disease at the time of study, different activities of the disease, the influence of therapies, for example, corticosteroids may increase the proportion of CD4+ CD25hi T cells and FoxP3 expression [111]. Further, the variable definition of surface and cytoplasmic markers of CD4+ Treg, and the heterogeneity of disease manifestations may also account for the inconsistent reports in the literature [112]. Further studies are required to confirm the exact role of CD4+CD25+ Treg in SLE.

Tr1 cells

In general, Tr1 cells are derived from naive CD4+ T cells in the presence of IL-10, by repeated stimulation with immature DC or by treatment with immunosuppressive drugs (e.g. vitamin D3 and dexamethasone) [52]. Tr1 cells are IL-10-producing Treg cells and suppress the proliferation of autoreactive Th cells in antigen-specific but cell contact–independent manner [113,114]. They also suppress naive and memory Th1 or

Th2 responses via production of IL-10 and TGF-β [115]. In the NZB/NZW mouse model of lupus, Riemekasten et al [116] found that high dose tolerance can delay the production of autoantibody, postpone the onset of LN, and prolong the survival mainly by inducing Tr1 cells. Furthermore, these IL-10+ / IFN-γ+ Tr1 cells could prevent autoantibody generation and anti-CD3-induced proliferation of naive T cells [116]. In addition, IL-10 can also down-modulate murine lupus through inhibition of pathogenic Th1 cytokine responses [117]. However, some studies suggest the contrary, and IL-10 has been reported to be involved in the pathogenesis of lupus, and the use of IL-10 antagonists may be beneficial in the management of refractory SLE [118].

Th3 cells

There is currently limited information on the role of Th3 cells in pathogenesis of SLE. Th3 cells are generated following oral administration of antigen or by stimulation in the presence of TGF-β [52]. The main suppressive mechanism of Th3 cells is via TGF-β, which is also involved in the suppression of Th1 and Th2 proliferation [119]. Weiner et al [120] propose that Th3 cells suppress CD4+T cells in antigen non-specific manner. The inhibitory effect of ex vivo Th3 cells is mediated by TGF-β, and in a partially contact-dependent manner [121]. A significant enhancement in the levels of CD4+CD25bright ,Th3 and Tr1, accompanied by an improvement in their suppressor

function, was observed in LN patients treated with anti-CD20 antibody [122], suggesting that the function of Th3 may be deficient in LN and the defect could be reverted by therapy.

CD8+ Ts

In addition to CD4+ regulatory T cells, the suppression of immune responses could also be mediated by CD8+ T cells. Indiveri et al [123] observed that CD8+ T suppressor cell function is impaired in patients with active SLE. Skaggs et al [112] suggest that CD8+ Ts could delay autoantibody production, inhibit Th cell proliferation, and mediate immune suppression, largely as a result of the expression of FoxP3 and secretion of TGF-β. The CD8+CD28− T-cell population has been reported to be reduced in patients with SLE as compared to healthy individuals, suggesting an impaired T-cell suppression [124].

CD8+ Ts may regulate immune system either by direct cytotoxicity toward T, B cells, and professional antigen-presenting cells (APCs), or secretion of TGF-β, IL-10, IFN-γ and TNF-α [95,125]. Cytotoxicity of CD8 T cells may also be an important mechanism for limiting expansion of autoreactive B cells. The process may be mediated by release of calcium ion, perforin, and granzyme polarized toward the target after CD8+ TCR

recognizes responsible antigen, or by the activation of apoptosis in the target via Fas / FasL pathway [95]. Failure of this mechanism allows persistent CD4-driven polyclonal B cell activation, resulting in clinical lupus. Some animal experiments supports the hypothesis that therapeutic cytotoxic T cell enhancement may be beneficial in lupus [126].

NK/T cells

Natural killer / T cells are a unique T cell lineage which plays a critical role in modulating the immune responses. The pathways include secretion of cytokines (IFN-γ, IL-4, TGF-β, IL-10), direct cytotoxic effect on immature immune cells, and induction of immune deviation in CD4+ T cells from Th1 to Th2 phenotype [95]. Three main subsets of NK/T cells have been identified, including type I, type II NK/T cells and NK/T-like cells. Type I or classic NK/T cells are invariant NK/T (iNK/T) cells. They are CD1d-dependent and α-GalCer reactive. Type II or nonclassic NK/T cells are variant NK/T cells. They are CD1d-dependent but α-GalCer unreactive. The third type, so-called NK/T-like cells, are CD1d-independent NK1.1+ T cells [127].

The association between NK/T cell deficiency and autoimmune disease provides direct evidence on the pathogenic role of this T cell subset [128]. Green et al [129] found that

the frequency of NK/T cells was lower in patients with SLE than in control subjects. The reduction of NK/T-cell numbers in patients with SLE could be due to an increased sensitivity of NK/T cells to apoptosis. Tao et al [130] suggested that NK/T cells from active SLE may have high sensitivity to anti-CD95-induced apoptosis due to deficient expression of CD226 and survivin. They also confirmed this hypothesis by demonstrating that CD226 and survivin expression increased while anti-CD95-induced apoptosis decreased in NK/T cells treated with prednisone. In contrast, Forestier et al [131] demonstrated a temporally variable role of iNK/T cells in the (NZB × NZW) F1 mice lupus model, with a protective role in the early stage and a pathogenic role in the later stage of active lupus.

Two major determinants of NK/T cell function is CD1d and α-galactosylceramide (α-GalCer). The MHC class I-like CD1d glycoprotein is a member of the CD1 family of antigen-presenting molecules, and is responsible for the selection of NK/T cells. A regulatory role of CD1d and NK/T cells during the development of lupus nephritis has been implied. For example, BWF1 mice with germline deletion of CD1d have accelerated lupus nephritis, which is associated with an increase in IFN γ-producing CD1d-independent NK/T-like cells [132]. In addition, the proportions and numbers of iNK/T cells are reduced at the early stages of lupus disease development [132].

The α-GalCer is a glycolipid isolated from marine sponge. It can bind to CD1d and specifically activates mouse and human NK/T cells, leading to a rapid and massive secretion of IL-4 and IFN-γ [133]. The role of α-GalCer in regulating NK/T and lupus is controversial, and the precise mechanisms involved remain unclear. Yang et al [134] found that repeated treatment of α-GalCer may alleviate dermatitis by increasing the number of Th2 cytokine producing NK/T cells in MRL-lpr lupus-prone mice, but has no influence on kidney disease. Some studies have also shown that α-GalCer reduces nephritis in young, prenephritic NZB/W F1 mice [128]. However, Zeng et al [135] reported that multiple injections of α-GalCer may exacerbate lupus by activating NK/T cells and induce an abnormal Th1-type immune response in NZB/W F1 mice. These findings suggest that the impact of α-GalCer treatment on mouse model of lupus depends on the types of mice model and the stage of the disease.

γδ T cells

The γδ T cells express the T-cell receptors γ and δ chains. Since they constitute a minor T-cell population in the peripheral lymphoid organs (less than 5% of total T cells), their functions in SLE are still enigmatic. They are enriched in many epithelial organs such as skin, lung, intestine, and genitourinary tract [136]. Human γδ T cells can secrete a range

of cytokines and chemokines including IFN -γ, TNF-α, IL-4, IL-10 and IL-17. Previous reports have suggested that γδ T cells have a negative regulatory function in LN. For example, lupus-prone mice lacking γδ T cells show an exacerbated lupus phenotype as compared to that of their T cell-intact counterparts, with augmented hypergammaglobulinemia and autoantibody production, severe renal disease, and a polyclonal expansion of conventional CD4+ αβ T cells [137]. Fujii et al [138] suggested that γδ T cells down-modulate autoantibody synthesis in MRL mice through exerting TNF-α-mediated cytotoxicity against the activated B cells, and secreting IL-10 to inhibit the Th cells. In contrast, Peng et al [139] found that γδ T cells-dependent helper function are capable of inducing LN in MRL mice via humoral autoimmunity. These data indicate that γδ T cell has the conflicting role of both regulation and propagation of murine lupus.

How about human lupus? Robak et al [140] found that there was an accumulation of γδ T cells in skin of SLE patients, and the percentage of these cells correlated with the activity of SLE. In contrast, other workers showed that the absolute number of γδ T cells in peripheral blood was lower in SLE patients than healthy controls, with an inverse correlation toward the disease activity [140,141]. In addition, the regulatory effects of γδ T cells may also mediate by their interaction with dentritic cells, which affects both

the innate and adaptive immune response [142].

In summary, T cells from SLE patients have several biochemical abnormalities that favor their pathogenic roles. Each subset of T cells has their unique functions, but different subsets also interact with each other. The result is a complicated combination of effector cells, cytokines and chemokines. While the full details of the pathogenesis of SLE remain to be elucidated, recent advances in the identification and characterization of T helper and T regulatory cells subsets deepen our understanding of the pathogenesis of SLE and may lead to the discovery of biomarkers that can be used as prognostic indicators or for the monitoring disease activity.

1.5.4.2 B lymphocytes

Similar to T cells, the function of B lymphocytes are commonly abnormal in SLE patients, and the defect is responsible for the production of a whole spectrum of autoantibodies, notably against common nuclear antigens [143]. The most remarkable B cell defect in SLE is probably the increase in numbers of activated B lymphocytes, which contributes to the hypergammaglobulinaemia with immunoglobulins reactive to self antigens. SLE is also characterized by the loss of B cell tolerance and a defective

censoring at tolerance points that normally delete autoreactive B cells. B cell hyperactivity in SLE has been related to several molecular mechanisms: i) an increase in plasmablasts and transitional B, with decrease in naïve B cells; ii) increased stimulation via toll-like receptor-9 (TLR9); iii) increase in B-cell activating factor promoting their longevity or activation; and iv) reduction in suppressive signals [144].

In addition, B lymphocytes also play an important role in back-activating T cells. For example, autoimmune-prone mice that have B cells defective in antibody secretion still develop interstitial nephritis, vasculitis, and glomerulonephritis [145], but elimination of B cells in lupus-prone mice results in a complete abrogation of pathology, including T cell interstitial infiltration [145]

Recently, another population of "pre-naïve" B cells is described, which is intermediate between recent bone marrow emigrant B cells and naïve ones. This subset, containing also B cells reactive to autoantigens, is expanded in SLE patients. CD5+ pre-naïve B cells are responsive to B cell receptor (BCR) stimulation and differentiate into antibody-secreting plasma cells, although the degree of activation is not as much as traditional naïve B cells [146] .

1.5.4.3 Other accessory cells

A number of studies have shown that SLE patients exhibit deficient APC function for the presentation of exogenous antigen, and reduced stimulation of autologous T cell in the autologous mixed lymphocyte reaction (MLR). The frequencies of DCs in peripheral blood are consistently reduced in SLE patients as compared to healthy controls [147].

Another two remarkable defects in SLE are the impairment in cytotoxicity of macrophage and NK cell, and the reduction in clearance of immune complexes by the mononuclear phagocytic system. For example, Munoz et al [148] reported a defective clearance of apoptotic material by monocyte / macrophages in SLE; these apoptotic materials are then overpressed by follicular dendritic cells (FDC) in secondary lymphoid tissues to B cells, eventually resulting in the production of autoantibody in susceptible individuals. Riccieri et al [141] reported that non-MHC-restricted cytotoxicity, shared by NK cells, NK-like cells (T cells positive for CD3, CD16, and CD56), and γ/δ T cells are down-regulated in SLE patients. More recently, Toll like receptors (TLR), which are receptors of the innate immunity and are present in many accessory immune cells, have been identified to play a key role in the

activation of DC and B cells. Specifically, endosomal TLR-9, and, to a lesser extent, TLR-7, recognize ribonuclear-protein complexes and is able to trigger the intracellular signalling cascade and cell activation in DC and B cells [149,150].

1.5.5. Cytokines

The aberrant cellular effector mechanisms seen in SLE hinges on the interaction between immune cells and their extracellular environment – a process that cytokines are heavily involved. The inflammatory process that occurs in SLE can thus be understood in terms of an imbalance in the cytokine system, which may determine lupus disease activity in general as well as the particular pattern of organ involvement. A list of key cytokines involved in the pathogenesis of SLE is summarized in Table 1-3 [22,151-154].

Table 1-3 Key cytokines that may be related to the pathogenesis of SLE

Cytokine	Cell type	Clinical and experimental observations
IL-1	Dendritic cells, TH-1, TH-17	↓ Production in lupus patients. T cells unresponsive, possibly defective IL-1R
IL-2	TH-1	Normal or ↓ production by CD4 and CD8, ↓ T cell maturation and ↓ CD8 suppressor function. Functional activity may reflect ↑ soluble IL-2R and CD4 cells have low-affinity IL-2 receptors
IL-4	TH-2, $CD4^+CD25^+$, NK/T, γδ T	↑ Secretion by antigen-primed T cells
IL-6	TH-2, TH-17	↑ Levels in sera and cerebral spinal fluid of lupus patients. Localized in nephritic kidneys and detectable in urine of these patients. ↑ mRNA in PBMCs from patients B cells have ↑ IL-6R and spontaneously produce IL-6 (autocrine) and ↑ Ab production
IL-10	FOXP3−Treg, TH-2, $CD4^+CD25^+$, Tr1, $CD8^+$ Ts, NK/T, γδ T	↑ In sera of patients with SLE, suppresses Th1 cells and impairs cell mediated immunity ↑ Production by macrophages and B cells → defective B7-1 expression → ↓ APC function ↑ B cell function and ↑ production of pathogenic autoantibodies, by autocrine pathway Anti IL-10 blocks anti-DNA Ab production by PBMCs from lupus patients in SCID mice IL-10 accelerated disease and anti IL-10 delayed disease onset in the NZB/W murine model Disease severity correlates with increased ratio of IL-10:IFNγ secretion in lupus PBMCs
IL-12	Dendritic cells, TH-1	↓ In patients (in most reports) especially in glomerulonephritis patients
IL-16	$CD8^+$	↑ In patients, correlates with disease activity
IL-17	TH-17, γδ T	↑ In patients
IL-18	Dendritic cells, TH-1, Macrophages	↑ In patients, lymphocytes hyper-responsive to IL-18
IFNα	Dendritic cells	May activate immature dendritic cells, ↑ APC function may drive B/T cell autoimmunity

IFNγ	TH-1, $CD4^+CD25^+$, $CD8^+$ Ts, NK/T, γδ T	Normal production in patients, but macrophage and NK cell-mediated cytotoxicity impaired. Recombinant IFNγ exacerbated disease in patients and lupus prone mice
TNFα	TH-1, TH-17, $CD8^+$ Ts, γδ T	Protective role in lupus. Produced by Th1 cells, B cells, NK cells, and mononuclear phagocytes MHC linked production: ↑ TNFα seen in haplotypes, DR3 and DR4 ↓ incidence of lupus nephritis ↓ TNFα seen in haplotypes, DR2 and DqW1, associated with lupus nephritis
TGFβ	TH-3, $CD4^+CD25^+$, $CD8^+$ Ts, NK/T	↓ In lupus, low level may contribute to renal pathology since TGFβ suppresses IgG production
BlyS	Monocytes, Dendritic cells, Activated T cells	↑ Serum level in patients. Inhibition of BlyS improves survival in murine lupus models

IL, interleukin; R, receptor; Ab, antibody; PBMC, peripheral blood mononuclear cell; APC, antigen presenting cell; IFN, interferon; NK, natural killer cell; SCID, severe combined immunodeficient mice; MHC, major histocompatibility complex; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; BlyS, B cell stimulator; NZB/W, New Zealand Black/New Zealand White F1. Th: T-helper cells. Tr1: T-regulatory 1 cells. $CD8^+$ Ts: $CD8^+$ lymphocyte suppressor cells. NK/T: natural killer / T cells.

1.5.6 Complement

The complement system consists of approximately 20 plasma proteins, which mediate the inflammatory response to immune complexes and opsonisation of infectious microbes. The major function of the complement system is recognition and elimination of pathogens via direct killing or stimulation of phagocytosis.

The role of complement in the pathogenesis of SLE is paradoxical. On the one hand, complement components appear to mediate tissue damage initiated by autoantibody. On the other hand, the complement system appears to have a protective role because hereditary deficiencies of some complement components are associated with an increased risk for SLE. Although decrease in serum complement levels were not consistently associated with SLE flares, progressive decline in serum complement levels were associated with corresponding increase in renal and haematologic disease activity [155]. Studies in complement-deficient (C1q-/-) mice found higher titres of autoantibodies and an excess mortality as compared to strain-matched control mice. More importantly, 25% of the C1q-/- mice developed glomerulonephritis with immune deposits and multiple apoptotic cell bodies. Even in C1q-/- mice with no glomerulonephritis, a large number of apoptotic bodies were detected in the glomeruli [156]. It has been suggested that congenital complement defects result in a

defective clearance of immune complexes [157], which may contribute to the pathogenesis of SLE. Moreover, C3b/C4b receptor (CR1, CD35) on erythrocytes takes part in the binding, transport, and endocytosis of circulating immune complex in the expression of the erythrocyte CR1 as well as the serum levels of soluble CR1 are both decreased in SLE patients [158]. In addition, defective clearance of apoptotic cells by the complement system may play a role in the development of accelerated atherosclerosis in SLE [159].

1.5.7 Apoptosis

Apoptosis appears to have a dual role in the pathogenesis of SLE. First, apoptosis is an important mechanism for the deletion of autoreactive lymphocytes. Secondly, apoptosis results in the availability of autoantigens that can be presented to the immune system. Normally, apoptotic cells are rapidly cleared by macrophages, but there is a profound defect in this clearance pathway in SLE patients [160]. Studies from a number of animal models support the idea that defective clearance of apoptotic cells has an essential role in the pathogenesis of SLE [161].

1.5.8 Conclusion on pathogenesis

Taken together, the pathogenesis of SLE is multifactorial. It involves genetic and environmental factors. Abnormalities in almost all components of the immune system have been described.

*1.6 Clinical Features*s

1.6.1 Constitutional Symptoms

Systemic symptoms during SLE disease flare are usually prominent [162]. Fatigue, malaise, weakness, fever, lymphadenopathy, anorexia, nausea, vomiting, and unintentional weight loss are common symptoms.

1.6.2 Mucocutaneous Involvement

Skin and mucous membranes are clinically involved in 60% to 80% of all SLE patients. Notably, mucocutaneous lesions may accompany or precede other signs or symptoms of SLE. The cutaneous lesions of SLE may be classified as acute, subacute, and chronic. The acute lesions generally present as malar rash or butterfly dermatitis. These lesions are photosensitive and are often associated with systemic manifestations. They are characterized by confluent, symmetric erythema and oedema centred over the malar eminence. The subacute lesions consist of nonscarring papulosquamous or annular lesions which are exquisitely photosensitive. Typically they present as erythematous macules or papules which evolve into scaly papulosquamous or annular, polycyclic plaques. Chronic lesions are mostly represented by discoid lupus with flat, erythematous, demarcated macules or papulae evolving into coin-shaped, demarcated plaques.

Merging of several discoid plaques may cause disfiguration. Hyperkeratosis, verrucous dermatitis, or panniculitis are rare forms of chronic lesions. The sun-exposed areas of the skin are the common sites of cutaneous lesions [163].

1.6.3 Musculoskeletal System

Arthralgia and arthritis are reported by almost all patients with SLE. Symptoms are often asymmetrical and migratory. All major and minor joints may be affected. Fingers, hands wrists, knees, ankles, elbows, and shoulders are the most affected joints. Pain is often out of proportion to physical findings. Deformities and erosions may occur. Myalgias occur in 40% to 80% of patients and are most marked in proximal muscles. Tendonitis and inflammatory myositis may also be present. As a result of musculoskeletal symptoms, up to 80% of SLE patients are treated with anti-inflammatory drugs.

1.6.4 Kidney involvement

Renal problem is frequent in SLE patients. Overt clinical evidence of renal disease is present in 35% to 75% of patients. Lupus nephritis usually develops within 3 years after diagnosis of SLE. However, clinical data probably underestimate the prevalence of

lupus nephritis. Studies with renal biopsies show that SLE involves the kidney in almost all cases if enough tissue is available for analysis. Even in the absence of proteinuria or abnormal urinary sediment, some abnormality is almost always present in renal biopsy specimens, especially if evaluated by immunofluorescence or electron microscopy. The details of lupus nephritis are further reviewed in a later chapter of this thesis.

1.6.5 Neuropsychiatric Manifestations

About 28% of SLE patients have definite central nervous system disease, which is associated with antiphospholipid antibodies in half of the cases. Any region of the brain can be involved. Headache is probably the most frequent neurological symptom in SLE. Neurological events may be isolated or multiple, but usually occur in the presence of active disease. Mild mental dysfunction is the most frequent manifestation. Seizures are frequent. Other neurological manifestations include acute delirium, transient confusional state, aseptic meningitis, mononeuritis multiplex, ascending or transverse myelitis, peripheral or cranial neuropathy, choreoid movement, cerebellar ataxia, and pseudotumor cerebri. Anxiety and depression are not uncommon in patients with SLE, and the problem be worsened by corticosteroid therapy. Frank psychosis may develop. Phobias and obsessive behaviour may even precede the diagnosis of SLE. Intravenous

cyclophosphamide appears to be an effective treatment for many patients with severe neuropsychiatric SLE. The differential diagnosis between organic brain disease and psychological reaction may sometimes be difficult. Psychological tests, magnetic resonance imaging, evoked potentials, and cerebrospinal fluid analysis can help to discriminate functional disturbance from organic problem.

1.6.6 Cardiovascular manifestations

Cardiac disease is common in patients with lupus. Pericardial effusion, usually clinically silent, may occur in up to half of the patients. Myocardial dysfunction may be present, resulting in cardiac arrhythmias and heart failure. Valvular disease may occur in one-fourth of patients, with mitral valve prolapse being the most frequent abnormality. Verrucous endocarditis, secondary either to infection or to antiphospholipid antibodies, can result in valvular defects but is often underdiagnosed. Transoesophageal echocardiography has been recommended to detect cardiac abnormalities, which may be a result factor of stroke. Coronary artery disease and myocardial infarction are also frequent complications. In general, atherosclerotic diseases are common after the use of corticosteroids and the presence of hypertension or hypercholesterolemia. Furthermore, accelerated atherosclerosis can also be present without traditional risk factors, suggesting a role of SLE *per se* in atherogenesis. In addition, recurrent deep vein or

arterial thrombosis are frequent in SLE patients, especially those with antiphospholipid antibodies.

1.6.7 Pulmonary manifestations

Pleurisy and pleural effusions are common. Lupus pneumonitis is a rare problem characterized by recurrent pulmonary infiltrates with fever, dyspnoea, and cough. Radiography shows acinar infiltrates, especially in the lower lobes. Late complications of lupus pneumonitis include diffuse interstitial infiltrate, pulmonary fibrosis, and progressive pulmonary dysfunction. Pulmonary hypertension, either as the result of interstitial lung disease or as a primary problem, may also be present.

1.6.8 Gastrointestinal manifestations

Gastrointestinal symptoms are common in SLE patients, and can be the result of SLE *per se*, complications of therapy, or other primary gastrointestinal disorders. Notably, a substantial proportion of patients complain of dysphagia, often without radiographic abnormality. Mesenteric vasculitis may rarely cause acute abdomen. Acute pancreatitis may be caused by vasculitis or the use of corticosteroid. Liver involvement is, however,

not common. The association of liver function abnormalities with positive antinuclear antibodies (the so-called lupoid hepatitis) is more consistent with autoimmune chronic active hepatitis, which is a distinct disease entity not related to SLE.

1.6.9 Ophthalmological disorders

Keratoconjunctivitis sicca is the most common ocular disorder of SLE. Retinal vasculitis with cotton wool exudates and fundal cytoid bodies is rare. Other uncommon manifestations include episcleritis, scleritis, and transient amaurosis fugax secondary to the presence of antiphospholipid antibodies.

1.6.10 Vascular manifestations

Raynaud's phenomenon and livedo reticularis may occur in patients with SLE. Recurrent thromboembolism and phlebitis are typically related to antiphospholipid antibodies, which activates the coagulation cascade. Cutaneous vasculitis may result in mucocutaneous purpura or ulcers, both are particularly common in the legs. Rarely, vasculitis can cause acute surgical abdomen.

1.6.11 Haematological disorders

Anaemia occurs in most patients with active SLE. Haemolytic anaemia with an elevated reticulocyte count, reduced haptoglobin concentrations, and a positive direct Coomb's test has been noted in 10% to 40% of patients. The titre of autoantibodies is usually higher in anaemic patients. In addition, leucopenia is common, which may result from anti-neutrophil antibodies, circulating immune complexes, or cytotoxic medications. Mild thrombocytopenia is common is active SLE, but severe thrombocytopenia with haemorrhage or purpura occurs in less than 5% of cases. Patients initially presenting as Evans' syndrome, characterized by the presence of haemolytic anaemia and immune thrombocytopenic purpura, also have SLE as the disease gradually unfolds.

1.7 Lupus Nephritis

1.7.1 Clinical Features

Lupus nephritis usually develops within 3 years after diagnosis of SLE. Around 25% to 50% of SLE patients develop lupus nephritis at the initial presentation [164]. Occasionally, renal disease develops five or more years after the diagnosis of SLE. Some patients with lupus nephritis have only modest urinary abnormalities, such as microscopic haematuria or asymptomatic proteinuria, initially. These abnormalities may be intermittent. Other patients present with frank nephrotic syndrome or an acute nephritic picture, with various degrees of renal function impairment. Less commonly, patients may present with a rapidly progressive glomerulonephritis or chronic progressive renal insufficiency. Active lupus nephritis is often associated with the presence of other signs and symptoms of active SLE, as well as typical serological abnormalities.

In general, it is difficult to predict the histological findings accurately from urinalysis, serological findings, and serum creatinine level. A kidney biopsy is usually essential for SLE patients with renal involvement in order to differentiate the specific type of lupus

nephritis (see below), which is important in the decision of appropriate therapy and the estimation of prognosis.

1.7.2 Prognosis

The natural course of untreated lupus nephritis is almost always progressive. Before the era of aggressive immunosuppressive therapy, most patients with lupus nephritis died or developed dialysis-dependent renal failure within a few years of initial presentation. Recent series, however, reported that over 80% of patients still have adequate renal function 10 years after diagnosis [164]. Nonetheless, despite active treatment, many patients have persistent proteinuria and microscopic haematuria.

Predictors of renal failure include elevated baseline plasma creatinine, nephrotic range proteinuria, male gender, young age, black race, and low socioeconomic class [165]. Repeated flares of active nephritis are associated with an increased risk of progressive renal disease [166]. There is also a strong association between antiphospholipid antibodies and the development of chronic renal insufficiency [167].

1.7.3 Histologic Features

Renal biopsy plays an important role in the evaluation of patients with lupus nephritis. The spectrum of pathological changes observed in renal biopsy is extremely broad, including a variable degree of glomerular inflammation and interstitial involvement. Pathological features of lupus nephritis can be categorized as either active or inactive, as summarized in Table 1-4 [168].

Hypercellularity of the glomerular tuft is frequent. It can be segmental or global, focal or diffuse, but characteristically is almost never uniform and may vary widely from one glomerulus to another. Hypercellularity may be due to proliferation of mesangial, endothelial, and epithelial cells, as well as monocytes and polymorphonuclear cell exudation. There are often areas of necrosis of the glomerular tuft, usually confined to one lobule. Necrosis is characterized by ill-defined granular material, weakly eosinophilic staining for fibrin (so-called "fibrinoid necrosis"), nuclear fragments, and intermingling polymorphs within the area. Proliferating epithelial cells may encircle the necrotic area and adhere to the Bowman capsule, resulting in crescent formation. In fewer than 10% of cases, haematoxylin bodies can be found in the areas of necrosis or inflammation, at times in the capillary lumen.

The presence of immune deposits in the glomeruli is the hallmark of lupus nephritis. The amount and location of deposits are closely related to the severity and pattern of glomerular changes [169]. In general, immunofluorescence reveals glomerular deposits of IgG, and, to a lesser extent, IgM and IgA. All complement components may be found and the most usual being C3, C1q, and C4. A "full house" deposition of these immunoreactants is found in some 25% of patients, and is highly characteristic of lupus nephritis [170].

Table 1-4 Histologic findings considered to reflect active and inactive lesions in lupus nephritis (adopted from [171]).

Active lesions	Inactive lesions
Glomeruli	
Local necrosis	Basement membrane thickening
Cellular proliferation	Fibrosis
Karyorrhexis	Adhesions
Fibrinoid	Fibrous crescents
Wire loops	
Hematoxyphil bodies	
Hyaline thrombi (rare)	
Cellular crescents	
Tubulointerstitium	
Inflammatory infiltrates	Fibrosis
Tubular necrosis	Tubular atrophy
Edema	
Arteries and arterioles	
Fibrinoid deposits	Arterial sclerosis
Fibrin / Platelet thrombi	Arteriolar hyalinosis
Necrosis	

1.7.4 Histologic Classification

Because of the highly variable histological features of lupus nephritis, the World Health Organization (WHO) first tried to provide a standardized histological assessment and classification of kidney lesions caused by SLE. The WHO classification was first established in 1975 [172], which was further modified in 1982 [173] and then 1995 [174] (summarized in Appendix II). The classification was then adopted and fine modified by the International Society of Nephrology / Renal Pathology Society (ISN/RPS), which was published in 2003 [175].

Based on the original 1982 classification, it was necessary to clarify the morphological class of lupus nephritis, and the significance of segmental glomerular capillary wall necrosis was emphasized. The 1995 modification stated that segmental glomerular necrosis is the defining feature of class III lesions, regardless of the percentage of glomeruli involved (Appendix II). Years later, Najafi et al [176] reported that the outcome of diffuse segmental necrotizing glomerulonephritis involving over 50% of glomeruli is more severe than other forms of class IV lupus nephritis. As a result, the

definitions of class III and IV lesions were further modified in 2003, with specification on the quantitative and qualitative differences of glomerular involvement.

Appendix III summarizes the ISN/RPS 2003 classification of lupus nephritis. In Class I, the light microscopy is normal and immunofluorescence shows minimal mesangial disease except accumulation of immune complexes. Unlike the previous definition, a complete lack of renal abnormalities by light microscopy, immunofluorescence and electron microscopy is not defined as class I.

Class II disease is also known as mesangial proliferative lupus nephritis. It reflects any degree of mesangial hypercellularity, defined as three or more mesangial cells per mesangial area in a 3 micron thick section, in association with mesangial immune deposits. Isolated small immune deposits involving the peripheral capillary walls may rarely be seen in some examples of class II n by immunofluorescence or electron microscopy. However, detection of any subendothelial deposits by light microscopy would be considered as class III or class IV nephritis (depending on the degree and pattern of these deposits). In addition, the identification of any global or segmental

glomerular scars would be considered as class III or class IV nephritis, again depending on the proportion of scarred glomeruli.

Class III nephritis is defined as focal lupus nephritis involving less than 50% of all glomeruli, with subdivisions for active, "active and chronic" or "inactive and chronic" lesions, all of which are usually segmental and rarely global. Segmental endocapillary proliferative lesions or inactive glomerular scars, with or without capillary wall necrosis and crescents, with subendothelial deposits, are seen in affected glomeruli. The degree of glomerular lesions will be accounted for by both active and sclerotic lesions. Focal or diffuse mesangial alterations, including mesangial proliferation or mesangial immune deposits, may persist with the focal glomerular lesions. Combined class III and class V is termed when light microscopy or immunofluorescence indicate the presence of membranous involvement in at least 50% of the glomerular capillary surface area of at least 50% of glomeruli.

Class IV nephritis is defined as diffuse lupus nephritis involving 50% or more of glomeruli in the biopsy, either with segmental (class IV-S) or global (class IV-G) involvement, and also with subdivisions for active and sclerotic lesions. The new

subdivision for segmental and global lesions is based on the evidence that diffuse segmental lupus nephritis may have a different outcome than diffuse global lupus nephritis. Segmental lesion of the affected glomeruli is defined as sparing at least half of the glomerular tuft. Global lesion is defined when more than half of the glomerular tuft are involved. Class IV lesions are subdivided into diffuse segmental lupus nephritis (class IV-S) when more than 50% of the involved glomeruli have segmental lesions, and diffuse global lupus nephritis (class IV-G) when more 50% of the involved glomeruli have global lesions. Class IV-S typically shows segmental endocapillary proliferation that intrudes upon capillary lumina, with or without necrosis. Class IV-G is characterized by diffuse and global endocapillary and/or mesangial proliferation, or widespread wire loops. Features of active lesion, including karyorrhexis, capillary loop necrosis, and crescent formation, may be seen with class IV-G disease. Rare examples of extensive diffuse or global subendothelial glomerular deposits with little or no proliferation are also classified in this category. Since scattered subepithelial deposits are commonly seen in class IV disease, a diagnosis of combined class IV and class V is warranted only if subepithelial deposits involve at least 50% of the glomerular capillary surface area in at least 50% of glomeruli by light microscopy or immunofluorescence microscopy. Both active and sclerotic lesions will be taken into account when defining the extent of the lesions.

Class V disease is defined as membranous lupus nephritis with continuous global or segmental subepithelial granular immune deposits, with or without mesangial alternations by immunofluorescence or electron microscopy, and may accompany any degree of mesangial hypercellularity. Combined designation of class V with class III or IV lupus nephritis is possible, depending on the distribution of subendothelial deposits and the glomerular scarring (see previous discussion).

Class VI disease is defined as advanced stage lupus nephritis with 90% or more global glomerulosclerosis, which is resulted from chronic class III, class IV, or class V lupus nephritis. It represents a scarred, end-stage kidney without residual or ongoing activity.

1.7.5 Histological Activity and Chronicity Indexes

In addition to the classification of lupus nephritis, scoring of the renal biopsy for parameters of activity and chronicity, by grading and adding the individual morphologic components in a given biopsy, provides a useful guide to the choice of treatment as well

as valuable prognostic information. It is highly recommended to describe the histological Activity and Chronicity Indexes in the renal biopsy report [176].

The histological Activity Index is derived from semiquantitative scores for glomerlular cell proliferation, leukocyte exudation, subendothelial hyaline deposits, necrosis, cellular crescents and interstitial inflammation. Necrosis and cellular crescents are each arbitrarily weighted double. The histological Chronicity Index is similarly defined by semiquantitative glomerular sclerosis, fibrous cresents, tubular atrophy and interstitial fibrosis. The individual histologic scores are added to give the respective Activity and Chronicity Indexes [177,178].

*1.8 Clinical Assessment of Lupus Disease Activity*y

More than 95% of patients with SLE have antinuclear antibodies, which may be present many years before the diagnosis of SLE [179]. However, antinuclear antibodies are not specific as they can be present in other rheumatic diseases, chronic infections, chronic liver diseases, neoplastic syndromes, as well as in the elderly. Antibodies to DNA can be divided into native (double-stranded) and denatured (single-stranded) DNA, corresponding to the two macromolecular forms of DNA [180]. Anti-dsDNA antibody has a 95% specificity for the diagnosis of SLE, although they can be negative in some patients with inactive lupus. Anti-ssDNA antibodies can also be found in patients with other connective tissue diseases or infection. In general, the titer of anti-dsDNA antibody changes with the disease activity and is a useful indicator of therapeutic response.

During active lupus, there is an activation of the classical pathway of the complement cascade, with consumption of the early components C1q, C4, and C3. Serum C4 generally decreases earlier and to a greater extent than C3 during lupus disease flares. The combination of low C3 and increased anti-DNA antibody titres is virtually

pathognomonic for SLE and the magnitude of abnormality correlates well with clinical disease activity [181].

Nonetheless, simple laboratory tests do not reliably reflect the entire picture of a multisystem disease like SLE. A systematic assessment tool is indispensable in evaluating the diverse clinical manifestations. Several reliable and validated global activity scores have been developed to assess the disease activity of SLE. The most commonly used scoring system include the Systemic Lupus Activity Measures (SLAM) [182], British Isles Lupus Assessment Group (BILAG) [183] and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [184]. In general, SLAM and SLEDAI sum up a global score for disease activity; while BILAG allows reporting disease activity in different organs or systems separately. Although there is no recommended frequency for the lupus activity measures, a number of studies on the reliability and validity of these scoring systems have shown that they are reliable [185-187]. In addition, comparative studies on their reproducibility and ability to assess the change in disease activity over time showed that the SLEDAI, SLAM and BILAG are comparable measures, although SLEDAI appears to be particularly sensitive to change in disease activity over time [188,189]. SLEDAI has an additional advantage because it been used successfully by both expert clinicians [186] and trainees [187], and

has been shown to be valuable in both research and clinical settings [190,191].

Apart from the current disease activity, SLE can results in irreversible damages to the body. The Systemic Lupus International Collaborating Clinics (SLICC) group devised a damage score for SLE to assess the permanent damages, which has been recognized by the American College of Rheumatology [192]. In this index, damage occurring since diagnosis of SLE is confirmed by clinical assessment and must be present for at least six months. Since the same lesion cannot be scored twice and repeat episodes need to be at least six months apart for scoring, this index gives a cumulative score of organ damage. Appendix I summarize the scoring parameters involved in SLEDAI and SLICC/ACR damage index.

1.9 Advances in Biomarkers of Lupus Nephritis

In recent years, besides the traditional cytokines that are described in the previous chapters, a number of new candidate genes appear to play important roles in the pathogenesis of lupus nephritis and have the potential of further development as biomarkers, as summarized in Table 1-5.

Table 1-5 Summary of novel biomarkers for lupus nephritis

Biomarkers	Cell type and tissue expression	Function
TWEAK / Fn14	monocytes and macrophages	Mediates important biological effects, including upregulation of multiple chemokines, cytokines and adhesion molecules, induction of apoptosis, and enhancement of cell survival
IP-10 / CXCR3	monocytes, neutrophils, endothelial cells, keratinocyte, fibroblasts, mesenchymal cells, dendritic cells, and astrocytes	Regulates immune responses by enhancing activation and recruitment of leukocytes.
NGAL	epithelial cell	Antimicrobial protein and siderophore which involved in cellular growth and survival
TLR9	B cell, T cell	Recognition of self DNA-antigens and the production of pathogenic auto-antibodies.

1.9.1 The TWEAK / Fn14 axis

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a 249-amino acid cytokine from the TNF-ligand superfamily. TWEAK is synthesized as a membrane-bound protein, but it is the soluble trimeric form that mediates most of its biologic activities [193]. Under physiological and pathological conditions, TWEAK mediates important biological effects, including upregulation of multiple chemokines, cytokines and adhesion molecules [194], induction of apoptosis [195], and enhancement of cell survival [196] via its sole receptor, Fn14 [197]. In SLE, activated T cells express TWEAK, which induces macrophage apoptosis, and thereby contributes to the burden of apoptotic material [198,199]. Previous studies showed that the TWEAK/Fn14 axis plays an important role in tissue inflammation, repair and regeneration in SLE[200]. Zhao et al [201] found that kidney disease was significantly less severe in Fn14 knockout mice; kidney IgG deposition and cytokine levels, as well as macrophage infiltration, were significantly decreased in Fn14-deficient mice with induced lupus; mice with induced lupus treated with an anti-TWEAK neutralizing antibody had significantly diminished kidney expression of inflammatory cytokines as well as proteinuria.. In human SLE, Schwartz et al [202] found that urinary TWEAK levels in patients with lupus nephritis were significantly higher than SLE patients without

nephritis, and urinary TWEAK level correlated with the degree of renal inflammation and systemic disease activity. In another study, the same group further showed that urinary TWEAK levels, but not the serum levels, reflect renal disease activity in longitudinal follow-up [203]. However, the role of the TWEAK/Fn14 axis in the development of human lupus nephritis remains elusive.

1.9.2 The IP-10 / CXCR3 axis

Interferon-γ-inducible protein 10 (IP-10, also called CXCL10) was initially identified as a chemokine that is induced by IFN-γ and secreted by various cell types, such as, monocytes, neutrophils, endothelial cells, keratinocyte, fibroblasts, mesenchymal cells, dendritic cells, and astrocytes [204]. IP-10 is a 10 kDa protein; it belongs to the CXC family of chemokines and is functionally categorized as an "inflammatory" chemokine. This molecule, together with monokine induced by gamma-interferon (Mig, also called CXCL9), and their receptor, CXCR3, regulates immune responses by enhancing activation and recruitment of leukocytes, including T lymphocytes, eosinophils, monocytes, and NK cells [205,206]. Notably, CXCR3 is expressed not only by immune-related cells, but also by many resident cells in the kidney, including endothelial cells, vascular pericytes, and mesangial cells. Recent reports have shown

that serum and tissue expressions of IP-10 are increased in various autoimmune diseases, such as SLE, rheumatoid arthritis, systemic sclerosis, multiple sclerosis, autoimmune thyroid diseases, type 1 diabetes mellitus, and Addison's disease [207-211]. More importantly, activation of the IP-10 / CXCR3 axis also promotes mesangial cell expansion in proliferative glomerulonephritis[212]. A number of human studies reported that blood levels of IP-10 are increased in SLE patients, and the level strongly correlates with systemic disease activity [208,213,214]. Another study also found that IP-10 mRNA level in the urinary sediment helped to distinguish diffuse proliferative lupus nephritis (class IV) from other classes of lupus nephritis [215].

It is important to appreciate that the TWEAK/Fn14 and IP-10/CXCR3 axis are closely interlinked. For example, it has been reported that activation of the TWEAK/Fn14 axis induces human kidney cells to express IP-10, and promotes kidney cell proliferation [216]. In contrast, blockade of the TWEAK/Fn14 axis, either by anti-TWEAK monoclonal antibodies or Fn14 knockout, significantly reduces kidney expression of IP-10[201,216].

1.9.3 NGAL

Neutrophil gelatinase associated lipocalin (NGAL) is a small molecule of a moleulcar weight around 25 kD. It is a member of the lipocalin superfamily, and was first identified in the specific granules of human neutrophil [217]. The most important ligands of NGAL are siderophores[218], which are small non-peptidic iron-containing molecules produced in bacteria, plants, and probably also mammals. Through iron transport and supply, siderophores are involved in cellular growth and survival. NGAL has been implicated in the pathogenesis of several kidney diseases, and is a promising biomarker for both acute kidney injury (AKI) and chronic kidney disease (CKD) [219]. In patients with lupus nephritis, Pitashny et al [220] reported that urinary NGAL levels had better correlation with disease activity than other standard disease markers, while Bolignano et al [221] reported that urinary NGAL concentrations correlated with renal function and proteinuria. Similarly, in another study of patients with childhood-onset SLE, Brunner et al [30] found that urinary NGAL showed a significant correlation with renal disease activity and damage. However, the intra-renal level of NGAL in human lupus nephritis has not been studied.

1.9.4 TLR9

Toll-like receptors (TLRs) are pattern-associated receptors of the innate immune system. These receptors activate multiple inflammatory pathways and coordinate systemic defense against many common pathogens. Toll-like receptor 9 (TLR9) recognizes unmethylated CpG sequences of DNA molecules. Since anti-DNA antibody is a serological hallmark of SLE, it has long been suspected that in SLE, TLR9 may be involved in the recognition of self DNA-antigens and the production of pathogenic auto-antibodies. In murine model of SLE, it has been reported that TLR9 is crucial in the regulation of anti-DNA autoantibody production [222], and the TLR9 signaling pathway has been shown to be essential for the switching of antibody class to pathogenic IgG2a and IgG2b [223].

In human, previous study showed that a higher expression of TLR9 on peripheral blood B cells from patients with active SLE correlated with SLEDAI, and TLR9-CpG ligation induces the production of anti-dsDNA antibody and IL-10 [224]. Machida et al [225] reported that in active lupus nephritis, injured glomerular podocytes expressed TLR9, which disappeared during disease remission, while TLR9 was not expressed in normal

control kidneys. Furthermore, TLR9 expression correlated with the degree of proteinuria and anti-dsDNA antibody titre. Similarly, Benigni et al [226] showed a robust TLR9 expression in the proximal tubular epithelial cells in animal model of lupus as well as patients with lupus nephritis, and TLR9 level correlated with the degree of proteinuria and tubular damage. The up-regulation of TLR9 in the renal parenchyma of active lupus nephritis may be a response to inflammatory cytokines or a direct reaction to the nucleosomes (which contain the ligand for TLR9) present within the lesion [227]. In return, TLR9 induces macrophages and B-cells to produce cytokines and chemokines, thus aggravates inflammation and tissue damage [228-230]. Recent studies in human SLE showed that internalization of immune complexes into subcellular lysosomes that contain TLR9 induces a signaling cascade, eventually leading to the activation of dendritic cells and production of multiple cytokines and chemokines [231], suggesting that TLR9 plays a critical role in the onset and progression of lupus nephritis.

It is important to appreciate that the NGAL and TLR9 pathways are interlinked. Notably, TLR9 causes the activation of NF-κB [232], which is recruited to the NGAL promoter and is essential for the transcriptional activation of NGAL promoters [233]. However, the interplay of the two systems in the pathogenesis of lupus nephritis has not been explored.

1.10 Micro-RNA in Lupus Nephritis

Micro-RNAs (miRNAs) was first discovered in 1993 [234]. They are 20 to 22 nucleotide long non-coding RNA molecules that bind to the 3'-untranslated regions (UTR) of target mRNAs in a sequence specific manner and influence the translation or stability of the mRNA transcripts [235,236]. Mature miRNAs are produced from the pre-micro-RNA transcripts by sequential action of two members of the RNase III family, Drosha and Dicer [236]. Currently, the miRNA system is known to regulate a wide range of cellular processes, such as apoptosis, cell cycle, differentiation, and immune functions. Dysregulation of miRNA has been reported in many human diseases, including cardiac diseases, various cancers, neurodegenerative diseases, and diabetes [237]. More recently, miRNAs have been found to be important players in the homeostasis of both innate and adaptive immunity [238,239].

The role of miRNA dysregulation in the pathogenesis of SLE was suggested by Dai et al in 2007 [240]. In that study, seven miRNA species (namely, miR-196a, miR-17-5p, miR-409-3p, miR-141, miR-383, miR-112, and miR-184) were down-regulated and nine other miRNA species (miR-189,miR-61, miR-78, miR-21, miR-142-3p, miR-342,

miR-299-3p, miR-198, and miR-298) were up-regulated in SLE patients as compared to healthy controls [240]. Later, the same group of investigators further identified several other miRNAs differentially expressed in kidney biopsies of patients with lupus nephritis as compared to normal controls [241]. Similarly, Tang et al [242] revealed differential expression of several miRNA species, notably miR-146a, in SLE patients as compared with normal controls using the TaqMan micro-RNA profiling assays. More recently, Te et al [30] identified five miRNA species (namely, hsa-miR-371-5P, hsa-miR-423-5P, hsa-miR-638, hsa-miR-1224-3P and hsa-miR-663) that were differentially expressed in lupus nephritis across different racial groups.

Taken together, these data suggest that miRNAs are important factors involved in the pathogenesis of lupus nephritis. However, previous studies had not distinguish between glomerular and tubulointerstitial expression of miRNA.

1.11 Laser-assisted microdissection

As briefly explained above, kidney is a complex organ with glomerulus and tubulointerstitium, each may be differently involved in SLE. Laser-assisted microdissection (LMD) is the microdissection of tissue using a laser source, with an aim to isolate small areas of tissue or single cells out of a histological section. It is a rapid and precise method of isolating and removing specified cells from complex tissues [243]. The first user-friendly instrument for laser-assisted microdissection was developed in 1996 at the National Cancer Institute, Maryland, USA [244].

There are several state-of-the-art systems for performing laser microdissection. For example, the PALM® Robot Microbeam laser microdissection system (P.A.L.M. Carl Zeiss, Bernried, Germany) provides a convenient, efficient, and precise method of selecting specified cell populations from complex tissues for subsequent analysis of their RNA, DNA, or protein content. Microdissection of cells defined under the microscope ensures pure samples of cells of interest for downstream molecular applications. Fortunately, molecular biological technology developments, particularly real time quantitative polymerase chain reaction (RT-QPCR), allow analyzing gene expression from only limited amounts of cells. As a result, the combination of

microdissection and RT-QPCR may allow the possibility of assessing the role of specialized cell type or tissue in in a complex organ such as the kidney [245-247].

In principle, the PALM system removes coherent cell fields by applying a pulsed ultraviolet laser through an inverted microscope to allow laser ablation of cells and tissue on a tissue section, either manually or automatically [248]. It belongs to laser cutting microdissection. Briefly, 3 steps involved: Firstly, the tissue is mounted on a membrane-cover slide and is viewed by computer. Subsequently the pulsed ultraviolet A laser beam (focal spot <1 μm) impacts the samples and break molecular bonds, resulting in "cutting". Finally, the interested cells are catapulted into the cap of a tube overlying the membrane slide. This procedure ensures that no unwanted elements reach the specimen. Since the laser in the process is only directed at the sample for about 1 nanosecond, it does not transfer any heat. Thus, it avoids the potential risk of molecule modification of interest by heating or cooling of the thermoplastic membrane usually used in laser capture microdissection. The process is completely contact and contamination-free and guarantees the best possible preservation of the material [249].

CHAPTER 2 – HYPOTHESIS

We hypothesize that the glomerular and tubulointerstitial expression of different target genes are related to:

1. the specific histological pattern of lupus nephritis;
2. the severity of inflammation;
3. the degree of chronic renal damage; and
4. the risk of disease progression and response to treatment.

Specifically, based on the available literature, we hypothesis that:

1. Intra-renal expression of TWEAK / Fn14 is related to the specific histological pattern of lupus nephritis.
2. Intra-renal expression of IP10 / CXCR3 is related to the severity of inflammation.
3. Intra-renal expression of TLR9 / NGAL is related to the degree of chronic renal damage.
4. Intra-renal expression of TLR9 / NGAL is related to the risk of disease progression and response to treatment.

CHAPTER 3 – GENERAL METHODOLOGY

3.1 Assessment of Lupus Disease Activity

The SLE disease activity was assessed clinically with the SLE Disease Activity Index (SLEDAI) scoring system [157], on the day of kidney biopsy. The details of the assessment are outlined in Appendix I. In addition to direct questioning of specific symptoms related to active lupus, urinalysis and blood tests were performed; the latter included complete blood count, renal function test, serum complement level and anti-ds DNA antibody titre. Urinalysis included both conventional urine multistix test and phase-contrast microscopic examination of the urinary sediment. The overall SLEDAI score ranged from 0 to 32.

3.2 Other Clinical Assessment

In this series of work, proteinuria of individual patient was quantified by full 24-hour urine collection. Glomerular filtration rate (GFR) was estimated from the standard Modification of Diet in Renal Disease (MDRD) equation. [274]:

$$\text{GFR (mL/min)} = 186 \times (\text{SCr} \div 88.4)^{-1.154} \times (\text{age})^{-0.203} \times (0.742 \text{ if female}) \times (1.21 \text{ if black})$$

Where SCr is serum creatinine concentration in μmol/L and age is in years.

After the renal biopsy, the clinical management was decided by individual nephrologist and not affected by this series of work. All patients were followed for at least 6 months. Therapeutic response to induction immunosuppressive therapy was assessed at 12 weeks after treatment and classified into complete remission (CR), partial remission (PR), and no response (NR) as defined previously [250]:

1. Complete remission was defined as a value for urinary protein excretion that was less than 0.3 g/24 hours with normal urinary sediment, a normal serum albumin concentration, and values for both serum creatinine and estimated GFR that were 15% or less above the baseline values.

2. Partial remission was defined as a value for urinary protein excretion that was between 0.3 and 2.9 g/24 hours with a serum albumin concentration of at least 30 g/dL and stable renal function.
3. No response (treatment failure) was defined as a value for urinary protein excretion that remained at or above 3 g/24 hours or a value of 0.3 to 2.9 g/24 hours but with a serum albumin below 30 g/dL, an increase in the serum creatinine concentration $\geq$ 50 μmol/l, or a value for estimated GFR that was more than 15% above the baseline value, or the discontinuation of treatment due to side effects.

3.3 Histological Assessment

Kidney biopsy specimen was evaluated and histological pattern classified according to the International Society of Nephrology (ISN) classification of lupus nephritis[175]. The detail of the classification scheme is described in Appendix III. In addition, we also assessed the degree of glomerulosclerosis by calculating the percentage of sclerosed glomeruli and tubulointerstitial scarring by subjective semi-quantitative examination of the silver-stained histology slide under light microscopy.

The histological activity index (AI) and chronicity index (CI) of each biopsy specimen were scored by standard methods[251]. All biopsy specimens were assessed by light microscopy. In short, AI is the sum of semi-quantitative manual scores (0 to 3 each) of the following parameters: endocapillary hypercellularity; leucocyte infiltration, subendothelial hyaline deposits; interstitial inflammation, necrosis and cellular crescents. Scores of the last two parameters are counted double, making a total AI of 0 to 24. Chronicity index is the sum of semi-quantitative manual scores (0 to 3 each) of the following parameters: glomerular sclerosis, fibrous crescents, tubular atrophy and interstitial fibrosis. The maximum score of the chronicity index is 12. Evaluation of biopsy specimens was blinded from the results of the SLEDAI score, blood and urinary tests.

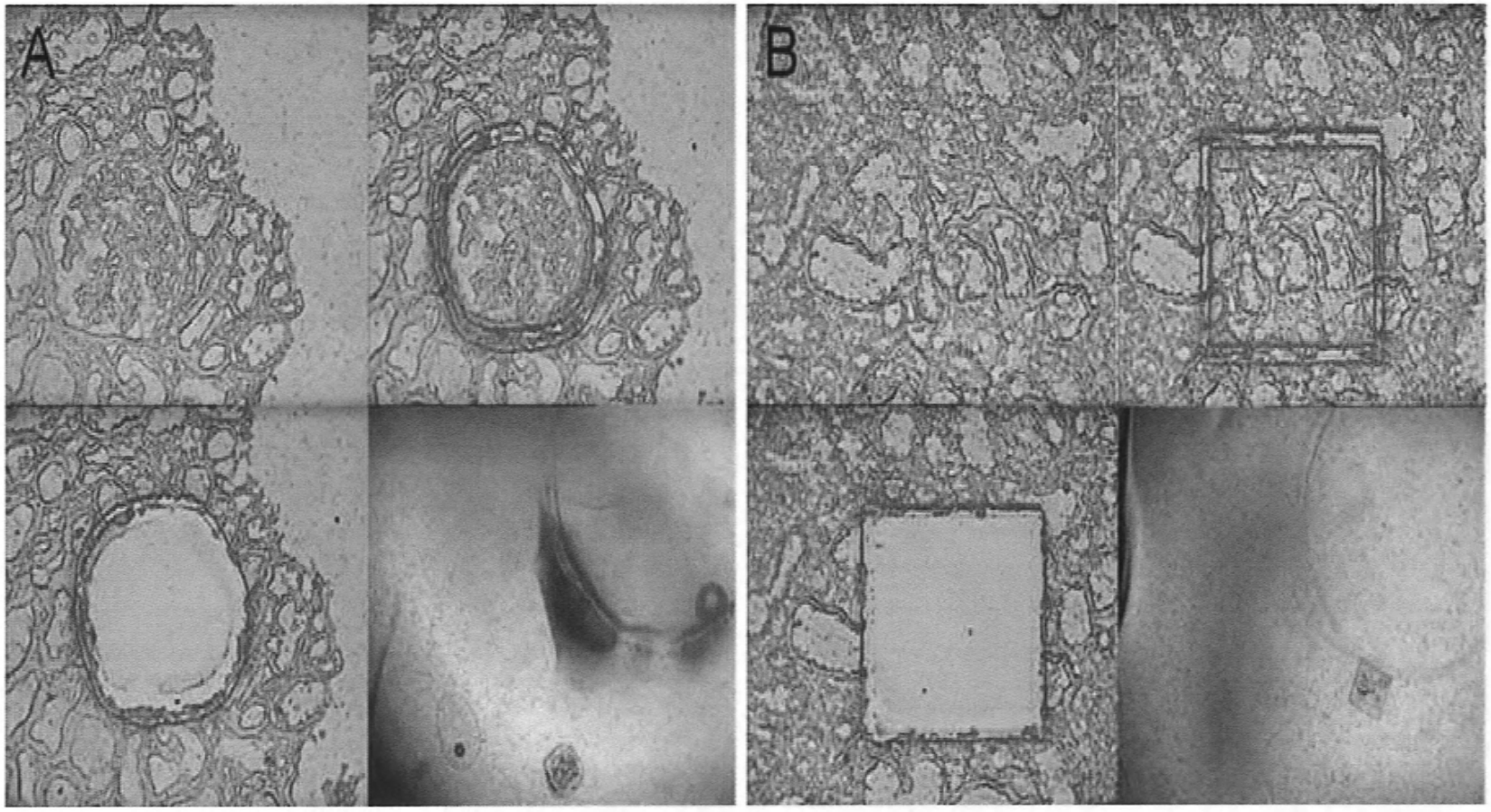
3.4 Laser microdissection

The method of laser microdissection has been described previously [246,251]. Briefly, cryo-sections of 10 μm thickness were prepared on a cryostat (Leica Microsystems, Wetzlar, Germany) using disposable microtome blades (Leica Microsystem) in RNase-free conditions, and were mounted on MembraneSlide 0.17 PEN slides (Carl Zeiss PALM Microlaser Technologies, Bernried, Germany). Immediately after taking

the slides out of the cryostat, the sections were fixed in 70% ethanol and dehydrated in 100% ethanol. Sections were air-dried at room temperature.

Laser microdissection of the snap-frozen kidney biopsy specimens was performed using the PALM Microlaser System (PALM Microlaser Technologies), which is equipped with a pulsed high-quality laser beam, computer-controlled microscope stage and micromanipulator. Under direct visual control, areas of interest in the histological specimens were selected through the PALM RoboSoftware (PALM Microlaser Technologies) by moving the computer mouse, and microdissected by cutting the contour of the selected areas with the adjusted laser beam. The isolated tissue was then laser-catapulted into a microcentrifuge cap filled with guanidine thiocyanate containing lysis buffer for the subsequent RNA isolation. Approximately 20–30 glomerulus and 20 randomly selected tubulointerstitial area were isolated from each specimen. The tissue lysate of glomerulus and tubulointerstitium were kept at –80°C until RNA extraction (Figure 3-1).

Figure 3-1 Glomerular areas (A) and proportional randomly selected tubulointerstitial areas (B) of each case were isolated by the focused laser beam and then catapulted into different micro-centrifuge caps



3.5 Quantification of Gene Expression

3.5.1 RNA extraction

RNA extraction was performed with the RNAqueous®-Micro Kit (Applied Biosystems, USA), following manufacturer's instruction. The procedure begins by lysing the sample in a lysis solution containing guanidinium thiocyanate, a strong chaotropic agent that disrupts cell membranes and rapidly inactivates ribonucleases. The lysate is then mixed with ethanol and applied to a silica-based filter that selectively binds RNA. A simple modification in the procedure allows the user to recover both large and small RNA species including tRNAs, 5S rRNA, and micro-RNAs. An important feature of this system is that the silica filter, on which the RNA is purified, is very small; this allows the RNA to be thoroughly eluted in a very small volume of only 10–20 μL. This allows for recovery of a more concentrated RNA solution than can be obtained by standard purification procedures, giving greater sensitivity in downstream assays (e.g. RT-PCR and linear amplification), without the need to further concentrate the RNA.

The details of procedure are shown here:

A. Preparation of Materials

Add 10.5 mL ethanol to Wash Solution 1 Concentrate

Add 22.4 mL 100% ethanol to Wash Solution 2/3 Concentrate

B. RNA Isolation Procedure

1. Drop sample into 100 µL Lysis Solution and incubate for 30 min at 42°C
2. Prewet the filter with 30 µL of Lysis Solution for ⩾5 min
3. Add 3 µL of LCM Additive to the lysate and mix
4. In order to recover all RNA (large and small RNA species), add 1.25 volumes (129 µL) of 100% ethanol to the lysate mixture, and mix by pipetting up and down or by gently vortexing.
5. Pass the lysate mixture through a prepared Micro Filter Cartridge Assembly
6. Wash with180 µL of Wash Solution 1
7. Wash the filter with 2 x 180 µL Wash Solution 2/3
8. Discard the flow-through and centrifuge the filter for 1 min
9. Elute the RNA in 2 x 10 µL of Elution Solution

C. DNase I Treatment and DNase Inactivation

1. Add 1/10 volume of 10X DNase I Buffer and 1 µL of DNase I
2. Incubate 20 min at 37°C
3. Add 2 µL or 1/10 volume DNase Inactivation Reagent, mix well, and leave at room temp for 2 min
4. Pellet the DNase Inactivation Reagent and transfer the RNA to a fresh tube

3.5.2 Reverse transcription

For the synthesis of cDNA for mRNA, 5 µl total RNA was mixed with 1 µl random primers (150 ng), 1 µl dNTP mix (10 mM each), 4 µl 5× first-strand buffer, 2 µl dithiothreitol (DTT) (0.1 M), 200 U Superscript II RNase H Reverse Transcriptase (all from Intrivogen™, Life Technologies, Philadelphia, USA) and made up to 20 µl with H_20. Reverse transcription was performed at 65°C for 5 minutes, 25°C for 10 minutes, 42°C for 50 minutes and then inactivate reaction at 70°C for 10 minutes. The resulting cDNA was stored in -80°C until use.

For the synthesis of cDNA for micro-RNA, 5 µl total RNA was mixed with 3 µl specific primers, 0.15 µl 100 mmol/l dNTPs (with dTTP), 1.5 µl 10× reverse transcription buffer, 1 µl (50 U) MultiScribe Reverse Transcriptase, 0.19 µl RNase inhibitor (20 U/µl) and made up to 15 µl with H2O. TaqMan microRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) were used for reverse transcription. Reverse transcription was performed at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min.

3.5.3 Quantification of gene expression

In this series of work, gene expression was quantified by RT-QPCR. Taqman primers and probes of each specific target gene were purchased from Applied Biosystems (Foster City, California, USA). The RT-QPCR was performed by ABI Prism 7900HT Sequence Detector System (Applied Biosystems), following the manufacturer's instruction. The mRNA expression of each signal was calculated by using the ΔCt procedure according to manufacturer's instruction, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as the housekeeping gene for normalisation among samples. All primers and probes were tested with purified DNA as the template in RT-QPCR to ensure that they do not amplify genomic DNA. All results were analyzed by Sequence Detection Software version 1.7 (Applied Biosystems).

For micro-RNA, 2.5 μl universal master mix, 0.25 μl primer, and probe set (all from Applied Biosystems), 0.33 μl cDNA and 1.92 μl H2O were mixed to make a 5-μl reaction volume. Each sample was run in triplicate. RT-QPCR were performed at 50°C for 2 min, 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Intrarenal expression of miR-146a, miR-155, miR-198 miR-638, and miR-663 were quantified by RT-QPCR with the ABI Prism 7900 Sequence Detection System (Applied

Biosystems). Commercially available TaqMan primers and probes, were used for all the targets (all from Applied Biosystems). RNU48 (Applied Biosystems) were used as housekeeping genes to normalize the miRNA expression [252]. Average expression level of normal renal tissue from patients with kidney clear cell cancer was used as calibrator for intrarenal expression, and the expression level of targets was a ratio relative to that of the controls.

Gene expression of each target was calculated by using the "difference in threshold cycle" (ΔCt) procedure, according to the manufacturer's instruction. For GAPDH and each target, the relative efficiency of amplification over various starting template concentrations was determined. Approximately equal efficiencies for other targets with GAPDH amplifications were verified by an absolute value of <0.1 for the slope of log input complementary DNA amount versus ΔCt, which was obtained by subtracting the threshold cycle (Ct) value of GAPDH from that of the target. Therefore, it was possible to detect GAPDH in the same tube with other targets. The relative quantification of using multiplex reaction with a comparative method was determined by the formula $2^{-(\Delta\Delta Ct)}$, where the ΔΔCt was calculated by the subtraction of ΔCt of the calibrator from ΔCt of the sample. Throughout this series of experiment, a group of normal kidney tissue, either from the normal pole of nephrectomy specimen or kidney transplant donor biopsy, was used as the calibrator. Gene expression level was a dimensionless number relative to that of the controls.

CHAPTER 4 – Intra-Renal Gene Expression of TWEAK / Fn14 and IP-10 / CXCR3 in Lupus Nephritis

4.1 Introduction

As outlined in the previous chapters, systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by aberrant cytokines milieu. Although almost all organs in the body could be involved, lupus nephritis (LN) remains the leading cause of morbidity and mortality in SLE. The precise pathogenic mechanism of SLE and LN, however, is not completely understood. Cytokine aberration is a cardinal phenomenon of LN. It is, however, important to realize that cytokines may not only be involved in the generation of aberrant immune regulation [253], but also participates in the local inflammatory processes that ultimately lead to tissue destruction. Unfortunately, most studies focused on the cytokine profile of peripheral blood in SLE patients, and the results are often inconsistent [254,255]. Since specific organ or tissue involvement in SLE probably involve local cytokine aberrations that do not appear in the systemic circulation, study of the immunopathogenesis should focus at the specific sites of disease involvement.

The biology of tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and interferon-inducible protein-10 (IP-10) axis have been reviewed in the previous chapters. Briefly, TWEAK is one of TNF ligand superfamily members; it mediates important biological effects, including up-regulation of multiple chemokines, cytokines and adhesion molecules [194], induction of apoptosis [195], and enhancement of cell survival [196] via its sole receptor, Fn14 [197]. IP-10 (also known as CXCL10) belongs to the CXC family of chemokines. IP-10, together with Mig and their receptor, CXCR3, promotes mesangial cell expansion in proliferative glomerulonephritis [212]. It has been reported that TWEAK/ Fn14 could induce human kidney cells to express IP-10 and promote kidney cell proliferation [216]. Although a number of in vitro and animal studies suggest a pathogenic role of the TWEAK/Fn14 and IP-10/CXCR3 axis in lupus nephritis, data in human lupus nephritis are scarce (see previous chapters).

4.2 Specific objective of this part

We began by examining the gene expression of TWEAK/Fn14 and IP-10/CXCR3 in the kidney tissue of LN patients. Our objectivs are:

1. To compare intra-renal TWEAK/Fn14 and IP-10/CXCR3 expression between LN patients and controls.
2. To compare intra-renal TWEAK/Fn14 and IP-10/CXCR3 expression between different histological classes of LN.
3. To correlate intra-renal TWEAK/Fn14 and IP-10/CXCR3 expression with clinical and histological severity of LN.

4.3 Patients and Methods

4.3.1 Patient selection

We studied 42 consecutive SLE patients with active renal disease and required kidney biopsy. All patients fulfilled the American College of Rheumatology diagnostic criteria of SLE [256]. The uninvolved pole of 10 kidneys that were removed for renal cell carcinoma and had entirely normal morphology by light microscopy and immunofluorescence study were used as control. Patients with fulminant lupus nephritis that required temporary dialysis support were excluded. All patients provided informed consent.

4.3.2 Clinical and histological assessment

The disease activity of SLE was assessed clinically by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [184] on the day of kidney biopsy by an independent physician. Baseline serum creatinine, urine protein, complement levels (C3 and C4) and anti-double strand (ds) DNA antibody titre were also measured.

Kidney biopsy specimen was evaluated according to the International Society of Nephrology (ISN) classification of lupus nephritis [15]. As described previously, the activity index (AI) and chronicity index (CI) of each biopsy specimen were scored by standard methods [15].

4.3.3 Laser microdissection

The method of laser microdissection has been described in Chapter 3. Approximately 20 to 30 glomerulus and 20 randomly selected tubulointerstitial areas were isolated from each specimen. The tissue lysate of glomerulus and tubulointerstitium were kept at –80°C until RNA extraction.

4.3.4 Quantification of intra-renal gene expression

The method of total RNA extraction, reverse transcription and real-time quantitative polymerase chain reaction (RT-QPCR) was described in Chapter 3. In the present work, we quantified the mRNA expression of TWEAK, Fn14, IP-10, and CXCR3 in

glomerulus and tubulointerstitium. Taqman primers and probes of each target were purchased from Applied Biosystems (Foster City, California, USA).

4.3.5 Statistical analysis

Statistical analysis will be performed by SPSS for Windows software version 15.0 (SPSS Inc., Chicago, IL). All the results from this series of experiments are quantitative. The results are presented as mean ± SD unless otherwise specified. Since the data on gene expression are highly skewed, they are compared between groups by Kruskall Wallis test or Mann-Whitney U test as appropriate. Since there were very few cases of class II or III nephritis, analysis that compared gene expression between groups focused on patients with diffuse proliferative (class IV), pure membranous (class V), and mixed proliferative / membranous nephritis. Correlations between continuous variables are calculated by Spearman's rank correlation coefficient. A p value of less than 0.05 is considered as statistically significantly. All probabilities are two tailed.

4.4 Result

4.4.1 General Description

We studied 42 SLE patients. Their baseline demographic and clinical data are summarized in Table 4-1. All patients had at least 5 mm of renal cortex and 5 non-sclerosed glomeruli for histological study. Their histological diagnoses were class II nephritis (3 cases), proliferative nephritis (ISN class III 2 cases; class IV, 7 cases), pure membranous (ISN class V) nephritis (9 cases), and mixed proliferative and membranous nephritis (i.e. both class IV and class III or IV lesions, 21 cases). The mean histological activity and chronicity indices were 7.05 ± 4.25 and 2.69 ± 2.15, respectively.

Table 4-1 Baseline demographic and clinical data*.

Histology class	class II	class III	class IV	class V	class III and V	class IV and V	P value
No. of case	3	2	7	9	16	5	
Sex (M:F)	1:2	0:2	0:7	0:9	1:15	0:5	p = 0.8
Age (years)	44.4 ± 3.8	37.6 ± 7.5	53.4 ± 14.4	38.1 ± 9.1	38.2 ± 10.1	35.5 ± 8.6	p = 0.031
SLEDAI score	5.3 ± 1.2	11.0 ± 1.4	10.6 ± 7.9	6.2 ± 2.9	6.4 ± 3.4	9.6 ± 3.0	
Serum creatinine (μmol/l)	72.0 ± 16.1	51.5 ± 3.5	244.7 ± 199.7	84.0 ± 56.6	119.8 ± 88.5	112.2 ± 83.7	p = 0.058
Proteinuria (g/day)	1.1 ± 0.7	3.1 ± 0.1	3.1 ± 2.6	2.0 ± 1.3	2.0 ± 2.0	3.2 ± 1.2	p = 0.5
GFR (mL/min/1.73m^2)	93.6 ± 23.9	123.8 ± 4.7	36.1 ± 32.6	90.3 ± 35.9	65.2 ± 31.8	71.0 ± 32.3	p = 0.007
Serum C3 (g/L)	0.70 ± 0.27	0.69 ± 0.05	0.45 ± 0.15	0.60 ± 0.17	0.53 ± 0.17	0.41 ± 0.11	p = 0.085
Serum C4 (g/L)	0.06 ± 0.05	0.14 ± 0.02	0.11 ± 0.08	0.16 ± 0.11	0.11 ± 0.08	0.11 ± 0.09	p = 0.5

Anti-ds DNA titre	99.3 ± 172.1	831.5 ± 238.3	472.6 ± 344.7	86.6 ± 99.1	625.9 ± 400.6	389.4 ± 321.2	p = 0.003
Renal histology							
activity index	2.0 ± 0.0	10.0 ± 2.8	11.6 ± 4.1	2.2 ± 2.3	8.3 ± 2.6	7.4 ± 1.3	p < 0.0001
chronicity index	4.0 ± 2.6	1.0 ± 0.0	3.9 ± 2.5	1.8 ± 2.2	2.8 ± 1.8	2.2 ± 2.3	p = 0.3
cortical fibrosis (%)	23.3 ± 23.6	3.5 ± 2.1	27.9 ± 20.6	9.1 ± 19.2	11.7 ± 13.9	8.0 ± 17.9	p = 0.2
glomerosclorosis (%)	20.2 ± 22.0	1.1 ± 1.6	25.2 ± 21.1	2.2 ± 2.7	10.3 ± 14.2	6.4 ± 10.6	p = 0.037
Prednisolone (mg/day)	6.7 ± 1.4	3.8 ± 5.3	4.6 ± 2.2	4.2 ± 2.5	5.3 ± 1.8	5.5 ± 1.1	p = 0.5
Azathioprine, no. of case	1	1	5	8	11	3	p = 0.7
ACEI / ARB, no. of case	2	2	5	7	11	5	p = 0.8

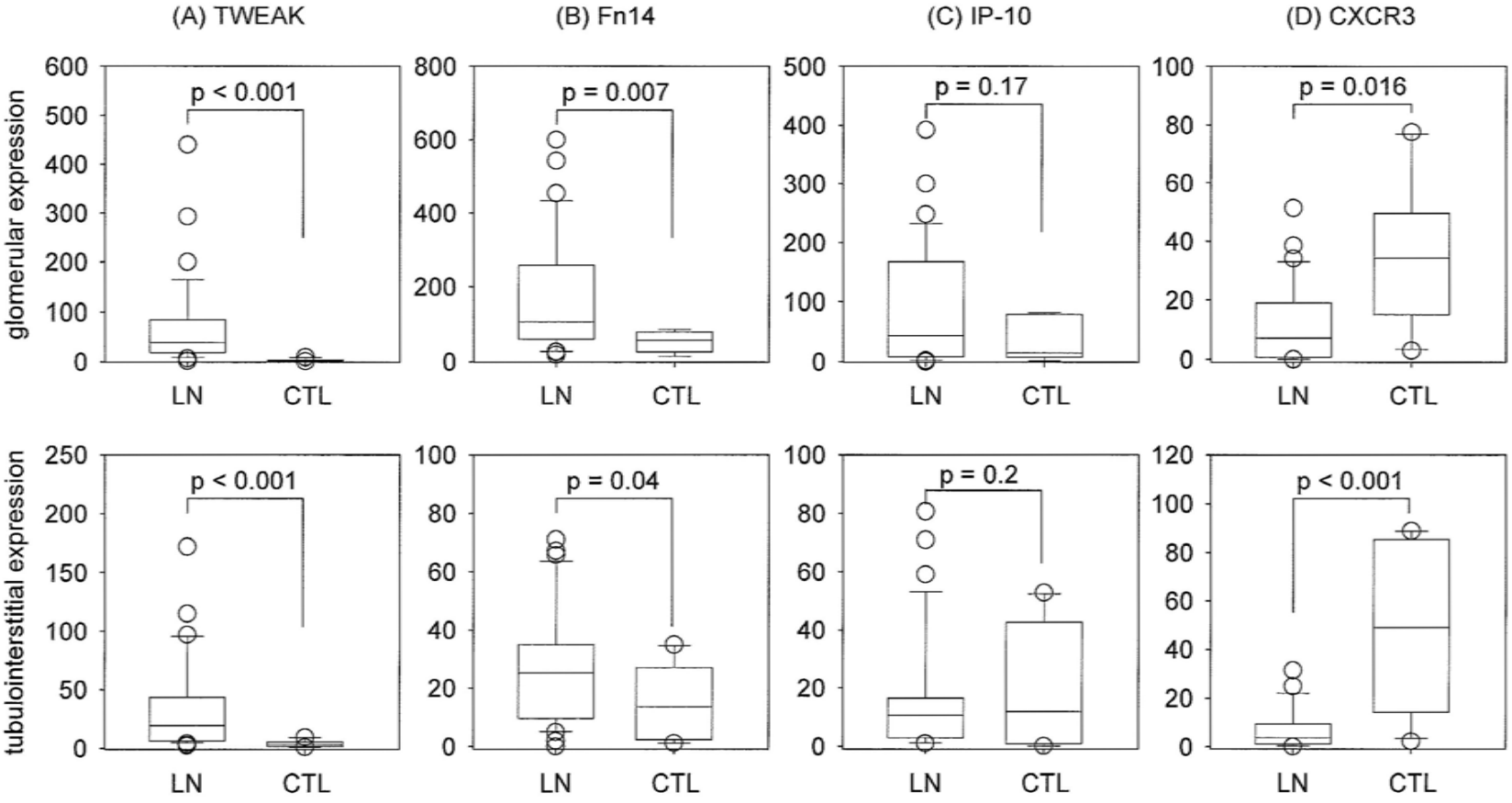
SLEDAI, Systemic Lupus Erythematosus Disease Activity Index; GFR, glomerular filtration rate; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.

*Data are represented as mean ± standard deviation.

4.4.2 Comparison of gene expression levels

The glomerular and tubulointerstitial mRNA expression levels of TWEAK, Fn14, IP-10 and CXCR3 are summarized in Figure 4-1. As compared to controls, LN patients had higher glomerular expression of TWEAK ($p < 0.001$) and Fn14 ($p = 0.007$), but lower glomerular CXCR3 expression ($p = 0.016$). Similarly, LN patients had higher tubulointerstitial expression of TWEAK ($p < 0.001$) and Fn14 ($p = 0.04$), but lower tubulointerstitial expression of CXCR3 ($p < 0.001$) as compared to controls. Glomerular and tubulointerstitial IP-10 expression was similar between LN patients and controls.

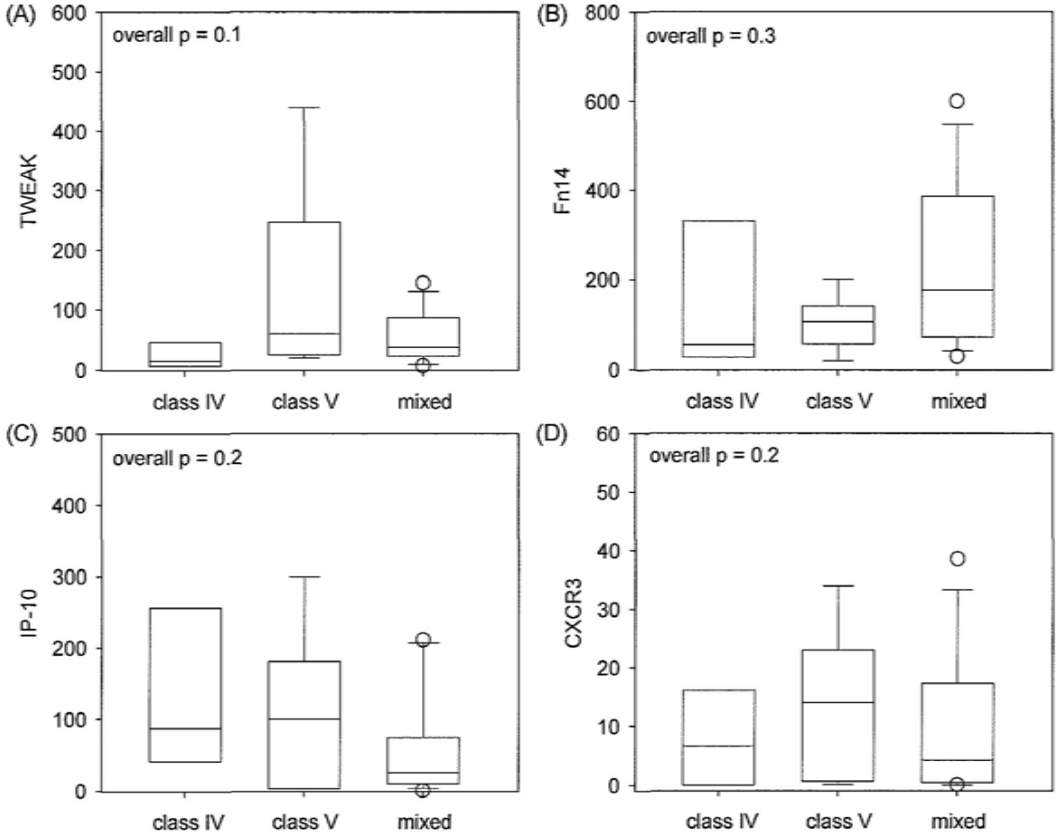
Figure 4-1 Comparison of glomerular and tubulointerstitial mRNA expression levels of (A) TWEAK; (B) Fn14; (C) IP-10; and (D) CXCR3 between LN and CTL. Whisker-box plot, with the boxes indicate median, 25 and 75 percentile; whisker caps indicate 5 and 95 percentile; circles indicate outliers. Data are compared by Mann-Whitney U test. (LN, lupus nephritis group; CTL, control group)



4.4.3Relation with histology

The glomerular expression of target genes are compared between ISN classes and summarized in Figure 4-2. In short, we found that glomerular TWEAK expression of pure membranous nephritis was significantly higher than class IV nephritis, and glomerular TWEAK expression (but not that of other target genes in this study) inversely correlated with the histological activity index ($r = -0.443$, $p = 0.021$). In contrast, glomerular expression of Fn14, IP-10 and CXCR3 was not significantly different between histological groups. Amongst patients with class IV nephritis, there was no significant difference in gene expression between patients with global and segmental lesion, or between those with active or chronic lesions. There was no significant difference in the tubulointerstitial expression of all four genes expression between histological groups. The result also remains similar when all proliferative lupus nephritis (class III and IV disease) was combined for analysis.

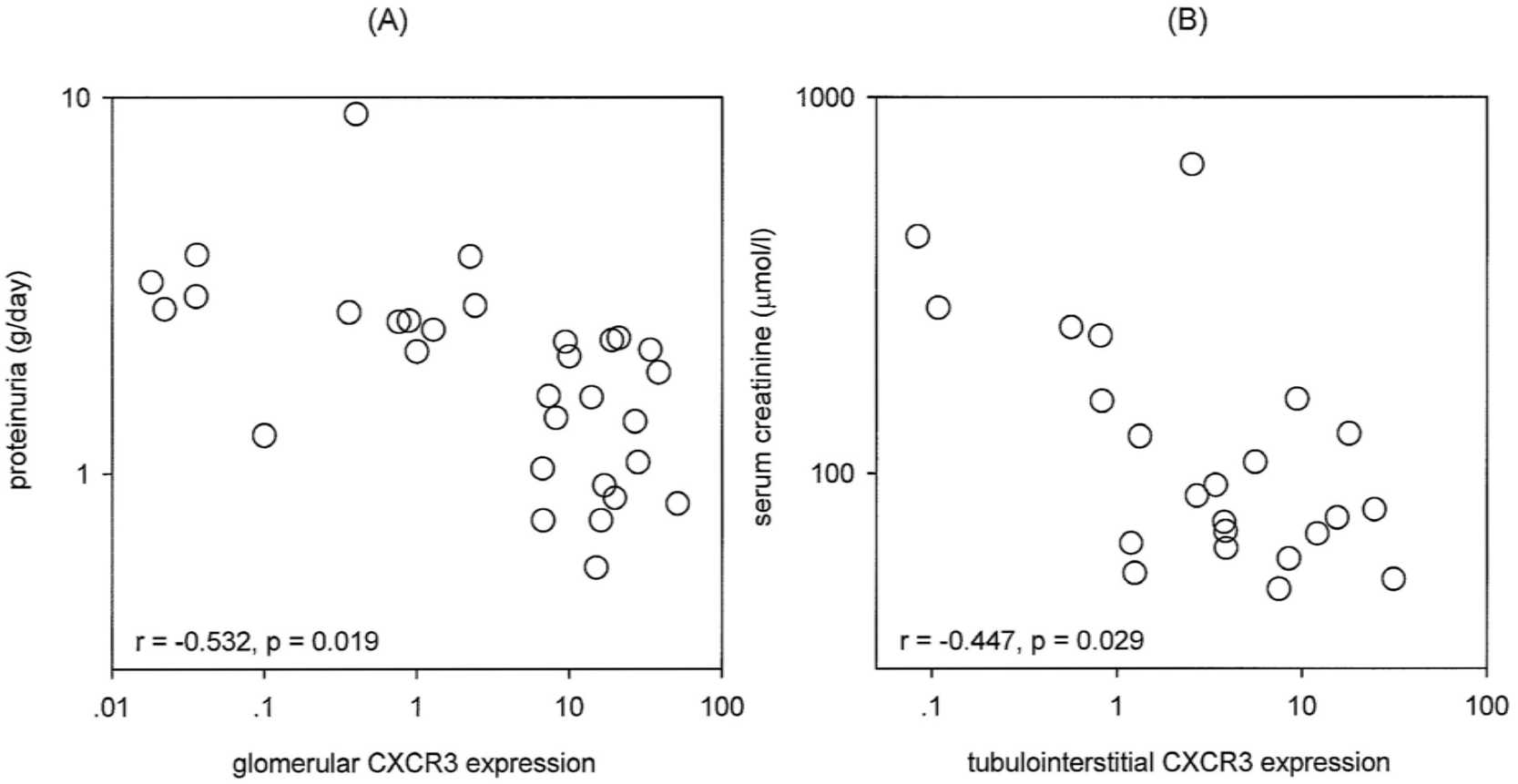
Figure 4.2 Comparison of glomerular mRNA expression levels of (A) TWEAK; (B) Fn14; (C) IP-10; and (D) CXCR3 between class IV and class V (pure membranous) nephritis. Whisker-box plot, with the boxes indicate median, 25 and 75 percentile; whisker caps indicate 5 and 95 percentile; circles indicate outliers. Data are compared by Mann-Whitney U test.



4.4.4 Relation with clinical parameters

We further explore the correlation between gene expression and baseline clinical and histological parameters. In essence, glomerular expression of CXCR3 inversely correlated with the degree of proteinuria ($r = -0.532$; $p = 0.019$), whereas tubulointerstitial CXCR3 expression inversely correlated with baseline serum creatinine ($r = -0.447$; $p = 0.029$) (Figure 4-3). In contrast, none of the gene expressions correlated with markers of systemic disease activity, including SLEDAI score, serum complement, or anti-ds DNA titre. Gene expressions did not correlate with the dosage of prednisolone or azathioprine, or the use of angiotensin converting enzyme inhibitor or angiotensin receptor blocker.

Figure 4-3 Correlation between (A) glomerular mRNA expression of CXCR3 and the degree of proteinuria; and (B) tubulointerstitial CXCR3 expression and baseline serum creatinine. Data are compared by Spearman's correlation coefficient.



4.5 Conclusion

In this part of experiment, we found an increase in intra-renal expression of TWEAK and Fn14 mRNA and decrease in CXCR3 mRNA in LN patients as compared to controls. Within the LN group, glomerular TWEAK expression was higher in pure membranous than proliferative nephritis. Besides, intra-renal expression of CXCR3 correlates with the degree of proteinuria and renal function. Our findings suggest that TWEAK/Fn14 and IP-10/CXCR3 axis may contribute to the pathogenesis of lupus nephritis.

CHAPTER 5 –Intra-Renal Expression of NGAL and TLR9 in Lupus Nephritis

5.1 Introduction

As discussed in Chapter 1, recent evidence suggests an important role of neutrophil gelatinase associated lipocalin (NGAL) and Toll-like receptor 9 (TLR9) in the pathogenesis of lupus nephritis (LN). NGAL is a member of the lipocalin superfamily, which is first found in the specific granules of human neutrophil [217]. TLR9 is a pattern-associated receptor functioning in innate immunity that recognizes unmethylated CpG sequences in DNA molecules; it may be involved in the recognition of self DNA-antigens and the production of pathogenic auto-antibodies. Previous study showed that a higher expression of TLR9 on peripheral blood B cells from patients with active SLE correlated with SLEDAI [224]. Notably, TLR9 causes the activation of NF-κB [232], which is recruited to the NGAL promoter and is essential for the transcriptional activation of NGAL promoters [233]. Taken together, it seems probable that intra-renal activation of TLR9 and NGAL are related to the development of LN.

5.2 Specific objective of this part

In this part of the work, we examined the intra-renal expression of TLR9 and NGAL in the kidney tissue of LN patients. Our objectivs are:

1. To compare intra-renal TLR9 and NGAL expression between LN patients and controls.
2. To compare intra-renal TLR9 and NGAL expression between different histological classes of LN.
3. To correlate intra-renal TLR9 and NGAL expression with clinical and histological severity of LN.
4. To correlate intra-renal TLR9 and NGAL expression with the therapeutic response of LN.

5.3 Patients and Methods

5.3.1 Patient selection

We studied 42 consecutive SLE patients with active renal disease and required kidney biopsy. All patients fulfilled the American College of Rheumatology diagnostic criteria of SLE [256]. The uninvolved pole of 10 kidneys that were removed for renal cell carcinoma and had no morphological evidence of renal disease were used as control. All patients provided informed consent.

5.3.2 Clinical and histological assessment

As described in the previous chapters, the disease activity of SLE was assessed clinically by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [184] on the day of kidney biopsy. Baseline serum creatinine, urine protein, complement levels (C3 and C4) and anti-double strand (ds) DNA antibody titre were also measured. Glomerular filtration rate (GFR) was estimated by a standard equation [257]. Kidney biopsy specimen was evaluated according to the International Society of Nephrology (ISN) classification of lupus nephritis [175]. The activity index (AI) and chronicity index (CI) of each biopsy specimen were scored by standard methods [251].

5.3.3 Laser microdissection and gene expression study

The method of laser microdissection, total RNA extraction, reverse transcription and real-time quantitative polymerase chain reaction (RT-QPCR) were described in Chapter 3. In the present work, we quantified the mRNA expression of NGAL and TLR9. Taqman primers and probes of each target were purchased from Applied Biosystems (Foster City, California, USA). Gene expression of each target was calculated by using the difference-in-threshold-cycle (ΔCt) procedure, according to manufacturer's instruction.

5.3.4 Clinical follow-up

After the renal biopsy, all patients were followed for at least 6 months. The clinical management was decided by individual nephrologist and not affected by the study. In general, patients were treated with corticosteroid, together with cyclophosphamide or mycophenolate according to published protocols. Therapeutic response to induction immunosuppressant was assessed at 12 weeks after treatment and classified into complete remission, partial remission, and no response as defined in Chapter 3.

5.3.5 Statistical analysis

Statistical analysis will be performed by SPSS for Windows software version 15.0 (SPSS Inc., Chicago, IL). All the results from this series of experiments are quantitative. The results are presented as mean ± SD unless otherwise specified. Since the data on gene expression are highly skewed, they are compared between groups by Kruskall Wallis test or Mann-Whitney U test as appropriate. Correlations between continuous variables are calculated by Spearman's rank correlation coefficient. A p value of less than 0.05 is considered as statistically significantly. All probabilities are two tailed.

5.4 Result

5.4.1 General Description

We studied 42 SLE patients. Their baseline demographic and clinical data are summarized in Table 5-1. The histological diagnoses were proliferative nephritis (class III or IV, 9 cases), pure membranous nephritis (class V, 9 cases), class II nephritis (3 cases), and mixed proliferative and membranous nephritis (21 cases). The mean histological Activity and Chronicity Indices were 7.1 ± 4.3 and 2.7 ± 2.2, respectively.

Table 5-1 Baseline demographic and clinical data.

Sex (M:F)	2:40
Age (years)	40.8 ± 11.4
SLEDAI score	7.6 ± 4.5
Serum creatinine (μmol/l)	125.4 ± 115.5
Proteinuria (g/day)	2.33 ± 1.84
Glomerular filtration rate (mL/min/1.73m^2)	71.2 ± 37.2
Renal histology	
interstitial fibrosis (%)	13.8 ± 17.9
glomerosclorosis (%)	10.8 ± 15.5

5.4.2 Gene expression level between groups

There was a modest internal correlation between glomerular and tubulointerstitial expression of NGAL ($r = 0.410$, $p = 0.030$), while those of TLR9 had no significant correlation ($r = 0.199$, $p = 0.388$) (Figure 5-1). There were no significant differences in glomerular or tubulointerstitial expression of TLR9 or NGAL between histological groups (details not shown).

The glomerular and tubulointerstitial mRNA expression levels of NGAL and TLR9 are summarized in Figure 5-2. As compared to controls, LN patients had higher glomerular expression of TLR9 ($p < 0.001$) but not NGAL. In contrast, LN patients had higher tubulointerstitial expression of TLR9 ($p = 0.011$) and NGAL ($p < 0.001$) as compared to controls.

Figure 5-1 Correlation between glomerular and tubulointerstitial expression of NGAL and TLR9.

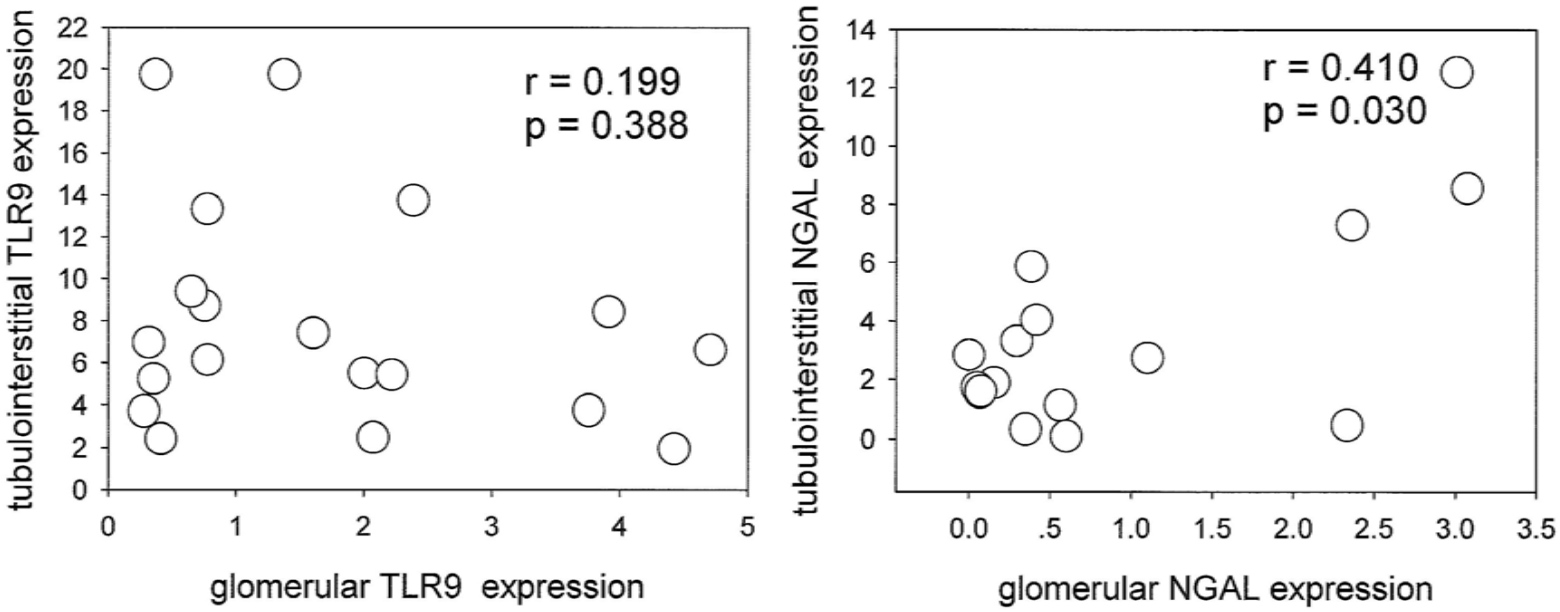
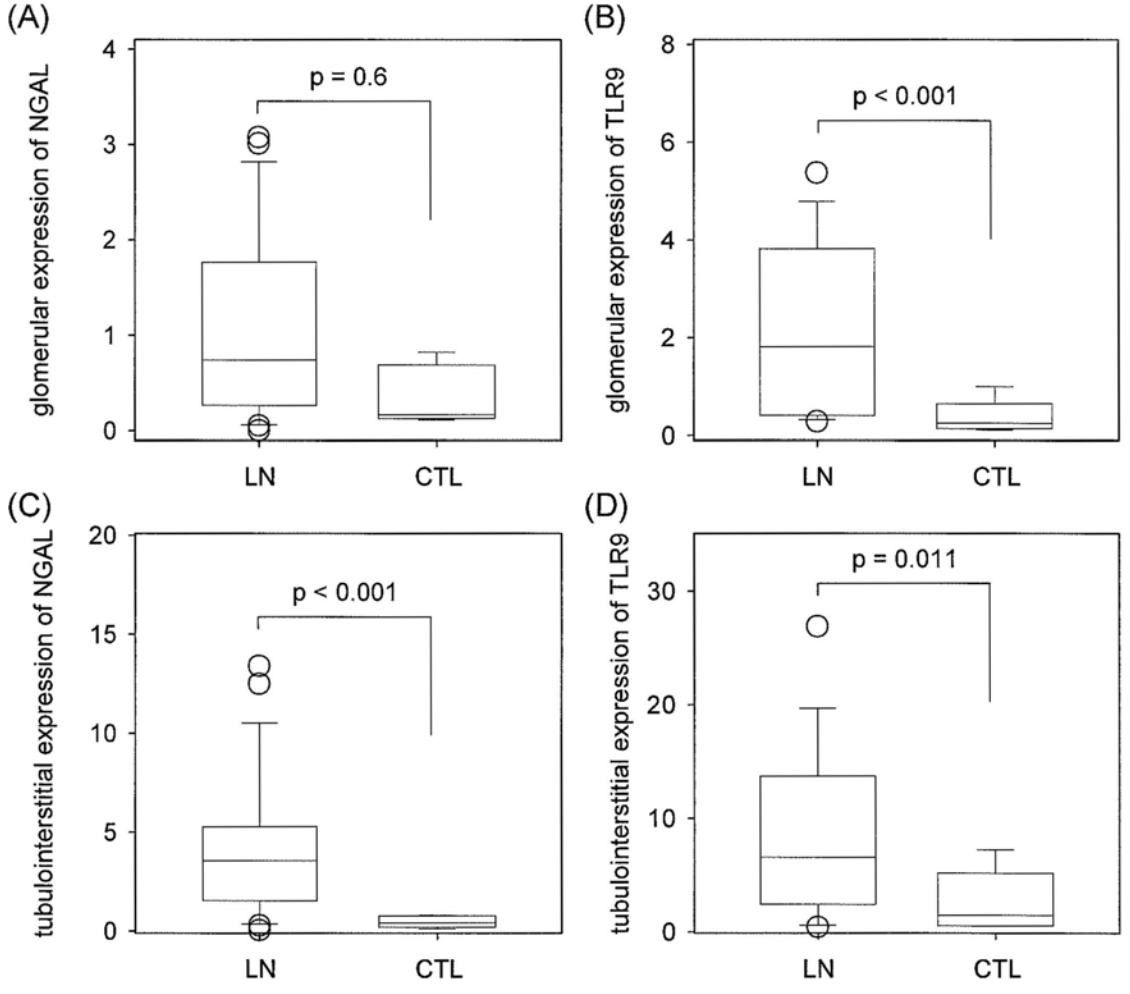


Figure 5-2 Comparison of glomerular expression of (A) NGAL and (B) TLR9, and tubulointerstitial expression of (C) NGAL and (D) TLR9, between groups. Whisker-box plot, with the boxes indicate median, 25 and 75 percentile; whisker caps indicate 5 and 95 percentile; circles indicate outliers. Data are compared by Mann-Whitney U test. (LN, lupus nephritis group; CTL, control group)



5.4.3 Relation with clinical and histological parameters

We further explore the correlation between gene expression and baseline clinical and histological parameters. We found that tubulointerstitial, but not glomerular, NGAL significantly correlated with proteinuria ($r = 0.492$; $p = 0.003$), estimated GFR ($r = -0.386$; $p = 0.022$) and histological CI ($r = 0.54$; $p = 0.004$) (Figure 5-3). In contrast, both glomerular ($r = 0.554$; $p = 0.001$) and tubulointerstitial ($r = 0.379$; $p = 0.043$) TLR9 expression significantly correlated with proteinuria, but not estimated GFR or histological CI (Figure 5-4). The glomerular or tubulointerstitial expressions of TLR9 or NGAL did not correlate with markers of systemic disease activity, including SLEDAI score, serum complement, or anti-ds DNA titre.

Figure 5-3 Correlation between (A) glomerular and (B) tubulointerstitial expression of NGAL and the degree of proteinuria, estimated glomerular filtration rate (GFR), histological activity index and chronicity index.

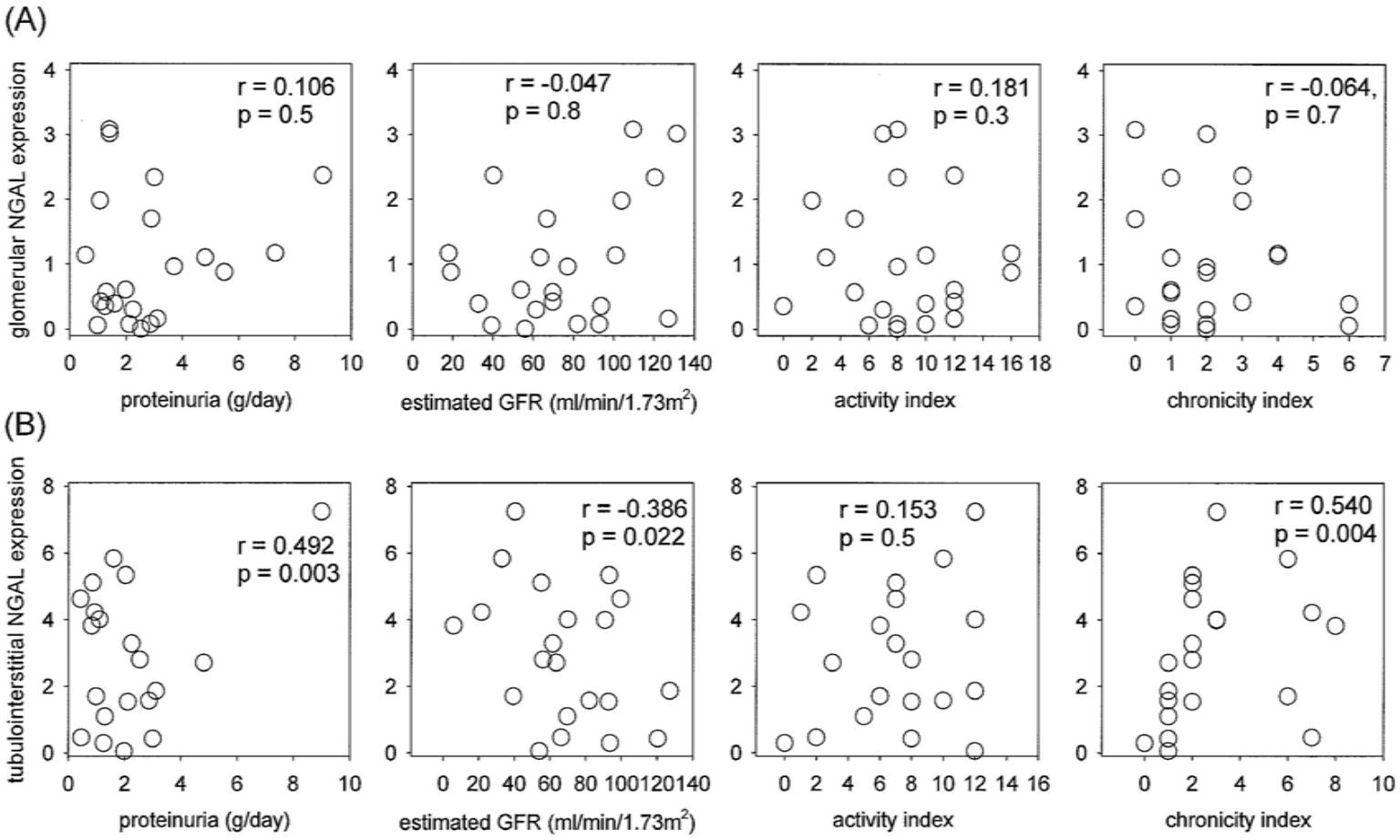
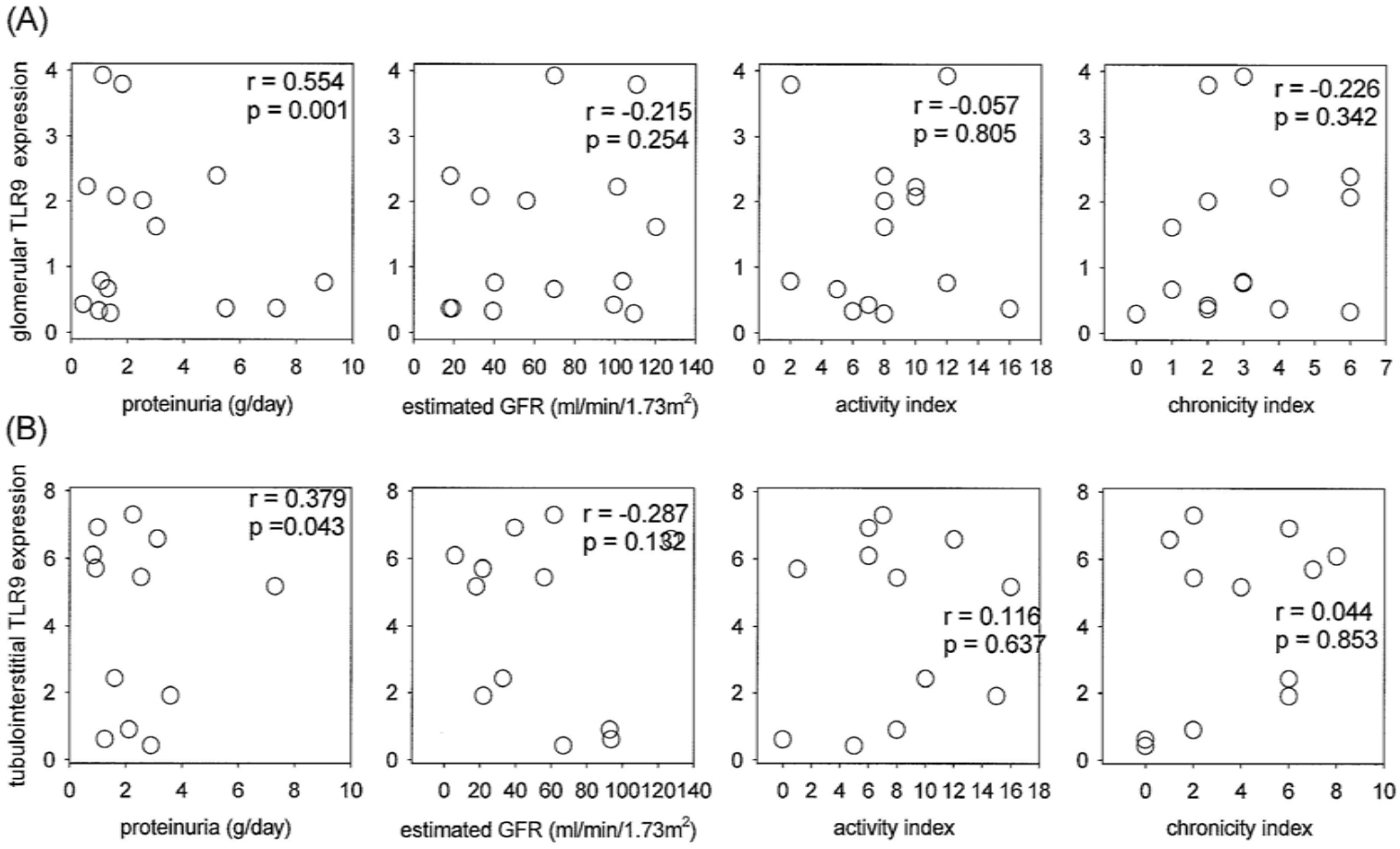


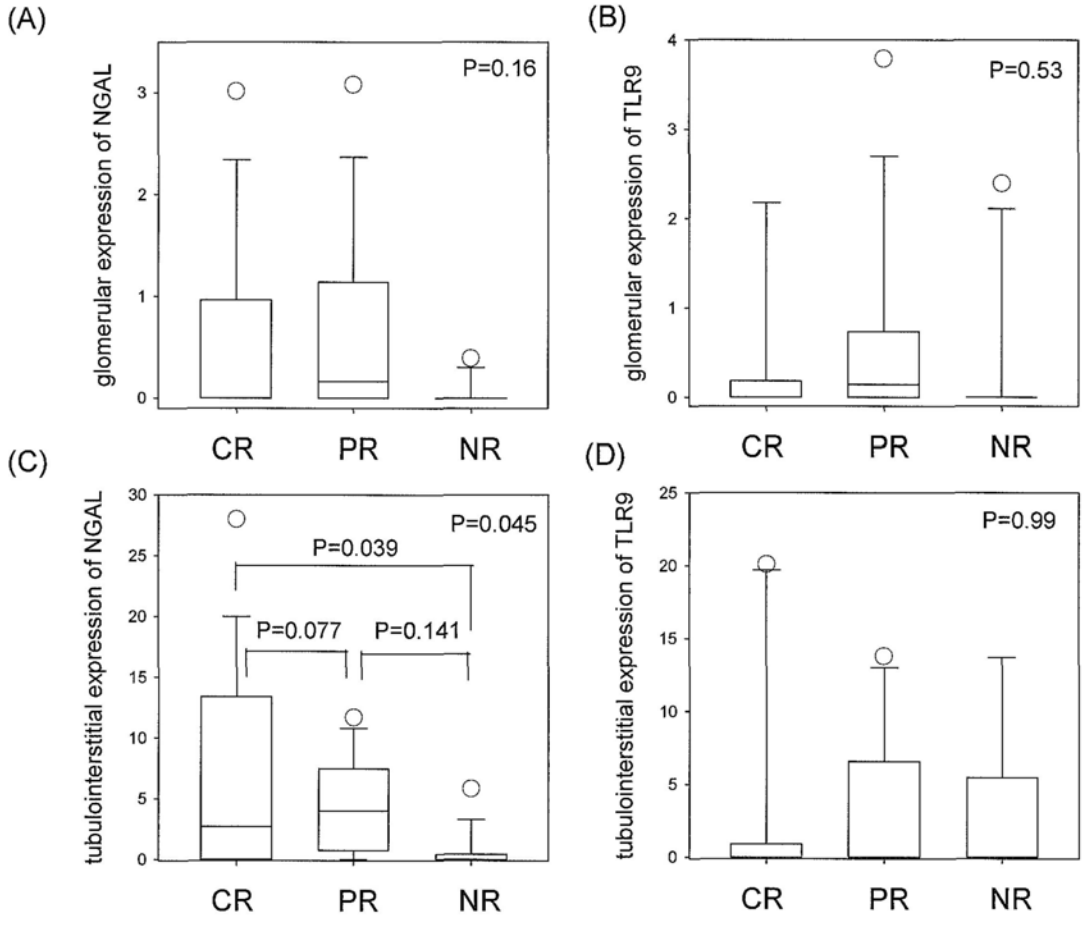
Figure 5-4 Correlation between (A) glomerular and (B) tubulointerstitial expression of TLR9 and the degree of proteinuria, estimated glomerular filtration rate (GFR), histological activity index and chronicity index.



5.4.4 Relation with therapeutic responses

After 6 months follow up, 14 cases had complete remission (CR), 21 cases had partial remission (PR) cases and 7 cases had no response (NR). The expressions of the two target genes are compared between treatment response groups (Figure 5-5). There was a significant difference in tubulointerstitial expression of NGAL between treatment response groups. Post hoc analysis showed that, as compared to NR group, CR group had higher tubulointerstitial expression of NGAL ($p = 0.039$).

Figure 5-5 Comparison of glomerular expression of (A) NGAL and (B) TLR9, and tubulointerstitial expression of (C) NGAL and (D) TLR9, between treatment response groups. Data are compared by Kruskal Wallis test. The box indicates median, 25 and 75 percentile; whisker caps indicate 5 and 95 percentile; outliers are represented as closed circles. (CR, complete remission; PR, partial remission; NR, no response.)



5.5 Conclusion

In this part of the work, we found an increase in intra-renal mRNA expression of NGAL and TLR9 in LN. Although tubulointerstitial expression of NGAL does not correlate with systemic disease activity, it closely correlates with the degree of proteinuria, renal function, and therapeutic response. The role of NGAL as a biomarker for lupus nephritis is worth further study.

CHAPTER 6 – Change in the Histological Pattern of Lupus Nephritis with Time

6.1 Introduction

The work described in Chapters 4 and 5 are cross sectional studies. Although they shed light on the pathogenesis of lupus nephritis, it is difficult to reach a definite conclusion because there is no internal control for the observations. Notably, the role of individual target gene in the pathogenesis of a specific histological class of lupus nephritis remains unclear. In this respect, we believe it would be informative if we compare the intra-renal gene expression of a cohort of patients who, at different times, has different histological class of nephritis.

To achieve this objective, we first have to examine the frequency and pattern of change in the histological class of lupus nephritis with longitudinal follow up. As discussed in Chapter 1, SLE is characterized by relapses and remissions. Previous study showed that SLE had around 20 to 30% chance of flare per patient-year of follow up [258]. There are several pathological classes of lupus nephritis [175] , each with a different clinical behaviour and probably immunopathogenesis, and the pathological class of lupus nephritis may change from one to another during disease flare of a patient [259].

A number of authors advise to perform repeat biopsies to determine the most effective treatment in the case of a lupus nephritis flare [259-263]. Unfortunately, there are few published data on the longitudinal change in the pattern of renal pathology in patients

with lupus nephritis and multiple flares. Some have suggested that the need for repeat biopsies in renal flares may depend on the type of lupus nephritis in the original biopsy and is not always necessary [264,265]. For example, a recent review of 35 patients with repeat renal biopsy for lupus nephritis showed that patients with proliferative lesions in the original biopsy rarely switch to a pure non-proliferative nephritis during a flare, and a repeat biopsy during a lupus nephritis flare is not necessary [264].

In our hospital, it is the policy for over 20 years to perform renal biopsy before treating renal flares. Although this practice may not be considered necessary by all authorities [264,265], it provides with us an excellent opportunity to examine the longitudinal change in renal pathology of a large unselected cohort of patients with lupus nephritis.

6.2 Specific objective of this part

In this part of the work, we reviewed the longitudinal change in renal pathology of a cohort of LN patients. Our objectivs are:

1. To determine the prevalence of change in histological class of lupus nephritis upon a renal flare.
2. To determine clinical and pathological factors that predict a change in histological class of lupus nephritis.
3. To identify suitable patients with paired renal biopsy for further experiments on intra-renal cytokine gene expression.

6.3 Patients and Methods

6.3.1Patient Selection

We reviewed 156 patients who had at least 2 renal biopsy performed in our hospital between 1989 and 2008. All patients fulfilled the American College of Rheumatology diagnostic criteria for SLE [256].

6.3.2 Collection of Clinical Data

We collected the clinical data by chart review, including baseline serum creatinine, albumin, urea, proteinuria, complement levels (C3 and C4), anti-double strand (anti-ds) DNA antibody titre, and the disease activity of SLE as represented by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [184,256], which is scored on the day of kidney biopsy by an independent physician. Glomerular filtration rate (GFR) was estimated from a standard prediction equation [257].

6.3.3 Assessment of Renal Pathology

Kidney biopsy was evaluated according to the International Society of Nephrology / Renal Pathology Society (ISN / RPS) classification of lupus nephritis [175]. The Activity Index (AI) and Chronicity Index (CI) of each biopsy were scored by standard

methods [266,267]. If patients had more than two biopsies, the second and third biopsies, the third and fourth biopsies, and so forth, were paired. In short, the last biopsy performed before the repeat biopsy served as the reference biopsy.

6.3.4 Clinical follow-up

After the renal biopsy, all patients were followed for at least 6 months. The clinical management was decided by individual nephrologist and not affected by the study. As described in Chapter 3, therapeutic response to induction immunosuppressant was assessed at 12 weeks after treatment and classified into complete remission (CR), partial remission (PR), and no response (NR).

6.3.5 Statistical Analysis

Data were analysed using the SPSS for Windows version 15.0 software (SPSS Inc., Chicago, IL). Results were presented as mean ± SD unless otherwise specified. Data were compared by Student's t test, or Kruskal Wallis test as appropriate. P-values of <0.05 were considered statistically significant. All probabilities were two tailed.

6.4 Result

6.4.1 General Description

We studied 156 lupus patients with serial renal biopsies. In total, there were 412 renal biopsies: 82 patients had two biopsies, 52 had three, 21 had four, and 1 patient had five biopsies. Their baseline demographic and clinical data, at the time of first renal biopsy, are summarized in Table 6-1.

Table 6-1 Basline demographic and clinical characteristics at the time of first renal biopsy.

Sex (M:F)	14:142
Age (years)	43.7 ± 10.8
Serum creatinine (μmol/l)	120.3 ± 65.3
Proteinuria (g/day)	3.28 ± 2.47
estimated GFR (mL/min/1.73m^2)	65.8± 33.2
SLEDAI score	9.5 ± 4.2
Renal histology	
activity index	6.8 ± 4.6
chronicity index	2.6 ± 2.4
interstitial fibrosis (%)	15.6 ± 15.2
glomerosclorosis (%)	17.7 ± 21.2

GFR, glomerular filtration rate; SLEDAI, Systemic Lupus Erythematosus Disease Activity Index.

6.4.2 Pattern of pathology

There were, in total, 253 pairs of renal biopsy; 9 were excluded from analysis because either of the specimens was insufficient for a proper assessment. Table 6-2 shows the ISN/RPS classification of the 244 pairs of renal biopsies. Overall speaking, the ISN/RPS class of repeat biopsy was the same as that of the reference biopsy in only 61 pairs (25.0%).

For simplicity of analysis, we further classified the pathological patterns into pure proliferative nephritis (class III or IV), pure membranous nephropathy (class V), and mix nephritis (class III or IV, plus class V disease); the result is further summarized in Table 6-3. In short, for the reference biopsy that showed pure proliferative, pure membranous, and mixed nephritis, the histological pattern in the repeat biopsy changed in 57.8%, 50.0%, and 60.4%, respectively ($p = 0.6$).

Table 6-2 ISN/RPS Classifications on repeat Biopsy.

	Reference Biopsy							
	I	II	III	IV	V	VI	III+V	IV+V
Repeat Biopsy								
I	0	0	0	1	0	0	0	0
II	0	2	4	16	1	0	0	2
III	0	5	3	5	2	0	1	3
IV	0	8	9	21	4	0	7	7
V	1	5	7	17	18	1	6	8
VI	0	0	0	8	0	1	0	1
III+V	0	5	7	13	10	0	10	4
IV+V	0	3	1	7	2	1	1	6

Table 6-3 Comparison of baseline clinical and histological characteristics.

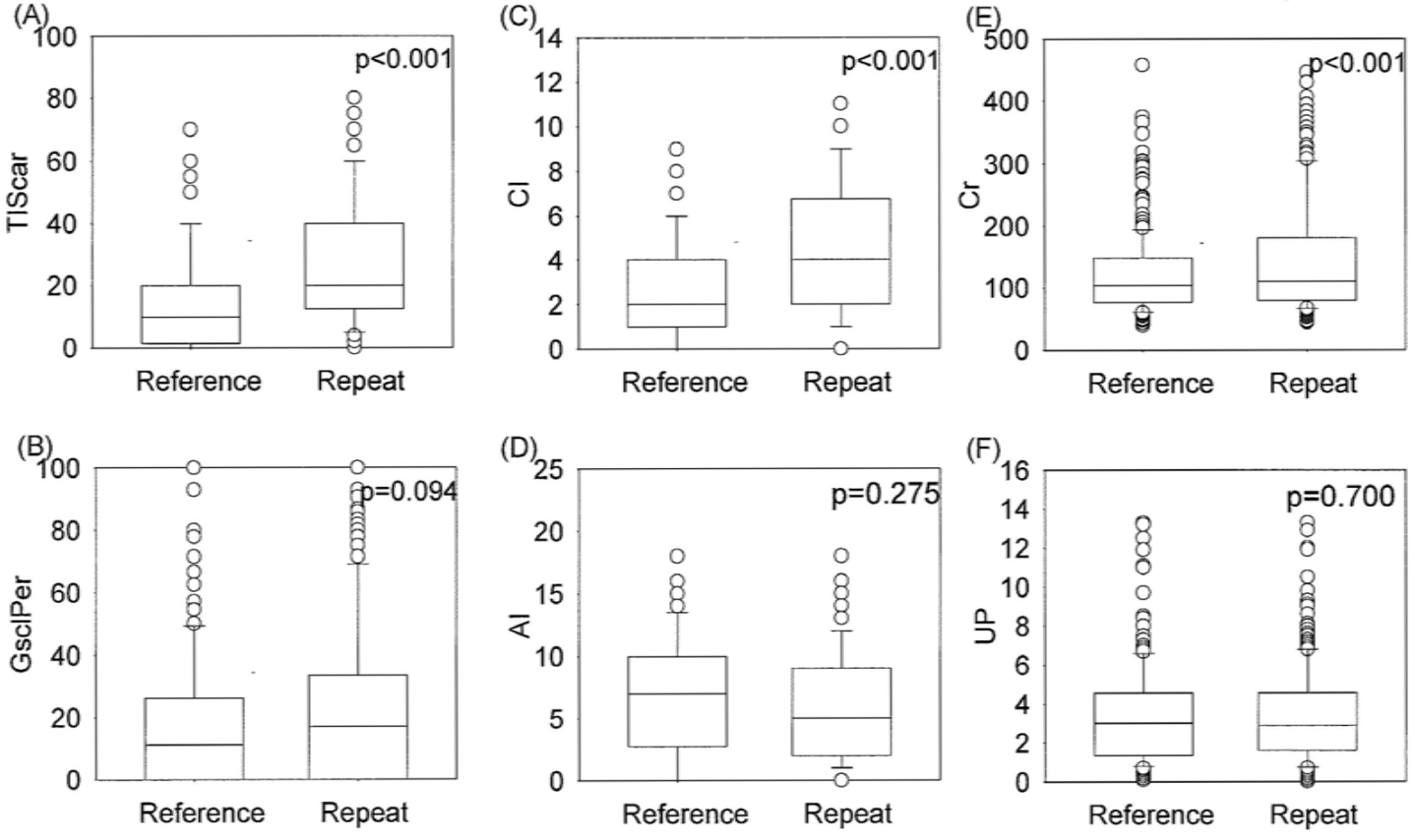
Reference biopsy	proliferative			membranous			mix		
Repeat biopsy	proliferative	membranous	mix	proliferative	membranous	mix	proliferative	membranous	mix
Number of case	38	24	28	6	18	12	18	14	21
Age (years)	30.3 ± 8.9	28.5 ± 9.4	29.0 ± 10.1	33.3 ± 10.6	32.3 ± 9.9	32.8 ± 12.4	29.8 ± 8.1	29.8 ± 7.9	29.3 ± 11.8
Sex (M:F)	0:38	4:20	3:25	0:6	2:16	1:11	1:17	1:13	4:17
Duration of SLE (months)	30.7 ± 9.1	28.9 ± 9.5	29.7 ± 10.3	33.8 ± 10.8	32.8 ± 10.0	33.3 ± 12.6	30.5 ± 8.4	32.0 ± 7.0	28.8 ± 12.0
Lapse between the biopsy (months)	38 ± 23.5	46.2 ± 37.9	55.3 ± 43.5	60.5 ± 30.3	49.0 ± 44.2	60.8 ± 43.9	35.3 ± 26.7	65.4 ± 53.7	34.3 ± 24.1
At the time of reference biopsy									
creatinine (μmol/l)	138.4 ± 76.6	120.6 ± 39.9	123.0 ± 66.7	99.0 ± 40.9	130.3±102.5	117.4 ± 63.3	96.1 ± 38.9	91.9 ± 40.2	108.9 ± 45.8
proteinuria (g/day)	3.0 ± 2.2	4.4 ± 3.2	2.9 ± 2.3	2.3 ± 1.2	3.8 ± 1.4	2.8 ± 1.3	3.4 ± 1.9	4.3 ± 3.1	4.4 ± 2.5
GFR (ml/min/1.73m2)	56.2 ± 28.6	59.7 ± 24.6	67.4 ± 38.1	68.8 ± 23.2	68.0 ± 32.3	64.1 ± 28.6	76.5 ± 32.2	82.7 ± 38.1	75.0 ± 43.2
SLEDAI	9.2 ± 4.5	10.5 ± 3.5	8.6 ± 3.1	7.7 ± 3.7	11.0 ± 4.3	11.3 ± 3.1	9.4 ± 4.1	10.6 ± 3.4	10.8 ± 4.6
Characteristics of the reference biopsy									
activity index	9.4 ± 3.7	8.2 ± 2.1	8.6 ± 4.7	0.7 ± 1.2	2.3 ± 1.7	2.2 ± 2.0	7.2 ± 3.3	8.0 ± 3.8	8.4 ± 3.5
chronicity index	2.3 ± 2.1	2.2 ± 2.0	1.8 ± 1.5	3.0 ± 3.0	4.0 ± 3.5	2.7 ± 3.0	3.6 ± 1.6	2.4 ± 1.7	2.4 ± 1.9
glomerulosclerosis (%)	20.8 ± 21.0	19.4 ± 23.4	19.8 ± 23.8	49.4 ± 31.2	14.6 ± 20.3	12.0 ± 20.1	13.3 ± 11.6	9.6 ± 8.7	26.5 ± 23.6
tubulointerstitial fibrosis (%)	15.0 ± 14.6	15.0 ± 16.5	11.1 ± 8.8	20.0 ± 20.0	21.7 ± 20.0	21.0 ± 22.0	22.9 ± 18.6	12 ± 7.9	12.1 ± 11.2
Treatment response (CR:PR:NR)	21:0:17	16:1:7	16:0:12	4:0:2	7:3:8	5:2:5	5:2:11	8:0:6	8:1:12

GFR, glomerular filtration rate; SLEDAI, systemic lupus erythematosus disease activity index; CR, complete response; PR, partial response; NR, no response.

6.4.3 Comparison with clinical and histological parameters

The degree of tubulointerstitial scarring, percentage of glomerulosclerosis, histological chronicity index, activity index, serum creatinine and proteinuria at the time of biopsy were compared between the reference and repeat biopsies and summarized in Figure 6-1. As compared to reference biopsy, repeat biopsy had higher degree of tubulointerstitial scarring ($p < 0.001$), chronicity index ($p < 0.001$) and serum creatinine ($p < 0.001$). In contrast, the degree of glomerulosclerosis, histological activity index and proteinuria were not significantly different between the two biopsies. There was also no significant difference in other markers of systemic disease activity, such as SLEDAI score, serum complement, or anti-ds DNA titre between the two groups.

Figure 6-1 Comparison of (A) percentage of tubulointerstitial scarring (TIScar); (B) percentage of glomerulosclerosis (GsclPer); (C) histological chronicity index (CI); (D) histological activity index (AI); (E) serum creatinine (Cr); and (F) proteinuria (UP) between the reference and repeat biopsy. Data are compared by Wilcoxon sign rank test.



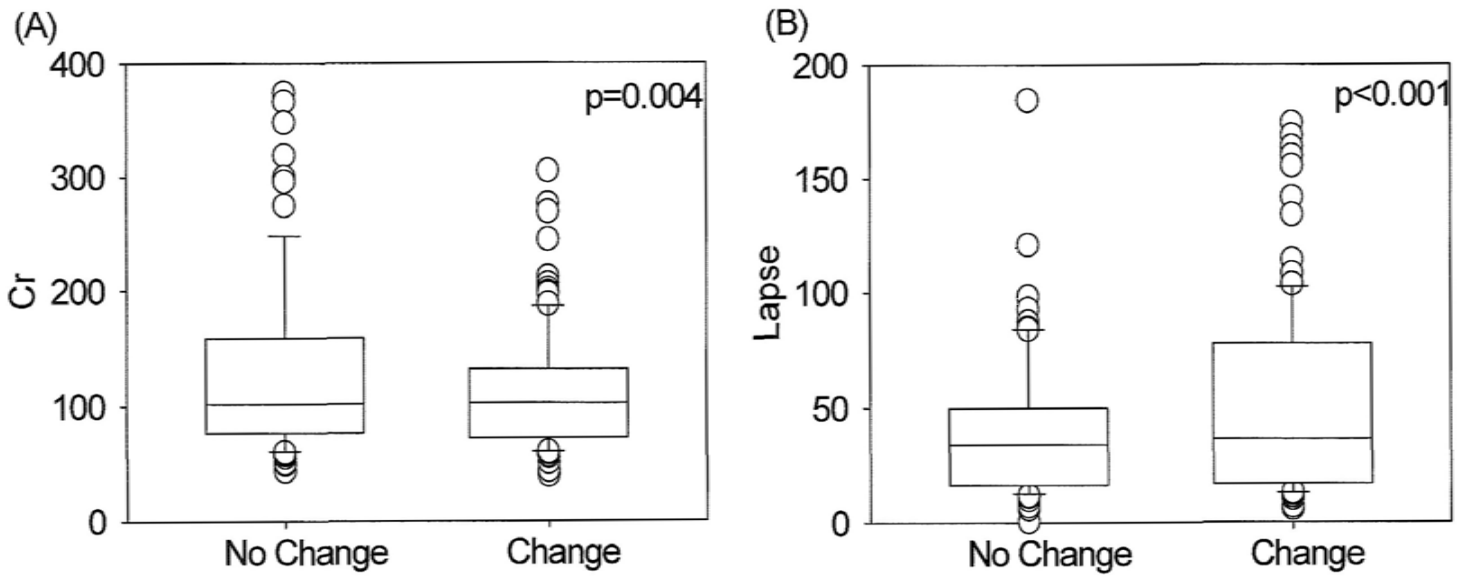
6.4.4 Predictor of change

We went on to compare the baseline clinical, biochemical, and pathological characteristics of patients who had a change in histological pattern upon repeat biopsy and those whose histological pattern remained the same. In short, baseline serum creatinine was significantly lower (111.33 ± 51.97 vs 128.44 ± 76.89 μmol/l, $p = 0.004$), and the time lapse between the two biopsies were significantly longer (51.96 ± 41.98 vs 39.56 ± 30.00 months, $p < 0.001$) amongst patients who had a change in the histological pattern upon repeat renal biopsy (Figure 6-2). In contrast, age, duration of SLE, baseline renal function, proteinuria, SLEDAI, histological activity and chronicity indices, the choice of immunosuppressive treatment, or initial therapeutic response did not predict a change in histological pattern in the repeat biopsy.

Baseline clinical and histological characteristics amongst subgroups based on the histology of reference and repeat biopsy were compared (please refer to Table 6-3). For patients with the reference biopsy showing pure proliferative nephritis, no clinical, biochemical, or pathological characteristics were found to predict the pathology of the repeat biopsy. However, in patients with the reference biopsy showing pure membranous nephropathy, baseline proteinuria was significantly higher among patients who had pure membranous nephropathy upon repeat biopsy than those who had a change in histological pattern (one way ANOVA, $p = 0.033$). In patients with the reference biopsy showing mix nephritis, the time lapse between the two biopsies ($p = 0.026$) was significantly longer in patients whose repeat biopsy became pure

membranous nephropathy, while the degree of glomerulosclerosis in the reference biopsy was significantly higher in patients whose repeat biopsy continued to show persistent mixed nephritis ($p = 0.031$).

Figure 6-2 Comparison of (A) baseline serum creatinine (Cr) and (B) the time lapse between the two biopsies (Lapse) between patients who had no change and had a change in the histological pattern upon repeat renal biopsy. Whisker-box plot, with the boxes indicate median, 25 and 75 percentile; whisker caps indicate 5 and 95 percentile; circles indicate outliers. Data are compared by Wilcoxon sign rank test



6.5 Conclusion

Our present study shows that change in the histological class of lupus biopsy is common in SLE patients with lupus flare, and the histological during disease flare could not be predicted by baseline clinical, biochemical, or pathological parameters. Our result argues for repeating renal biopsy to guide the treatment when there is lupus flare with renal involvement.

CHAPTER 7 – Relation Between Intra-Renal Gene Expression and Histological Class of Lupus Nephritis – Insight from Serial Renal Biopsy

7.1 Introduction

Our previous study showed that there is an increase in intra-renal expression of TWEAK and Fn14, and a decrease in CXCR3 expression in lupus nephritis (see Chapter 4). However, the relation between intra-renal TWEAK, Fn14, IP-10 and CXCR3 expression and the histological class of lupus nephritis remains unclear.

7.2 Specific objective of this part

In this part of the work, we compare the intra-renal expression of TWEAK/Fn14, IP-10/CXCR3, TLR9 and NGAL in a nested cohort of LN patients who had two renal biopsies during renal flare of lupus. By using each individual patient as their own control, our objectivs are:

1. Compare the intra-renal gene expression between different histological class of lupus nephritis.

2. Correlate the change in intra-renal gene expression with the duration of disease.

7.3 Patients and Methods

7.3.1Patient Selection

We reviewed 55 SLE patients, each with two kidney biopsies for disease flare. All patients fulfilled the American College of Rheumatology diagnostic criteria for SLE [256]. These patients were randomly selected from the cases reviewed in the work described in Chapter 6. We performed chart review to collect clinical data, which included baseline serum creatinine, albumin, urea, proteinuria, complement levels (C3 and C4), anti-double strand (anti-ds) DNA antibody titre, and the disease activity of SLE as represented by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [184,256]. Glomerular filtration rate (GFR) was estimated from a standard prediction equation [257].

7.3.2 Assessment of Renal Pathology

Kidney biopsy was evaluated according to the International Society of Nephrology / Renal Pathology Society (ISN/RPS) classification of lupus nephritis [175]. The Activity Index (AI) and Chronicity Index (CI) of each biopsy were scored by standard methods [266,267].

7.3.3 Laser microdissection and gene expression study

The method of laser microdissection, RNA extraction, reverse transcription, and RT-QPCR has been described in Chapter 3. In this part of the work, we quantified the mRNA expression of TWEAK, Fn14, IP-10, CXCR3, TLR9 and NGAL in glomerulus and tubulointerstitium. Taqman primers and probes of each target were the same as those we used in Chapters 4 and 5.

7.3.4 Statistical Analysis

Data were analysed using the SPSS for Windows version 15.0 software (SPSS Inc., Chicago, IL). Results were presented as mean ± SD unless otherwise specified. Data were compared by paired or unpaired Student's t test, Kruskal Wallis test, Mann-Whitney U test, or Wilcoxon sign rank test as appropriate. P-values of <0.05 were considered statistically significant. All probabilities were two tailed.

7.4 Result

7.4.1 General Description

We studied 55 lupus patients with repeat renal biopsies. They were divided into 7 groups according to the patterns of histology in the first and second renal biopsy specimens. Their baseline demographic and clinical data are summarized and compared in Table 7-1.

Table 7-1 Comparison of baseline clinical and histological characteristics.

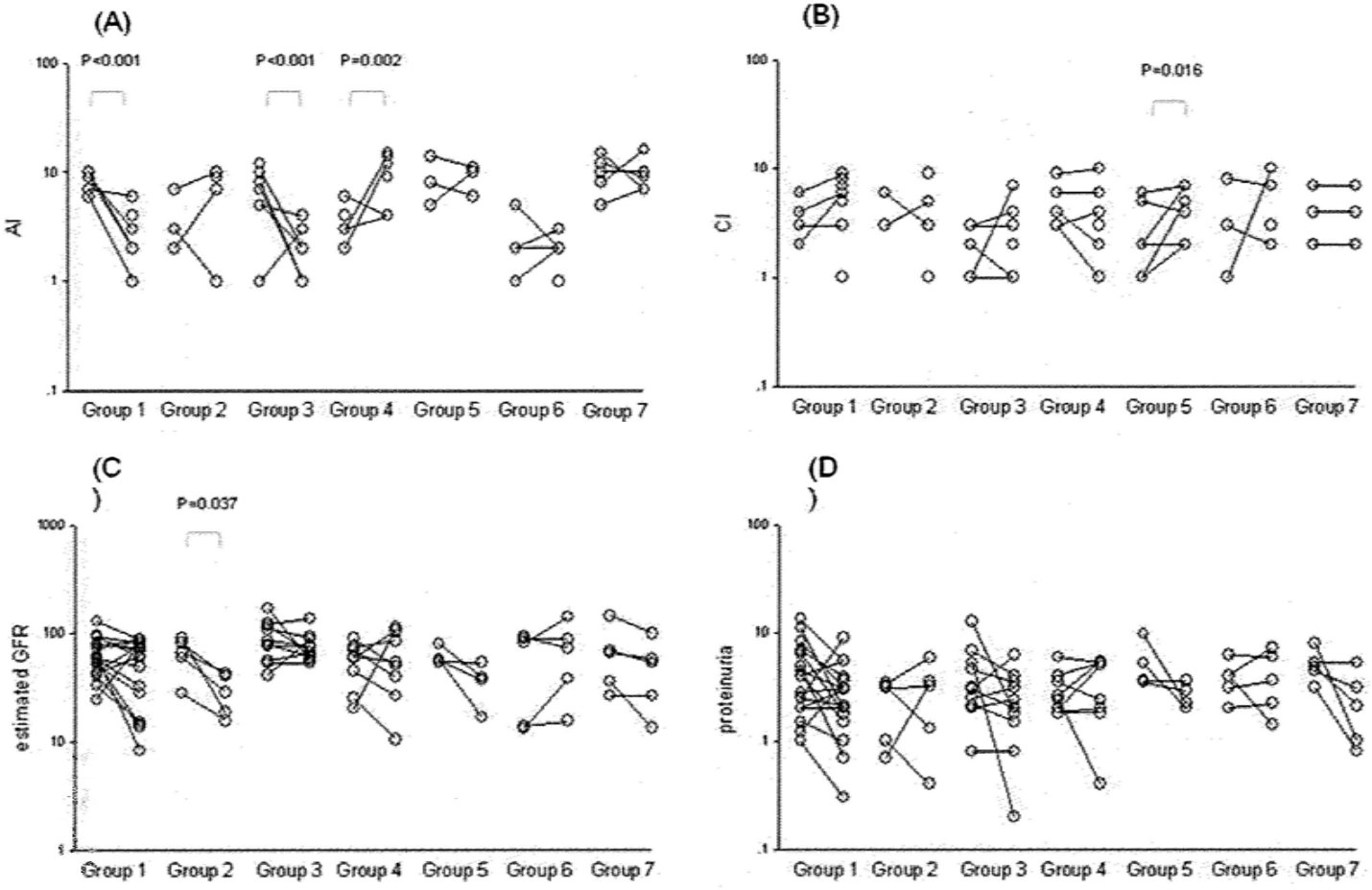
Group	1	2	3	4	5	6	7
First biopsy	proliferative	membranous	mixed	membranous	proliferative	membranous	mixed
Repeat biopsy	membranous	proliferative	membranous	mixed	proliferative	membranous	mixed
Number of case	18	5	10	9	4	4	5
Age (years)	44.46±10.87	46.6±15.24	42.44±8.89	45.33±12.44	45.75±14.97	39.8±5.4	47±19.38
Sex (M:F)	3:15	0:5	2:8	1:8	0:4	0:4	1:4
Duration of SLE (months)	31.7 ± 8.1	29.8 ± 7.4	28.7 ± 11.2	32.9 ± 9.8	32.0 ± 7.0	32.3 ± 11.5	27.2 ± 6.4
Lapse between the biopsy (months)	43.69±37.96	55.97±31.52	63.88±55.54	66.59±45.79	26.61±14.18	55.92±45.44	30.37±21.33
At the time of first biopsy							
creatinine (μmol/l)	125.32±43.66	103.6±43.78	90.08±41.24	120.2±67.35	151.11±87.89	143.1±108.0	103.37±46.13
proteinuria (g/day)	4.11±3.48	2.28±1,31	4.07±3.14	3.03±1.32	3.78±2.54	3.88±1.38	4.53±1.92
GFR (ml/min/1.73m2)	57.25±26.70	65.22±23.99	85.34±38.3	64.33±29.22	51.03±25.55	62.48±31.93	79.43±47.86
SLEDAI	9.89±3.51	7±3.74	10.23±3.27	11.9±2.72	10.61±5.14	11.13±4.65	11.18±4.59
Treatment response (CR:PR:NR)	11:6:1	3:2:0	4:6:0	4:4:1	2:2:0	2:1:1	1:3:1

GFR, glomerular filtration rate; SLEDAI, systemic lupus erythematosus disease activity index; CR, complete response; PR, partial response; NR, no response.

7.4.2 Change in clinical and histological parameters

The degree of histological chronicity index, activity index, estimated GFR and proteinuria at the time of two biopsies were compared and summarized in Figure 7-1. As expected, activity index generally increased when the pattern of lupus nephritis changed from membranous to mixed nephritis ($p = 0.002$), and decreased when change from proliferative and mix to membranous nephritis ($p < 0.001$ for both). In contrast, we did not observe any change in the estimated GFR or histological chronicity index from the first renal biopsy to the second.

Figure 7-1 Comparison between the first and second renal biopsies between different groups: (A) histological activity index (AI); (B) chronicity index (CI); (C) estimated glomerular filtration rate (GFR); and (D) proteinuria.



7.4.3 Comparison of gene expression levels

The glomerular mRNA expression levels of TWEAK, Fn14, IP-10, and CXCR3 are summarized in Figure 7.2. In short, glomerular Fn14 expression tends to decrease when change from proliferative or mixed nephritis to membranous nephropathy ($p = 0.019$ and $p = 0.014$, respectively), while the glomerular Fn14 expression remained static when the first and second renal biopsy showed the sample histological pattern. There was no consistent pattern of change in the glomerular expression of TWEAK, IP-10, or CXCR3 (see Figure7-2).

The tubulo-interstitial mRNA expression levels of TWEAK, Fn14, IP-10, and CXCR3 are summarized in Figure 7-3. In short, tubulo-interstitial TWEAK expression tends to decrease when change from proliferative or mix nephritis to membranous nephropathy ($p = 0.043$ and $p = 0.034$, respectively), while tubulointerstitial TWEAK expression increases when change from membranous nephropathy to proliferative nephritis ($p = 0.047$), and remained static when the first and second renal biopsy showed the sample histological pattern.

The glomerular and tubulo-interstitial mRNA expression levels of NGAL and TLR9 are summarized in Figure 7-4. There was no consistent pattern of change in the glomerular expression of NGAL and TLR9. However, tubulo-interstitial TLR9 expression trends to increase when change from proliferative to membranous ($p = 0.018$) while decrease when change from membranous to mix ($p = 0.020$).

Figure 7-2 Comparison of Glomerular mRNA expression levels of (A) TWEAK; (B) Fn14; (C) IP-10; and (D) CXCR3 between the first and second biopsy in different groups. Data are compared by Mann-Whitney U test.

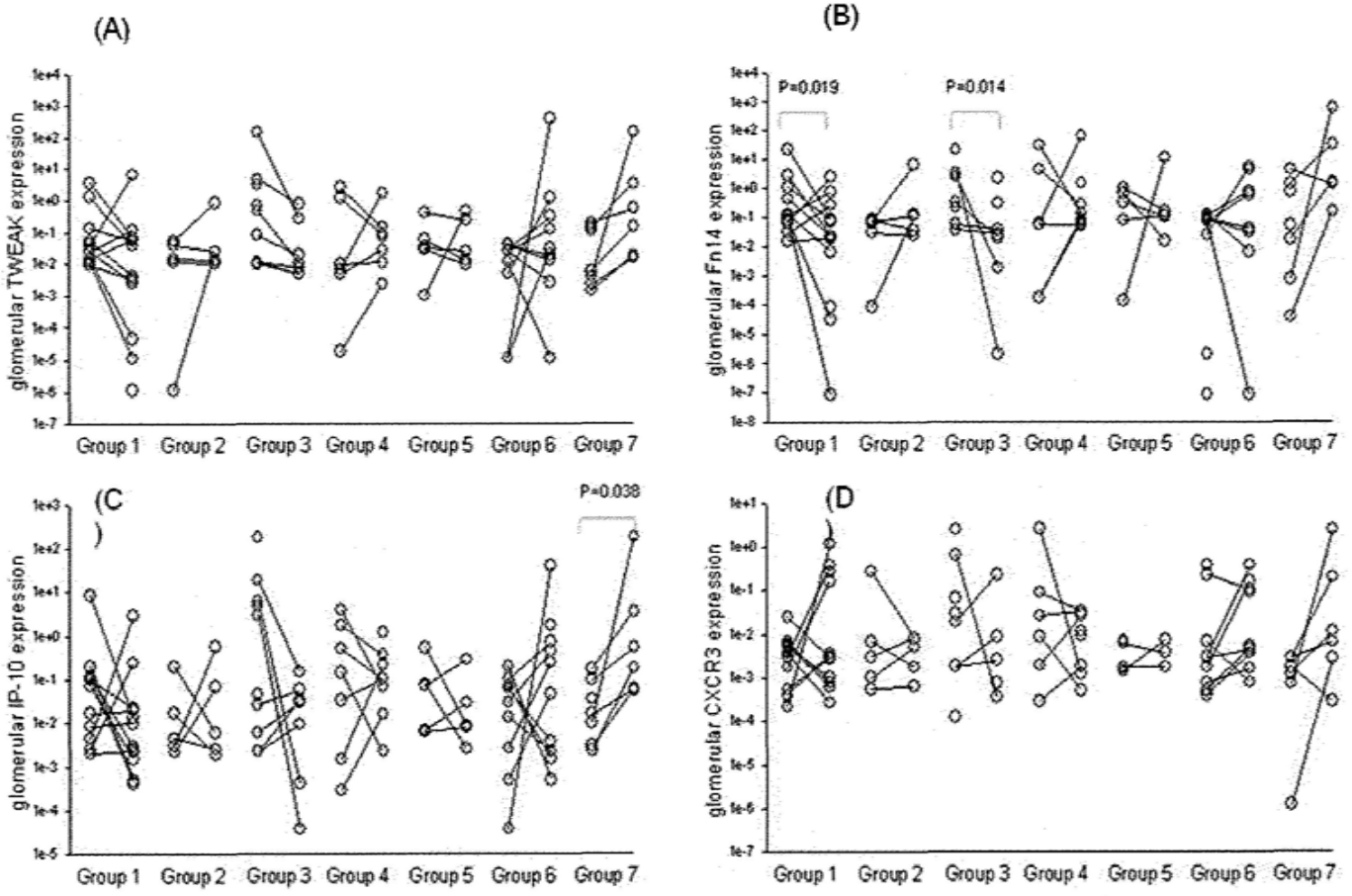


Figure 7-3 Comparison of tubulointerstitial mRNA expression levels of (A) TWEAK; (B) Fn14; (C) IP-10; and (D) CXCR3 between the reference and repeat biopsy among different groups.

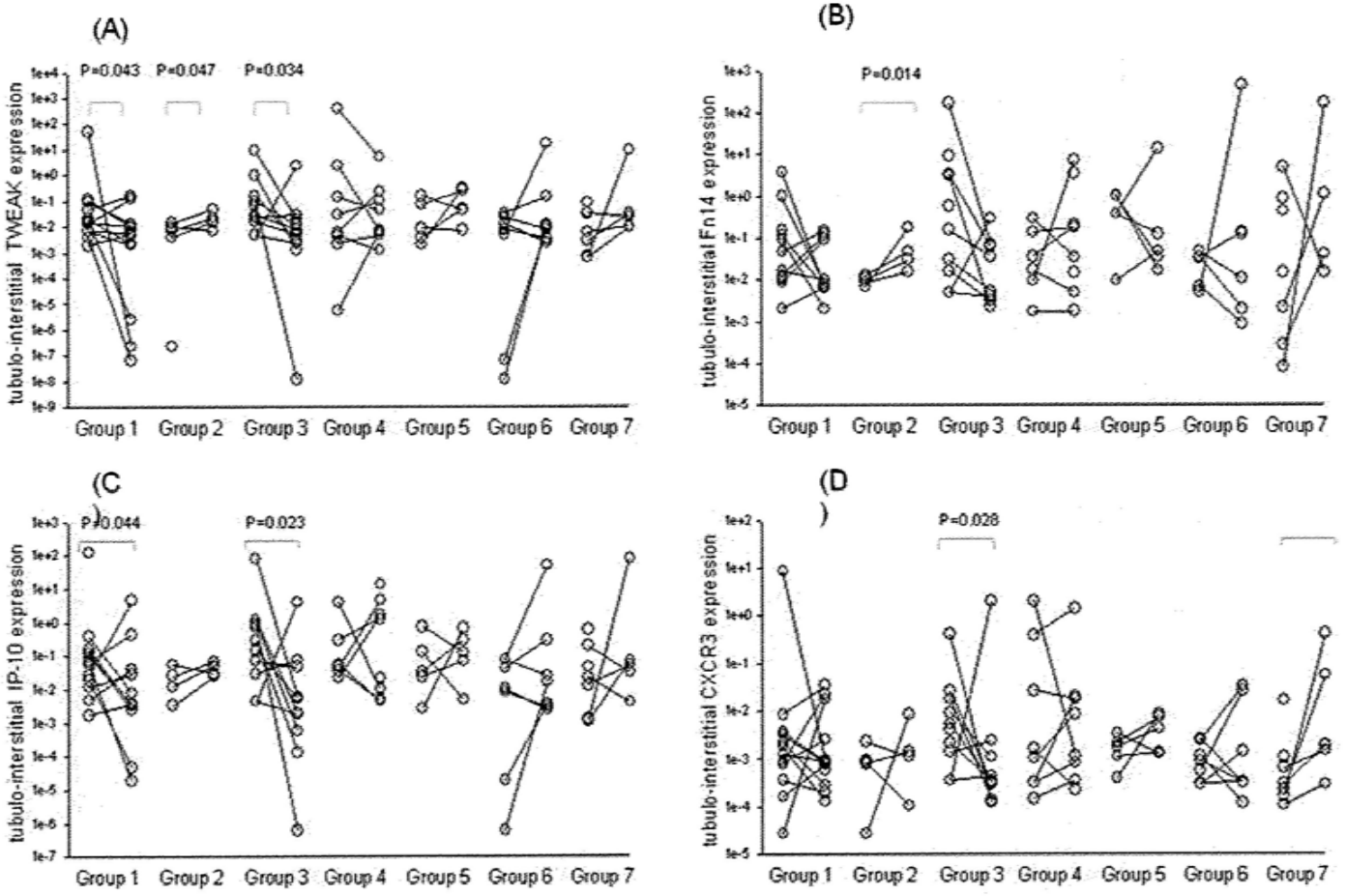
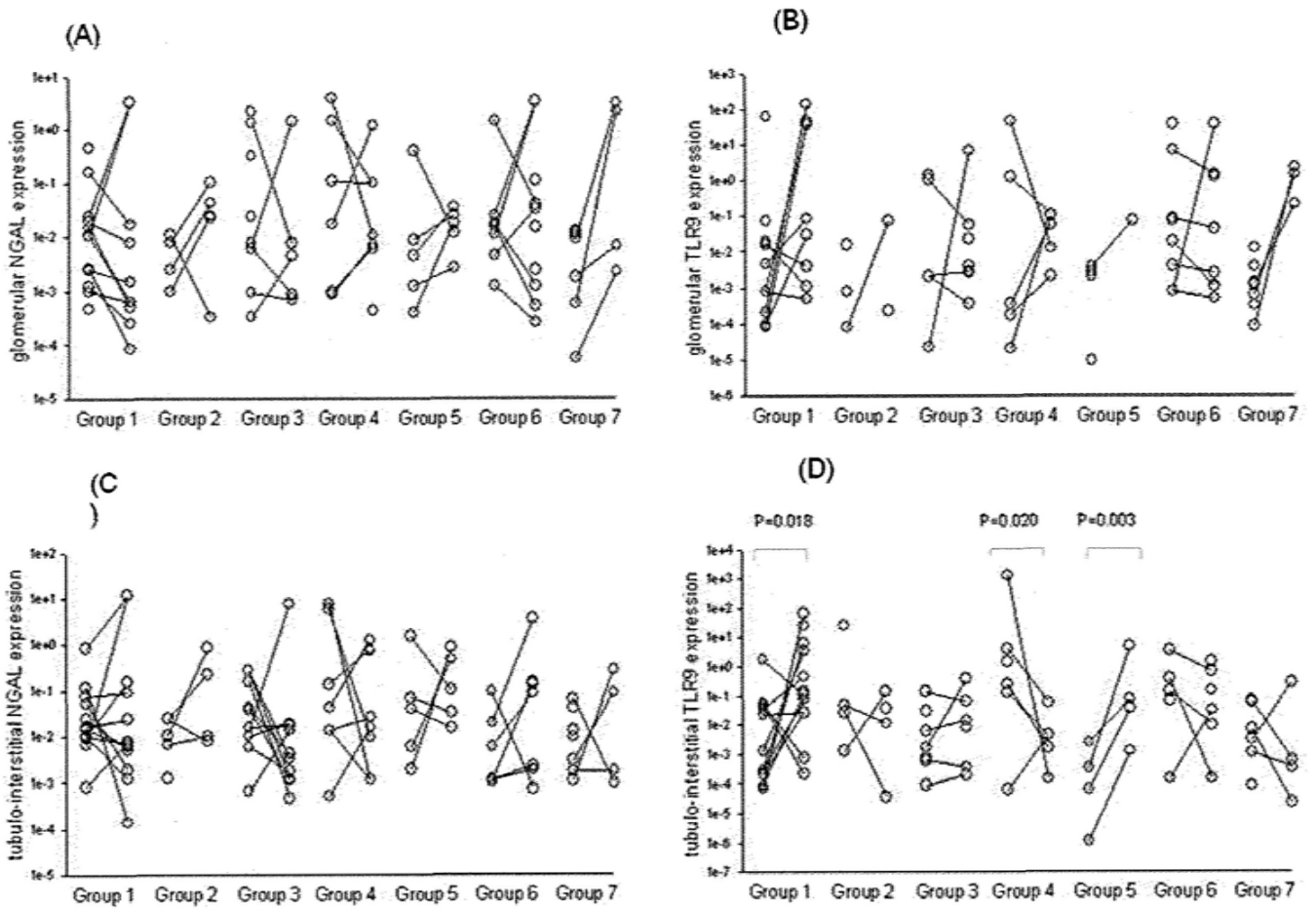


Figure 7-4 Comparison of Glomerular mRNA expression levels of (A) NGAL; (B) TLR9; tubulointerstitiale mRNA expression levels of (C) NGAL; and (D) TLR9 between the first and second biopsy in different groups.



7.5 Conclusion

In summary, the present study suggests that tubulo-interstitial IP-10 expression consistently decreased when the histology changed to membranous nephritis in the second biopsy. Tubulo-interstitial TLR9 expression tends to increase when change to membranous and decrease when change from membranous nephritis. In contrast, tubulo-interstitial TWEAK expression tends to decreased when change to membranous and increased when change from membranous. Our findings suggest that TWEAK and TLR9 appeared to have a consistent change in expression in parallel with the alteration in special histological class of lupus nephritis, suggesting a specific role in pathogenesis.

CHAPTER 8 – Intra-renal micro-RNA expression in lupus nephritis

8.1 Introduction

Micro-RNA (miRNA) are small noncoding, single-stranded RNA molecules that regulate gene expression at the posttranscriptional level by degrading or blocking the translation of messenger RNA (mRNA) [268]. Several miRNA species, notably hsa-miR-638, hsa-miR-663, hsa-miR-198, miR-146a and miR-155, are reported to be involved in the pathogenesis of SLE [30,240,242]. Previous studies from our center found that miR-146a and miR-155 in the serum and urinary supernatant are differentially expressed between patients with lupus nephritis and normal controls [269]. Recently, there are emerging evidence that miRNA species may regulate the inflammatory cascade via TWEAK/Fn14 and IP-10/CXCR3 axis. For example, Panguluri et al [270] showed that TWEAK affects the expression of several genes and miRNAs involved in inflammatory response, fibrosis, extracellular matrix remodeling, and proteolytic degradation. Similarly, Imaizumi et al [271] reported that miR-155 may play a role in the regulation of inflammatory and immune reactions in the kidney by lowering IP-10 expression, and Liu et al [272] found that miR-148/152 expression was up-regulated in dentritic cells on maturation and activation induced by TLR9 agonists. However, there are few data on the intra-renal miRNA expression in patients with lupus nephritis.

8.2 Specific objective of this part

In this part of the work, we studied the intra-renal expression of miR-146a, miR-155, miR-198 miR-638, and miR-663 in LN patients. Our objectivs are:

1. To compare intra-renal miRNA expression between LN patients and controls.
2. To compare intra-renal miRNA expression between different histological classes of lupus nephritis.
3. To correlate intra-renal miRNA expression with clinical and histological severity of lupus nephritis.
4. To correlate intra-renal miRNA expression with intra-renal expression of TWEAK/Fn14, IP-10/CXCR3, TLR9 and NGAL.

8.3 Patients and Methods

8.3.1 Patient Selection

We studied 42 consecutive SLE patients with active nephritis and required kidney biopsy; they were the same 42 patients as we studied in Chapter 4 and 5. All patients fulfilled the American College of Rheumatology diagnostic criteria of SLE [256]. The uninvolved pole of 10 kidneys that were removed for renal cell carcinoma and had no morphological evidence of renal disease were used as control. All patients provided informed consent.

8.3.2 Clinical and histological assessment

The disease activity of SLE was assessed clinically by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [184] on the day of kidney biopsy. Baseline serum creatinine, urine protein, complement levels (C3 and C4) and anti-double strand (ds) DNA antibody titre were also measured. Glomerular filtration rate (GFR) was estimated by a standard equation[257]. Kidney biopsy specimen was evaluated according to the International Society of Nephrology (ISN) classification of

lupus nephritis [175]. The activity index (AI) and chronicity index (CI) of each biopsy specimen were scored by standard methods [251].

8.3.3 Laser microdissection and gene expression study

The method of laser microdissection has been described in Chapter 3.The RNAqueous®-Micro Kit (Applied Biosystems, USA) was used for the extraction of total RNA. TaqMan microRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) were used for reverse transcription. Intrarenal expression of miR-146a, miR-155, miR-198 miR-638, and miR-663 were quantified by RT-QPCR with the ABI Prism 7900 Sequence Detection System (Applied Biosystems). Commercially available TaqMan primers and probes were used for all the targets (all from Applied Biosystems). RNU48 (Applied Biosystems) were used as housekeeping genes to normalize the miRNA expression [252]. Average expression level of normal renal tissue from patients with kidney clear cell cancer was used as calibrator for intrarenal expression, and the expression level of targets was a ratio relative to that of the controls. In this part of the work, we quantified the mRNA expression of TWEAK, Fn14, IP-10, CXCR3, TLR9 and NGAL in glomerulus and tubulointerstitium. Taqman primers and probes of each target were the same as those we used in Chapters 4 and 5.

8.3.4 Statistical analysis

Statistical analysis will be performed by SPSS for Windows software version 15.0 (SPSS Inc., Chicago, IL). All the results from this series of experiments are quantitative. The results are presented as mean ± SD unless otherwise specified. Since the data on gene expression are highly skewed, they are compared between groups by Kruskall Wallis test or Mann-Whitney U test as appropriate. Correlations between continuous variables are calculated by Spearman's rank correlation coefficient. A p value of less than 0.05 is considered as statistically significantly. All probabilities are two tailed.

8.4 Result

8.4.1 General Description

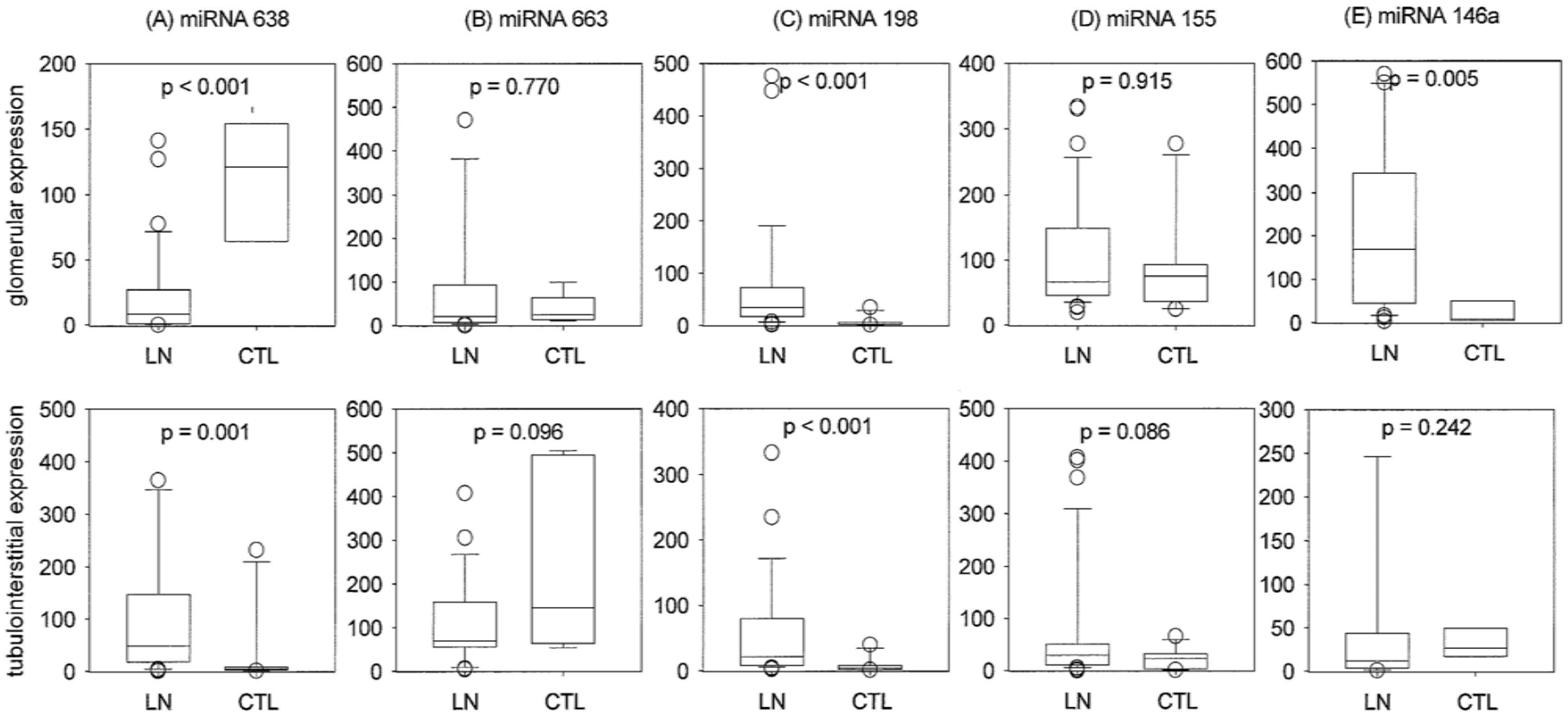
We studied the same 42 SLE patients that we examined in Chapter 5. Their baseline demographic and clinical data are summarized in Table 5-1. Briefly, the histological diagnoses were proliferative nephritis (class III or IV, 9 cases), pure membranous nephritis (class V, 9 cases), class II nephritis (3 cases), and mixed proliferative and membranous nephritis (21 cases). The mean histological Activity and Chronicity Indices were 7.1 ± 4.3 and 2.7 ± 2.2, respectively.

8.4.2 Gene expression level between groups

The glomerular and tubulointerstitial miRNA expression levels of miR-638, miR-663, miR-198, miR-155 and miR-146a are summarized in Figure 8-1. In short, as compared to controls, LN patients had lower glomerular expression of miR-638 ($p < 0.001$) but higher tubulointerstitial expression of this target ($p = 0.001$). Both of glomerular and tubulointerstitial expression of miR-198 are higher in LN patients than controls ($p < 0.001$). For miR-146a, LN patients only have higher expression in

glomerulus ($p = 0.005$) but not in tubulointerstitium. There were no significant differences in glomerular or tubulointerstitial expression of miR-663 or miR-155 between two groups.

Figure 8-1 Glomerular and tubulointerstitial mRNA expression levels of (A) miR-638; (B) miR-663; (C) miR-198; (D) miR-155; and (E) miR-146a. Data are compared by Mann-Whitney U test. (LN, lupus nephritis group; CTL, control group)



8.4.3 Relation with clinical and histological parameters

We further explore the correlation between gene expression and baseline clinical and histological parameters. We found that glomerular miR-638 expression significantly correlated with activity index ($r = -0.393$; $p = 0.024$), while tubulointerstitial miR-638 significantly correlated with proteinuria ($r = 0.404$; $p = 0.022$) and SLEDAI score ($r = 0.454$; $p = 0.008$) (Figure 8-2). In addition, the degree of proteinuria, but not estimated GFR or histological AI, significantly correlated with both glomerular expression of miR-663 ($r = 0.514$; $p = 0.001$) and miR-198 ($r = 0.325$; $p = 0.034$) (Figure 8-3 and 8-4). In contrast, the glomerular expressions of miR-146a correlated with both estimated GFR ($r = 0.253$; $p = 0.028$) and histological AI ($r = 0.494$; $p = 0.027$) (Figure 8-6). The glomerular or tubulointerstitial expressions of miR-155 did not correlate any clinical or histological parameter of lupus activity (Figure 8-5).

Figure 8-2 Correlation between (A) glomerular and (B) tubulointerstitial expression of miR-638 and the degree of proteinuria, estimated glomerular filtration rate (GFR), histological activity index, and SLEDAI score. Data are compared by Spearman's correlation coefficient.

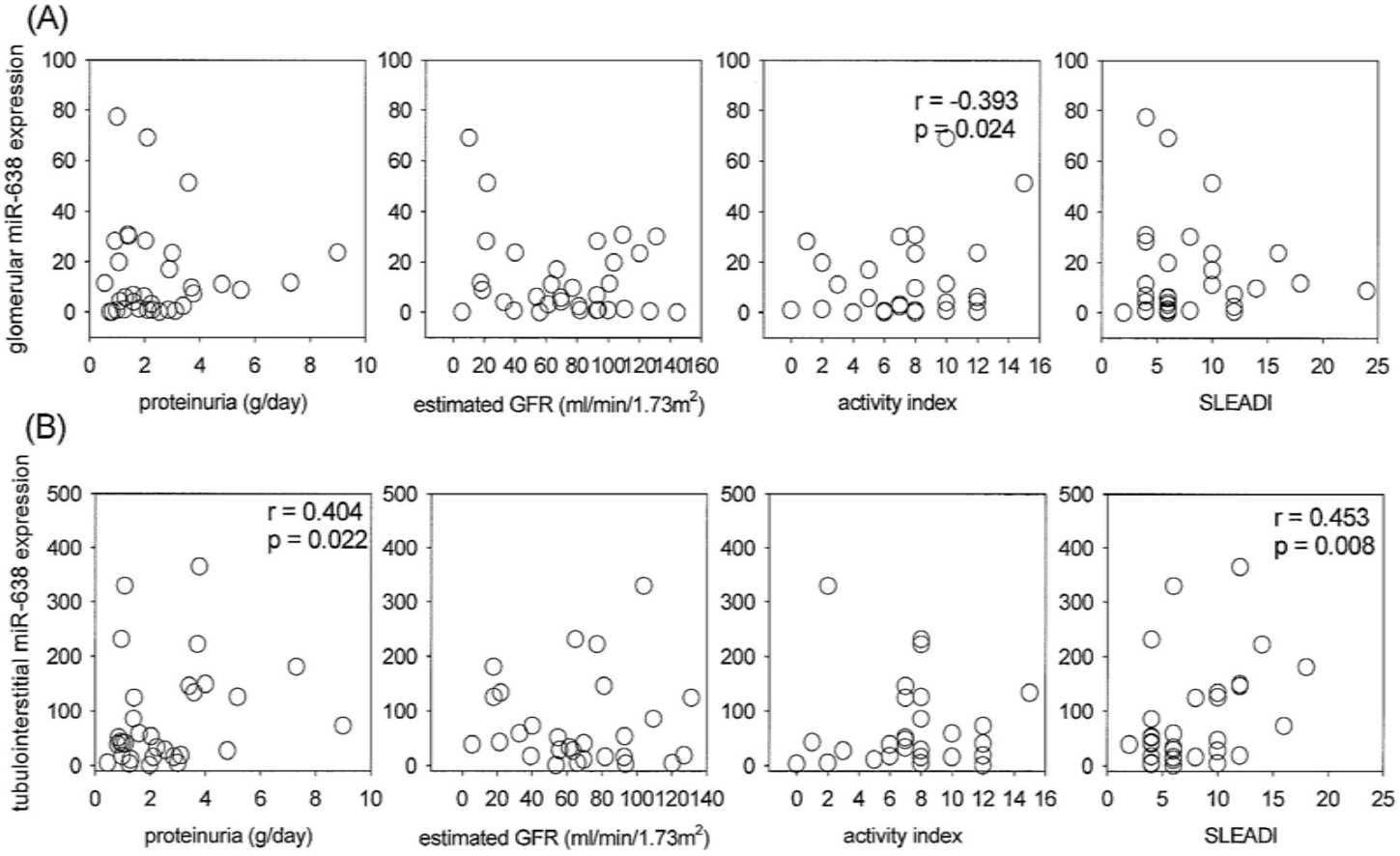


Figure 8-3 Correlation between (A) glomerular and (B) tubulointerstitial expression of miR-663 and the degree of proteinuria, estimated glomerular filtration rate (GFR), histological activity index and SLEDAI score. Data are compared by Spearman's correlation coefficient.

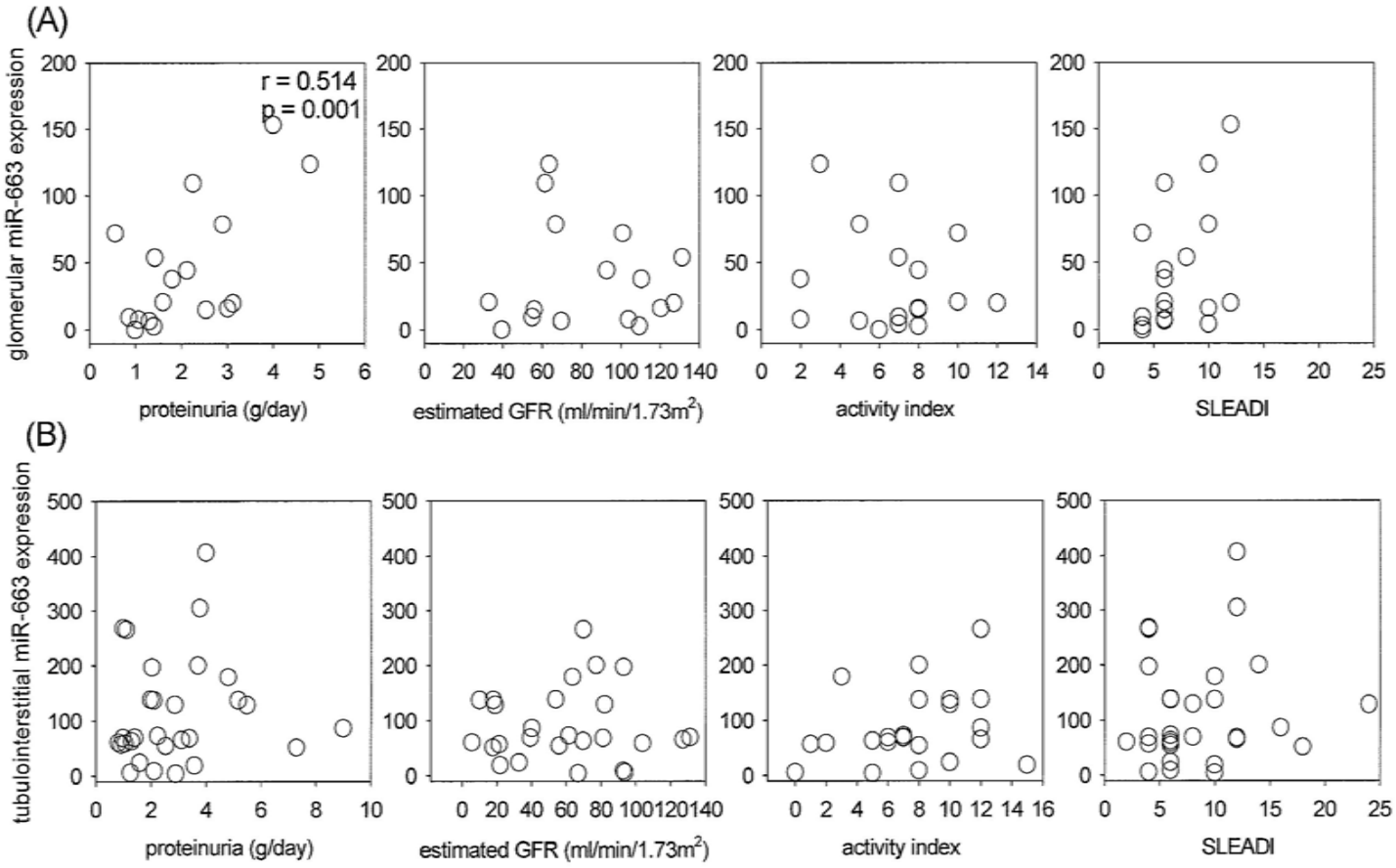


Figure 8-4 Correlation between (A) glomerular and (B) tubulointerstitial expression of miR-198 and the degree of proteinuria, estimated glomerular filtration rate (GFR), histological activity index and SLEDAI score. Data are compared by Spearman's correlation coefficient.

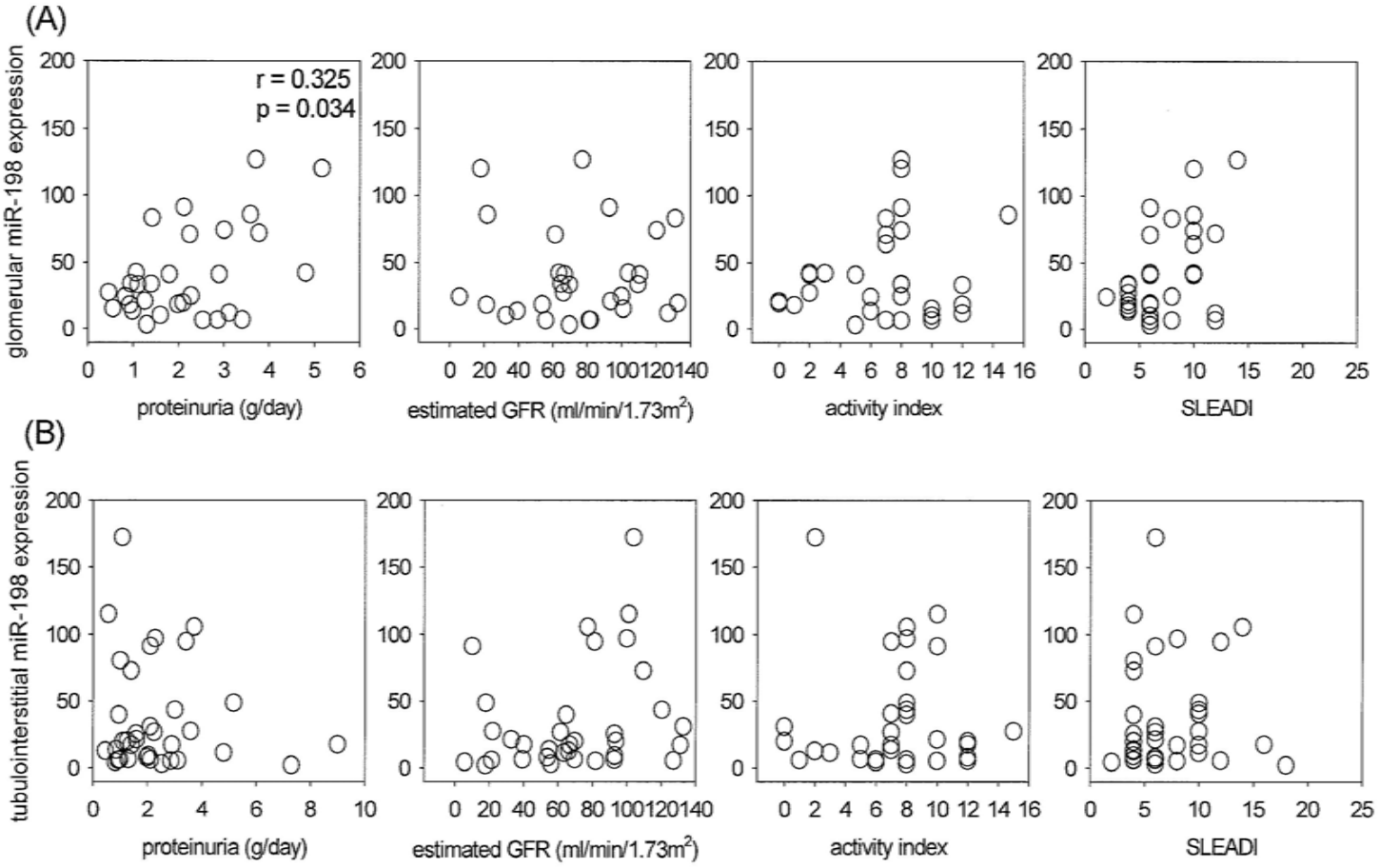


Figure 8-5 Correlation between (A) glomerular and (B) tubulointerstitial expression of miR-155 and the degree of proteinuria, estimated glomerular filtration rate (GFR), histological activity index and SLEDAI score. Data are compared by Spearman's correlation coefficient.

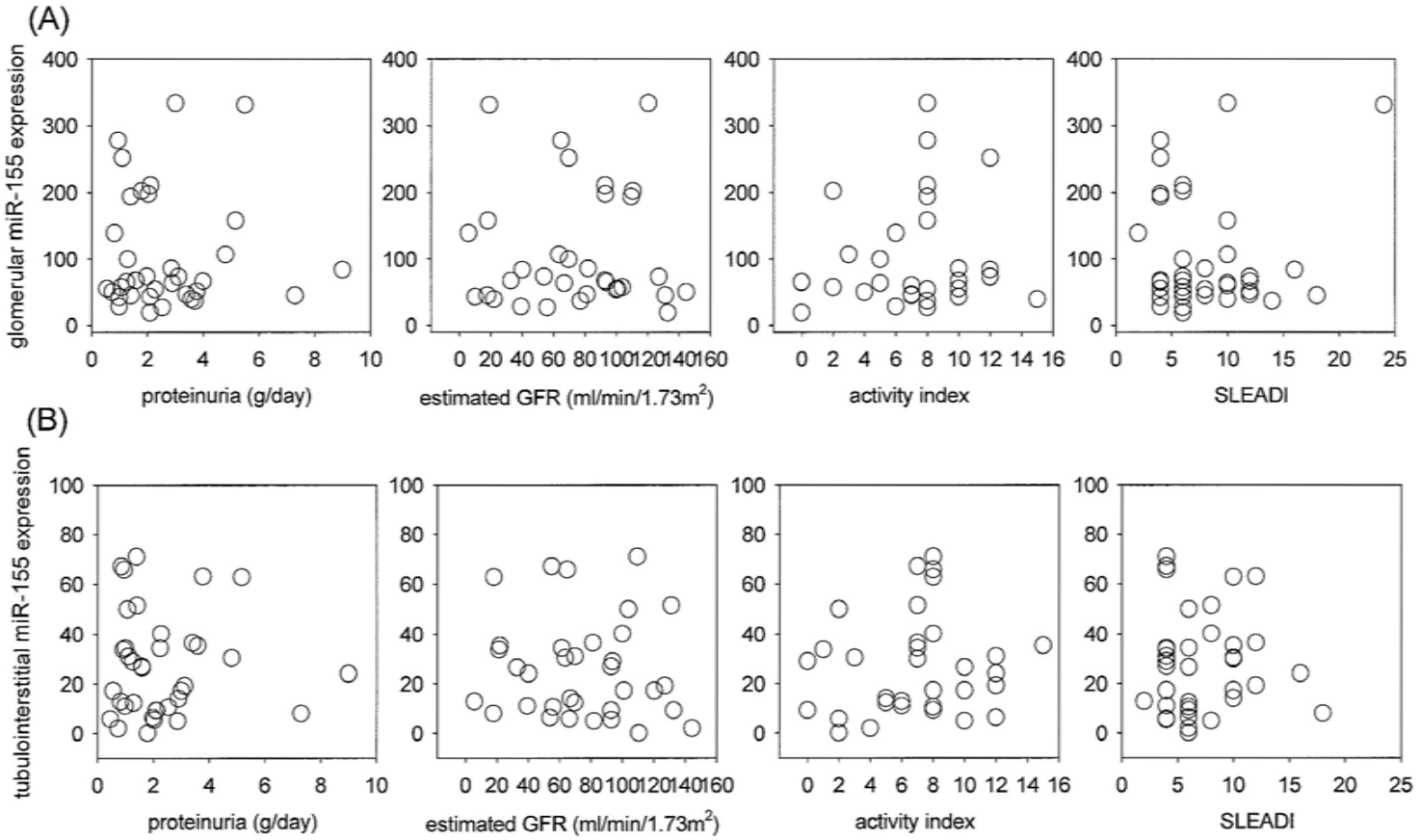
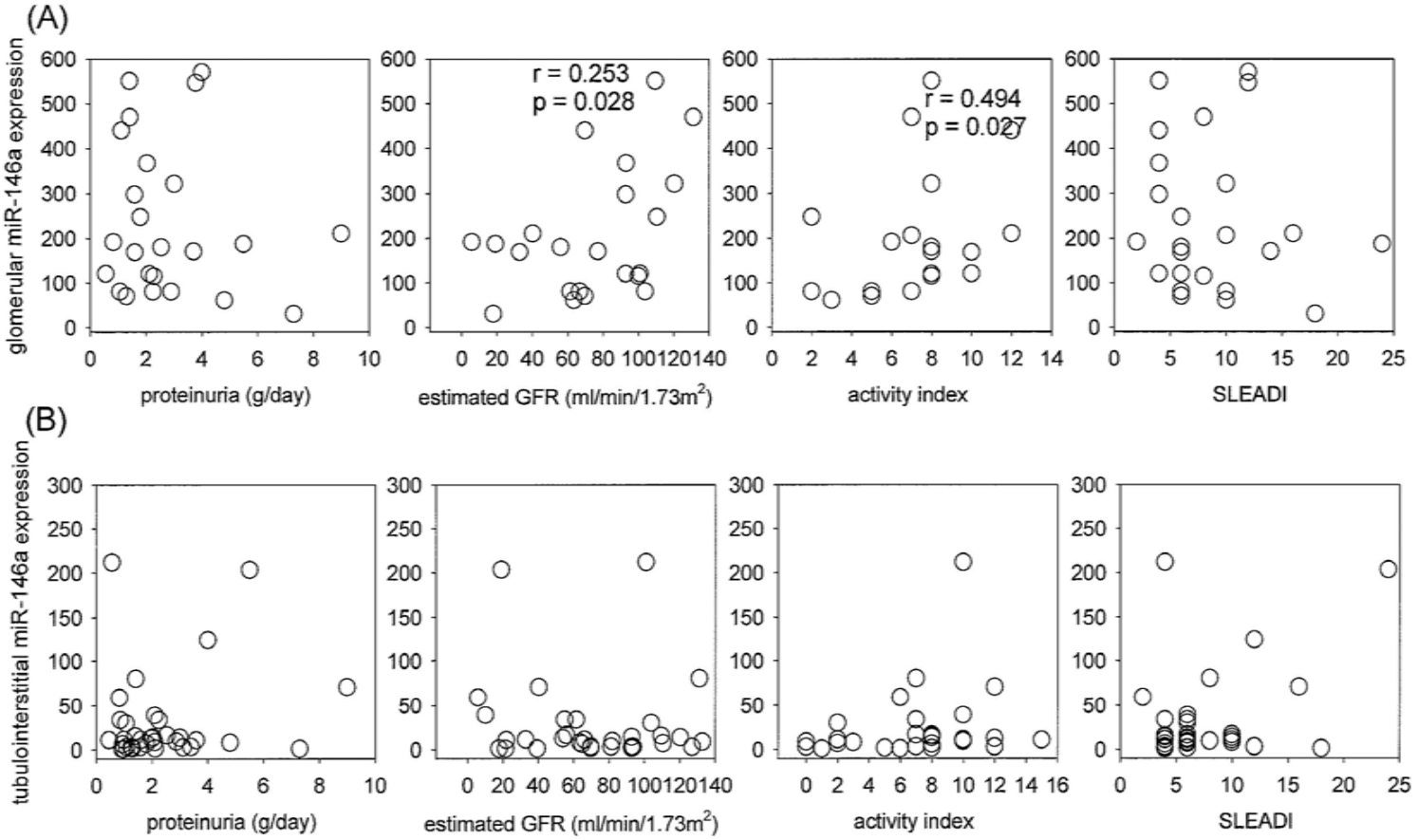


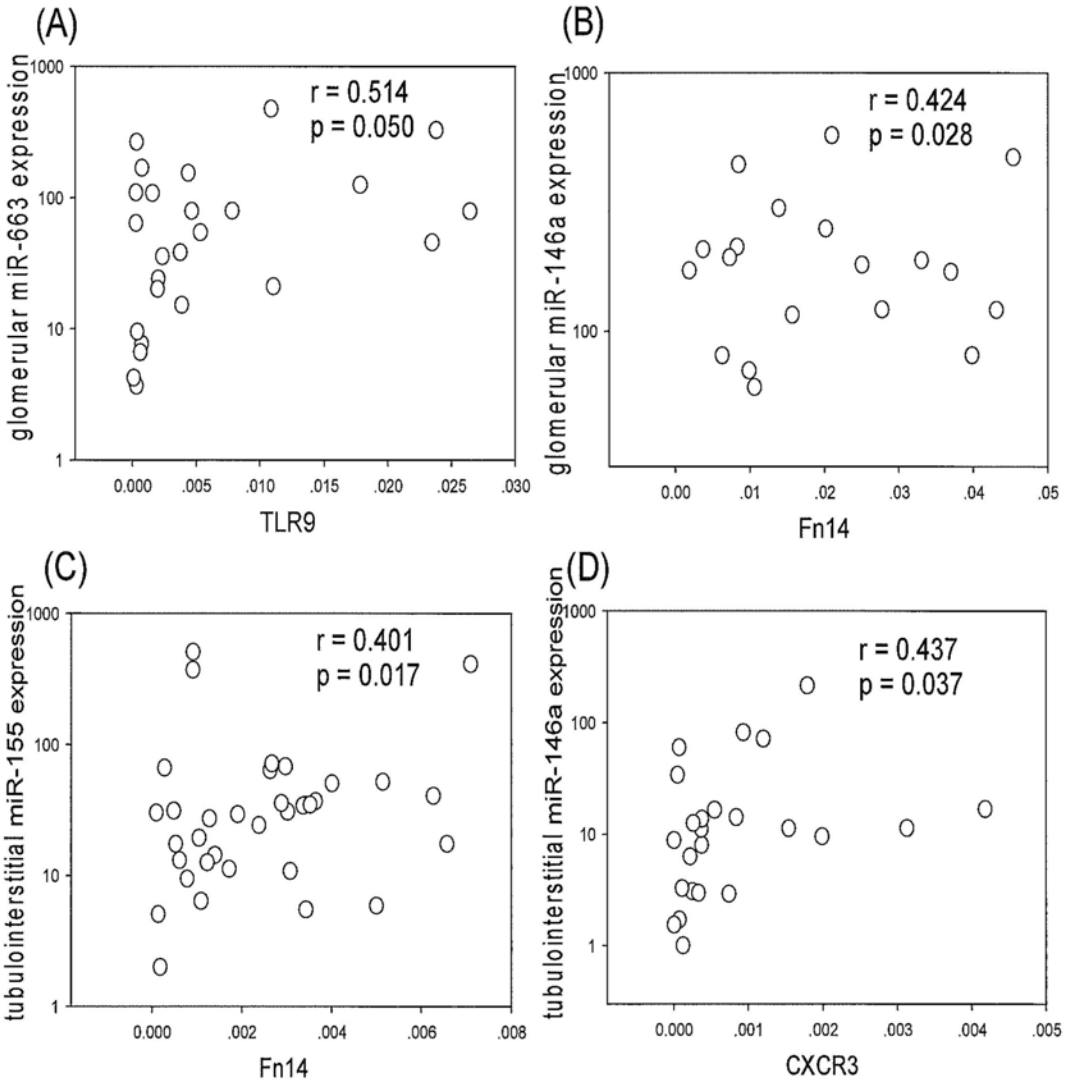
Figure 8-6 Correlation between (A) glomerular and (B) tubulointerstitial expression of miR-146a and the degree of proteinuria, estimated glomerular filtration rate (GFR), histological activity index and SLEDAI score. Data are compared by Spearman's correlation coefficient.



8.4.4 Correlation between miRNA levels and mRNA levels

We explored the relation between intrarenal miRNA level and gene expression of TWEAK, Fn14, IP10, CXCR3, TLR9 and NGAL. In short, glomerular expression of TLR9 correlated with glomerular expression of miR-663 ($r = 0.514$, $P = 0.050$), while glomerular expression of Fn14 correlated with glomerular expression of miR-146a ($r = 0.424$, $P = 0.028$). In contrast, tubulointerstitial expression of Fn14 correlated with that of miR-155 ($r = 0.401$, $P = 0.017$). Similarly, tubulointerstitial expression of CXCR3 correlated with that of miR-146a ($r = 0.437$, $P = 0.037$). The result is summarized in Figure 8-7.

Figure 8-7 Correlation between glomerular expression of (A) miR-663 and TLR9, (B) miR-146a and Fn14, tubulointerstitial expression of (C) miR-155 and Fn14, (D) miR-146a and CXCR3. Data are compared by Spearman's correlation coefficient.



8.5 Conclusion

In the present study, we found that intra-renal expression of miR-638, miR-663, miR-198, miR-155 and miR-146a are differentially expressed between patients with lupus nephritis and normal controls. Furthermore, the degree of change correlated with disease severity. The results suggested that these miRNA species may play a role in the pathogenesis of lupus nephritis. Our data also suggest a regulatory role of miR146a, miR155, and miR663 in the expression of inflammatory genes such as TLR9, CXCR3, and Fn14.

CHAPTER 9 – DISCUSSIONS

9.1 Methodology

9.1.1 Classification of lupus nephritis

The histological classification of lupus nephritis should be straight forward and has little dispute. The 2003 International Society of Nephrology (ISN) / Renal Pathology Society (RPS) Classification of lupus nephritis (LN) was designed to standardize definitions, to eliminate ambiguities, to emphasize clinically relevant lesions, and to improve interobserver reproducibility. This revised classification, as compared to the original one by the World Health Organization (WHO), introduced several important modifications concerning quantitative and/or qualitative differences between Class III, IV, and V lesions. Multiple changes from the 1982 Modified WHO Classification include the removal of the normal biopsy category (WHO class I) and the subcategories of membranous Class V (i.e. Va to Vd), the introduction of a sharp distinctions between the classes, the addition of subcategories within diffuse LN (class IV) based on whether the endocapillary involvement is predominantly segmental (LN IV-S) and global (LN IV-G) lesions, and clear definitions for all classes.

Since its publication, the ISN/RPS classification has been used successfully in a number of clinicopathologic studies. It is generally believed to have a high level of inter-observer reproducibility [273,274], although it causes a substantial increase in the number of class IV cases and a reciprocal decline in class III disease [273]. As it gains widespread acceptance, the ISN/RPS classification has provided a standard approach to renal biopsy interpretation for the comparison of outcome data across centers.

In this series of work, the ISN/RPS classification was used for all of our samples. However, we did not compare and analyze the subcategories within LN (for example, segmental versus global lesions). The major reason is that our sample size is small and further subgroups analysis would not have sufficient statistical power. On the other hand, there remains some confusion about the degree of active and chronic lesions that is needed for the designation of 'A' or 'C' sub-category. According to the definitions proposed, a single glomerular lesion with any feature of activity is enough to assign 'A' for active while a single segmentally or globally sclerotic glomerulus is 'C'. It is not uncommon to have these lesions coexist and it becomes difficult to distinguish 'A' and 'C' in reality.

9.1.2 Assessment of SLE disease activity

The optimal method to quantify SLE disease activity is more controversial. Standardised and objective assessment of disease activity is important, as this allows comparison of results between different centers and is necessary for multicentre studies. However, there are reasons that make it challenging to assess SLE disease activity: (1) the multisystem nature of SLE; and (2) involvement of one system could lead to many manifestations. In fact, the disease may present in variable combinations of organ system manifestations that may vary between patients and within the same patient over time. In addition, there is no reliable laboratory measurement or biological marker that uniformly reflects disease activity.

In this series of work, we used the systemic lupus erythematosus disease activity index (SLEDAI), which was developed in 1985 from the consensus of 15 lupus experts in Toronto [184]. SLEDAI has 24 items, of which 16 are clinical, and a manifestation is recorded if it has been present over the past 10 days, regardless of the severity or whether it has improved or deteriorated. Weighting (ranging from 1 to 8) is used for each of the items and all the individual item scores are summated to provide a global score, with a possible maximum score of 105. Nonetheless, a score of 8 to 12 usually

indicates active disease already. We use SLEDAI because it is a single parameter measurement that allows quantitative statistical analysis. However, it should be noted that SLEDAI only focus on new or recurrent manifestations, while ongoing disease activity is usually overlooked. On the other hand, it is often difficult to differentiate manifestations of disease activity from chronic damage or other concomitant illnesses such as infection. The scoring is further complicated in situations where both disease activity and chronic damage are occurring simultaneously.

We also quantify the histological disease activity and chronic damage by the activity index (AI) and chronicity index (CI), which provide a semiquantitative assessment of the acute and chronic renal injury, respectively (Table 9-1) [178,275]. Markers of AI include intracapillary proliferation, epithelial crescents, glomerular polymorph infilltration, wire loop lesions, intracapillary thrombi, fibrinoid necrosis and/or karyorrhexis, hematoxylin bodies, vasculitis and diffuse interstitial inflammation. CI include glomerular obsolescence, segmental glomerular hyalinosis, interstitial fibrosis, tubular atrophy, and arteriolar hyalinosis. Reproducibility of the activity and chronicity indices has been considered excellent [276], although this is not agreed by all authorities [277]. The prognostic relevance of AI is also controversial (after controlling for the histological class of nephritis), but, to the best of our knowledge, there is no other

readily available instrument for the quantification of histological lupus disease activity in renal biopsy.

After this series of work, we perceive there is a great need for a reliable method to distinguish whether (and by how much) a manifestation is due to disease activity, chronic damage, or concomitant illness – preferably without the need of multiple biopsies. Further research on novel biological marker may be fruitful. Appropriate clinical training is also essential to ensure that physicians are competent in using clinical disease activity indices.

Table 9-1 Activity and chronicity indexes

Activity index (lesions are scored 0 - 3. with maximum score 24 points)
▪ Hypercullularity: endocapillary proliferation compromising glomerular capillary loops ▪ Leukocyte exudation: polymorphonuclear leukocytes in glomeruli ▪ Karyorrhex /fibrinoid necrosis (weighted × 2): necrotizing changes in glomeruli ▪ Cellular crescents (weighted × 2): layers of proliferating epithelial cells and monocytes lining Bowman's capsule ▪ Hyaline deposits: eosinophilic and PAS-positive materials lining (wire loops) or filling (hyaline thrombi) capillary loops ▪ Interstitial inflammation: infiltration of leukocytes (predominantly mononuclear cells) among tubules
Chronicity index (lesions are scored 0 - 3. with maximum score 12 points)
▪ Glomerular sclerosis: collapse and fibrosis of capillary tufts ▪ Fibrous crescents: layers of fibrous tissue lining Bowman's capsule ▪ Tubular atrophy: thickening of tubular basement membranes, tubular epithelial degeneration, with separation of residual tubules ▪ Interstitial fibrosis: deposition of collagenous connective tissue among tubules

9.1.3 Renal biopsy

Kidney biopsy is an accurate method to determine the histology and activity of lupus nephritis. Renal biopsy is necessary in patients with lupus and evidence of renal disease because histological class and activity cannot always be predicted from the clinical presentation. In patients with suspected lupus nephritis, the renal biopsy may be used to: (1) confirm the diagnosis, (2) evaluate disease activity, (3) determine prognosis, and (4) determine appropriate therapy. In our center, it is performed in almost all new SLE patients who have clinical or laboratory evidence of renal involvement (for example, abnormal proteinuria, active urine sediment, elevated serum creatinine, or decreased GFR) to determine the histologic class of lupus nephritis as well as to guide the therapy.

Since renal biopsy provides information regarding acute and chronic renal injury at a single time point, transformation of renal lesions from one class to another can occur, both spontaneously and as a result of treatment [259,278,279]. In other center, the indications for a repeat biopsy include the emergence of active sediment, a new elevation in serum creatinine, suspicion of possible renal disease unrelated to lupus, or worsening of proteinuria. On the whole, our practices conform to the usual recommendation [263,280-282]. However, renal biopsy is an invasive procedure, with

the risk of gross hematuria, need for blood transfusion, and even nephrectomy [283]. As a result, it is not suitable for serial monitoring. For that reason, we do not generally perform repeat biopsy after treatment and clinical remission. In this series of work, we do not have a group of "quiescent" kidney tissue after treatment of nephritis for comparison.

9.1.4 Laser-assisted microdissection

Laser-assisted microdissection (LMD) has been developed to procure precisely the cells of interest from a tissue specimen in a rapid and practical manner. Together with the RT-QPCR techniques, it is now feasible to study gene expression and protein level in defined cell populations from complex normal or diseased tissues.

LMD has many advantages: precisely isolating the cells of interest, avoidance of contamination, non-destructive transferring process, efficiency of the procedure, and reliable nucleic acid recovery for downstream molecular applications [243]. On the other hand, LMD is very expensive (microscope, laser, computer hardware and software, membranecoated glass slides, etc). More importantly, the absence of a cover slid inevitably results in a non-optimal microscopic visualisation. Even if the LMD systems

are rapid, it remains time-consuming and labour-intensive to obtain a large number of samples. The complicated process from sample collection to the final quantitative results implies that many technical variables may potentially affect the result. These include tissue handling (i.e. fixation, storage, and staining), consumables (e.g. choice of slide), staining reagents (conventional hematoxylin and eosin staining versus fluorescence), and extraction methods. Each of these requires a careful decision in order to facilitate the study of gene expression study from the small amount of material obtained [284].

9.2 Result

9.2.1 Gene expression of TWEAK / Fn14 and IP-10 / CXCR3

In this part of study, we found an increase in the expression of TWEAK and Fn14 in both glomerulus and tubulointerstitium of lupus nephritis. In contrast, CXCR3 expression was down-regulated.

Through activation of the Fn14 receptor, TWEAK regulates cell proliferation, cell death and inflammation. Available evidence indicates that TWEAK might be implicated in the pathogenesis of SLE, and could be a target for therapeutic intervention in renal and vascular injury of lupus patients [19]. Our findings agree with the report of Schwartz et al, which showed that urinary levels of TWEAK in LN patients are much higher than those without LN, and urinary TWEAK level correlated with the degree of renal inflammation [202]. Notably, the same group subsequently showed that serum TWEAK levels did not correlate with the presence lupus nephritis or the degree of nephritis activity [203]. Our result could be regarded as complementary to that of Schwartz et al, and the up-regulation of TWEAK within kidney tissue is likely the origin of urinary TWEAK.

We found that glomerular TWEAK expression, but not other target genes tested in this part of the work, was significantly higher in pure membranous than proliferative nephritis. Our result seems to indicate that glomerular TWEAK may contribute to the pathogenesis of membranous nephritis. In contrast, previous studies showed that TWEAK/Fn14 axis plays a role in tissue inflammation, repair and regeneration in SLE [200]. In a study of mouse SLE model, the degree of renal inflammation and proteinuria reduced with the removal of Fn14 or treatment with an anti-TWEAK antibody [201]. However, inflammatory changes are generally more prominent in proliferative lupus nephritis than pure membranous ones. On the first glance, our findings may appear to contradict with previous reports. However, it seems possible that in pure membranous lupus nephritis, TWEAK is up-regulated in renal parenchymal cells, while in proliferative lupus nephritis, there is a relatively specific increase in TWEAK expression in the infiltrating inflammatory cells.

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Although some authors reported that serum or plasma levels of IP-10 are increased in SLE patients and the level strongly correlated with systemic disease activity [208,213,214], and a previous study found that IP-10 mRNA from urinary sediment helped to distinguish diffuse proliferative lupus nephritis from other histological classes

[215], we did not find any difference in intra-renal IP-10 expression between LN patients and normal control. The reason for this discrepancy remains unclear.

We found that intra-renal CXCR3 expression was significantly lower in LN patients than controls. More importantly, intra-renal CXCR3 expression inversely correlates with the degree of proteinuria and renal function (see Figure 4-3), suggesting that the alteration is functionally relevant. Our findings seem somewhat different from previous studies, which showed that in proliferative lupus nephritis, CXCR3 was mainly expressed by T cells infiltrating the tubulointerstitial compartment and correlated with renal function, proteinuria, and the percentage of globally sclerosed glomeruli [285]. However, the apparent discrepancy could be explained because previous report also found very low intra-glomerular CXCR3 expression in lupus nephritis [285], and a substantial proportion of our patients did not have proliferative disease.

There are a number of inadequacies in this part of the work. First, the target genes that we studied are limited, and a comprehensive elucidation of all pathogenic pathway was not possible. Moreover, the data from this study are largely descriptive in nature. Although it is well described that TWEAK could induce human kidney cells to express multiple inflammatory mediators, including IP-10 [216], we did not test the effect of

TWEAK on the up-regulation of other cytokines and chemokines, or its relation with apoptosis in the renal biopsy specimens because of the limitations in the original research design. Further studies are needed to elucidate the mechanism of TWEAK/Fn14 and IP-10/CXCR3 axis in the pathogenesis of lupus nephritis.

In this work, we did not determine the cellular origin for the altered gene expression. Since there is generally very little inflammatory cell infiltrate in class V nephritis, the altered gene expression observed likely came from resident kidney cells. On the other hand, the change in gene expression observed in proliferative nephritis could originate from both renal parenchymal as well as inflammatory cells. The exact origin would require further study to clarify.

Finally, the sample size of our study was small, so that subgroup comparison of gene expression between different histological classes of lupus nephritis may not be reliable. It was probably that type I error may be present. However, we would study the gene expression in different biological samples (for example, urine) in the future to confirm our findings. The purpose of our study was hypothesis generation and to explore the role of individual cytokine in the pathogenesis of lupus nephritis. Moreover, the sample size of many individual groups were small. As a result, we did not perform multivariate

analysis. As discussed previously, we could not examine renal tissue of lupus patients without kidney problem, or the longitudinal change of intra-renal gene expression profile or response to immunosuppressive therapy by serial renal biopsy.

Nonetheless, taken together, from this part of the work we found an increase in intra-renal expression of TWEAK and Fn14 mRNA and decrease in CXCR3 mRNA in LN patients as compared to controls. Within the LN group, glomerular TWEAK expression of was higher in pure membranous than proliferative nephritis. More importantly, intra-renal expression of CXCR3 correlates with the degree of proteinuria and renal function. Our findings suggest that TWEAK/Fn14 and IP-10/CXCR3 axis may contribute to the pathogenesis of lupus nephritis.

9.2.2 Gene expression of NGAL and TLR9

In this part of the work, we found that tubulointerstitial NGAL expression was increased in LN. Our result is similar to that of Brunner et al, which reported significantly greater urinary NGAL levels in individuals with active renal disease as compared to others [286]. Based on Figure 5-2, our findings also suggest that NGAL expression was nearly 6-8 times higher in tubulointerstitium than in glomerulus, which is consistent with the

prediction from the biology of NGAL.

We found that tubulointerstitial NGAL expression significantly correlated with proteinuria, GFR and histological chronicity index. The result agrees with the report of Bolignano et al, which also showed that urinary NGAL concentrations correlated with renal function and proteinuria [221]. In addition, we found that the tubulointerstitial expression of NGAL is related to treatment response. Our results is consistent with the report of Hinze et al [287], which also showed that urinary and plasma NGAL levels are predictors of the course of lupus nephritis in patients with childhood-onset SLE. Taken together, the data suggest that the alteration of tubulointerstitial NGAL in LN is functionally relevant.

The site of renal TLR9 expression has been a matter of debate. In a recent study, Machida et al [225] reported that injured glomerular podocytes in active LN expressed TLR9, which disappeared during disease remission, and TLR9 did not express in a normal control kidney. Furthermore, TLR9 expression correlated with the degree of proteinuria and anti-dsDNA antibody titre. On the other hand, Benigni et al. [226] showed a robust TLR9 expression in the proximal tubular epithelial cells in an animal lupus model as well as patients with LN, and TLR9 level correlated with proteinuria and

tubular damage. Our result showed that TLR9 expression increased in both glomerulus and tubulointerstitium of LN, which is consistent with the report of Papadimitraki et al [227].

The role of TLR9 in LN is relatively well studied. The up-regulation of TLR9 in the renal parenchyma of LN may be a response to inflammatory cytokines or the nucleosomes present within the lesion [227]. In return, TLR9 induces macrophages and B-cells to produce cytokines and chemokines, thus aggravates inflammation and tissue damage [228-230]. Studies showed that internalization of SLE immune complexes into subcellular lysosomes containing TLR9 induced a signaling cascade that led to the activation of dendritic cells and production of cytokines and chemokines [231], suggesting that TLR9 may have a role in the onset and progression of LN. In the present study, we found a modest, although statistically significant, correlation between tubulointerstitial expression of NGAL and TLR9. Our result indicates that tubulointerstitial NGAL expression is governed by factors other than TLR9.

Several limitations in this part of the work need to be addressed. First, the sample size of our study was small, so that subgroup comparison of gene expression between different histological classes of lupus nephritis may not be reliable. Because of the

limitation in the original design, we did not quantify intra-renal TLR9 or NGAL at protein level (e.g. by immunohistochemistry). As emphasized previously, we could not examine renal tissue of lupus patients without kidney problem as control. Furthermore, we do not examine the longitudinal change of intra-renal gene expression or the response to immunosuppressive therapy by serial renal biopsy. Further studies in these areas are required.

In summary, from this part of the work, we found an increase in intra-renal mRNA expression of NGAL and TLR9 in patients with LN. Although tubulointerstitial expression of NGAL does not correlate with systemic disease activity, it correlates with proteinuria, renal function, and therapeutic response. The roles of NGAL in the pathogensis in LN, as well as its application as biomarker for lupus nephritis, require further study.

9.2.3 Change in histological pattern of lupus nephritis with time

In this retrospective study, we reviewed the renal biopsy of 156 LN patients. In short, we found a high frequency of transformation of histological pattern upon repeat biopsy for disease flare. The incidence of transformation that we observed is similar to previous

reports [259,278,279]. In fact, histological class switch is thought to be a characteristic feature of lupus nephritis [265]. Studies that assessed biopsy specimens according to the original WHO classification showed a class switch in 26 to 50% of repeated renal biopsies [263]. A recent study that used the revised ISN/RPS classification showed that the incidence of histological class switch was 49% [264]. However, both of these studies had relatively small sample size, so that subgroup analysis for specific patterns of change was not possible.

In our present study, the most frequent transitions occurred between proliferative and mix nephritis, which accounted for around 20% of the cases. The result seems different from several previous studies, which all reported a predominance of transitions between classes III and IV disease, with or without an additional class V involvement, and the overall incidence varies from 36 to 54% [263,264,288]. Some other studies also showed a high frequency of transitions from class III or IV to class II disease [261,262,278,279,289], which was not observed in our study. It is, however, important to note that under the current ISN/RPS definition, class III and IV disease are both proliferative nephritis, differ only by the percentage of glomerular involvement. As a result, in our present analysis, we treat class III and IV nephritis as a single entity and did not consider any change from class III to IV or vice versa. More importantly, we

have a liberal indication of performing renal biopsy for lupus disease flare, and, as a result, tends to pick up the renal involvement early. For that reason, class III nephritis may be more common than class IV in our cohort.

Although we identify a few clinical predictors for a particular pattern of change in the repeat renal biopsy, our result showed that by and large the pathological class of LN upon repeat biopsy could not be predicted by baseline clinical, biochemical, or pathological parameters. Our observation highlights the importance of repeat renal biopsy in SLE patients with disease flare and renal involvement.

Several limitations of this part of the work need to be addressed. First, the sample size of our study was limited for many subgroups, and the follow-up of some patients was short, so that subgroup comparison between different histological classes of lupus nephritis may not be reliable. Since the treatment regimen was heterogeneous over the years, we could not comment specially on the role of therapy in the longitudinal change of histological pattern. Further studies in this area were required.

In summary, this part of our work suggests that a change in the histological class of lupus biopsy is common in SLE patients with lupus flare, and the histological pattern of

lupus nephritis during disease flare could not be predicted by baseline clinical, biochemical, or pathological parameters. Our result indicates that when there is lupus flare with renal involvement, repeat renal biopsy is often necessary to guide the treatment.

9.2.4 Intra-renal Gene Expression – Study on Paired Renal Biopsy

In this study, we identified some target genes that appeared to have a consistent change in expression in parallel with the alteration in histological pattern of lupus nephritis. Specifically, glomerular Fn14 and tubulo-interstitial TWEAK expression tend to decrease when change from proliferative or mixed nephritis to membranous nephropathy, while an opposite pattern of change was observed in tubulo-interstitial TWEAK expression.

The result of our present study is consistent with our previous work (see Chapter 4), which showed an increase in intra-renal expression of TWEAK and Fn14 in lupus nephritis. Taken together, the TWEAK/Fn14 pathway may play an important role in the determination of specific histological pattern of lupus nephritis, with high TWEAK/Fn14 expression is necessary for proliferative nephritis. Our result is also

consistent with previous animal experiments, which showed that lupus mice treated with anti-TWEAK neutralizing antibody had reduce proliferative glomerular lesions [201].

In this study, we found no consistent pattern of change in glomerular or tubulo-interstitial CXCR3 expression in relation to the histological pattern of lupus nephritis. The result may seem contradictory to our previous work (see Chapter 4), which showed a reduced glomerular or tubulo-interstitial CXCR3 expression in lupus nephritis. A more careful examination of the data showed that intra-renal expression of CXCR3 correlates with proteinuria and renal function [290] rather than the histological pattern of nephritis. Our findings seem to suggest CXCR3 pathway is important in the determination of the clinical severity rather than histological pattern of lupus nephritis.

The histological class switch is thought to be a characteristic feature of lupus nephritis [265]. There is a high frequency of transformation of histological pattern upon repeat biopsy [259,278,279]. However, the pathogenesis of the transformation remains elusive. It is important to note that our present study only takes the advantage of studying SLE patients with repeated renal biopsy so that gene expression profile between different histological types of lupus nephritis could be compared by an

"internal control". Other studies are needed to determine the factors that govern the longitudinal change of histological pattern, as well as the effect of time *per se* (i.e. the effect of the duration of disease) on lupus nephritis.

Several limitations of the current study need to be addressed. First, the sample size of many groups was small, so that there is not sufficient statistical power to compare all histological classes of lupus nephritis. In fact, a much large sample size would be necessary in order to allow sophisticated statistics for the dissection of the effects of histological pattern and the effect of time (i.e. duration of disease) on the gene expression profile. Second, the cytokines we selected are largely based on our previous studies and are limited; many other possibly involved pathways are not examined. Unfortunately, since the amount of RNA available from tissue microdissection is limited, it is difficult to perform microarray or other hypothesis-free technology to explore the gene expression profile of the specimen.

In summary, the present study suggests that tubulo-interstitial IP-10 expression consistently decreased when the histology changed to membranous nephritis in a second biopsy. Besides, tubulo-interstitial TLR9 expression trends to increase when change to membranous nephritis while decrease when change from membranous nephritis. On the

contrary, tubulo-interstitial TWEAK expression trends to decreased when change to membranous nephritis while increased when change from membranous nephritis. Our findings suggest that TWEAK, IP-10, and TLR9 may contribute to the pathogenesis of specific histological pattern of lupus nephritis, while CXCR3 is more important in the determination of the severity of disease.

9.2.5 Intra-renal micro-RNA expression

In this part of the work, we investigated the intrarenal expression of miR-638, miR-663, miR-198, miR-155 and miR-146a in patients with LN. We found that as compared to normal controls, LN patients had lower glomerular expression of miR-638 but higher tubulointerstitial expression of this target. Both of glomerular and tubulointerstitial expression of miR-198 are higher in LN patients than controls. For miR-146a, LN patients have a higher expression in glomerulus but not in tubulointerstitium. More importantly, there are significant correlations between miRNA species and activity index, SLEDAI score, proteinuria and estimated GFR, suggesting a dose-response type of relationship between intrarenal miRNA expression and the severity of LN.

MiRNAs have been suggested to play important roles in a variety of kidney diseases

including polycystic kidney disease, acute kidney transplant rejection, IgA nephropathy, hypertensive nephrosclerosis, and, more recently, lupus nephritis [240,241,291-294]. Dysregulation of specific miRNAs had been suggested to be related to the pathogenesis of SLE [30,240,242]. Several miRNA species may induce renal fibrosis through glomerular and tubular injury [295-297]. In line with these previous studies, we observed increased glomerular expression of miR-198 and miR-146a whereas lower glomerular expression of miR-638. In tubulointerstitium, both of miR-638 and miR-198 are higher in LN patients than controls. In addition, their expression correlated with disease severity, suggesting that these miRNA species may play a role in the pathogenesis of renal injury.

It should be emphasized that the causal relationship between studied miRNAs and the pathogenesis of LN remains to be elucidated, but there are emerging evidence that he biological effects of several miRNA species are mediated via the TWEAK/Fn14 axis. For example, the expression of miR-146a in C2C12 myotubes significantly increased in response to TWEAK treatment [270]. In our study, the glomerular expression of miR-146a was also found to correlate with that of Fn14, i.e. the receptor of TWEAK. Some authors suggest that TWEAK affects the expression of miR-146a, which is involved in renal inflammation, fibrosis, extracellular matrix remodeling, and

proteolytic degradation of collagen fibres through the down-regulation of Numb, TNF receptor-associated factor 6 (TRAF6), and activation of NF-kB [270,298]. MiR-146a may also negatively regulate the gamma interferon (IFN-γ) pathway, indirectly contributing to the "interferon signature" of SLE [242]. Taken together, our result is consistent with the hypothesis that miRNA plays a functionally important role in the pathogenesis of LN.

One reason for the difficulty in defining the relationships between miRNA expression and LN is that there are multiple mRNA targets for one particular miRNA, and the effect on translation could be variable [299,300]. In addition, it seems possible that miRNA may behave differently in glomerulus and tubulointerstitium, because the same miRNA show tissue-specific changes, and the same messenger RNA are often regulated by different miRNA in glomerulus and tubulointerstitium. The correlations between the expression of miRNAs and the clinical data of patients with LN observed in the present study could be regarded as the integrative effect of these miRNA species on their mRNA targets that are involved in the pathogenesis of LN.

There are a number of inadequacies in this part of the work. First, the choice of miRNA target is difficult. Our group had reported the role of miR-146a and miR-155 in LN

patients before [269]. We selected miR-638, miR-663, miR-198, miR-146a and miR-155 for this study because they were reported to be involved in the pathogenesis of SLE [30,240,242].

Furthermore, the present study is only cross-sectional, and it is possible that miRNA expression levels may alter with disease progression and therapy. Future studies are needed to investigate the serial change in urinary expression of miRNAs as the disease progresses or following various therapy strategies.

In summary, we found in this part of work that intra-renal expression of miR-638, miR-663, miR-198, miR-155 and miR-146a are differentially expressed between patients with lupus nephritis and normal controls. Furthermore, the degree of change correlated with disease severity. The results suggested that these miRNA species may play important roles in the pathogenesis of LN.

9.2.6 Impact of findings

In this serial work, we study the gene expression of TWEAK/Fn14, IP-10/CXCR3, NGAL, TLR9 and micro-RNAs in glomerulus and tubulointerstitium of lupus nephritis.

We found they probably contribute to the pathogenesis and progression of lupus nephritis. Most importantly, they have a consistent change in expression in parallel with the alteration in histological pattern of lupus nephritis. Our data seem to suggest that the TWEAK/Fn14 axis and IP10 are important in the determination of specific histological class of lupus nephritis, CXCR3 is involved in governing the severity of disease, while NGAL is related to therapeutic response irrespective to histological class. In addition, a panel of micro-RNA seems to be involved in the pathogenesis of lupus nephritis, possibly via their effect on the transcription of TWEAK/Fn14 and IP10/CXCR3 axis.

All of these findings expand our understanding of pathogenesis of lupus nephritis. These target genes have the potential of being further developed as biomarkers of lupus nephritis. They may help physicians in monitor of disease activity, assess severity of disease flare, adjust treatment, and predict prognosis of lupus nephritis.

9.3 Further direction of research

In the series of work, measurement of gene expressions in renal biopsy was used as the tool to explore the immunological aberration in lupus nephritis. However, biopsy is still an invasive procedure with possible risk. It also has the technical difficulty for serial monitoring. In other words, it is good for diagnosis, but not particularly ideal or practical for monitoring of disease progression. In contrast, urine collection is convenient and non-invasive. Our group had shown previously that cytokine gene expression in urinary sediment is a potential tool for the study of lupus nephritis. Further studies should be conducted to see the gene expression of TWEAK, Fn14, IP-10, CXCR3, TLR9 and NGAL in urinary sediment of SLE patients, and explore whether their expression in urinary sediment correlate with those in kidney tissue.

Since the target genes we selected were largely based on literature search and were limited, many other possibly involved pathways are not examined. In this respect, microarray analysis would be ideal in allowing simultaneous quantification of a large numbers of genes and investigation of the role of all inflammatory mediators in a

hypothesis-free setting. Unfortunately, the amount of RNA available from tissue microdissection is too small for traditional microarray analysis, which represents a bottleneck of further study along this line. Similarly, the small amount of RNA available limits the use of immunohistochemical study to confirm our findings at protein level.

Multiple previous studies showed that treatment of lupus nephritis affect gene expression levels in peripheral blood mononuclear cells. It would be interesting to examine the longitudinal change of intra-renal gene expression or the response to immunosuppressive therapy by serial renal biopsy. In addition, it would be informative to quantify intra-renal cytokines at protein level (for example, by immunohistochemistry) and correlate with the level of mRNA expression as determined by RT-QPCR. Other *in vitro* study (for example, cell culture) would also be necessary to delineate how specific miRNA species affect the expression of inflammatory genes.

9.4 Conclusion

In conclusion, the findings from this series of studies suggest that the TWEAK/Fn14 and IP10/CXCR3 axis, as well as TLR9 and NGAL may play important roles in the pathogenesis of lupus nephritis. Our data seem to suggest that the TWEAK/Fn14 axis and IP10 are important in the determination of specific histological class of lupus nephritis, CXCR3 is involved in governing the severity of disease, while NGAL is related to therapeutic response irrespective to histological class. In addition, a panel of miRNA seems to be involved in the pathogenesis of lupus nephritis, possibly via their effect on the transcription of TWEAK/Fn14 and IP10/CXCR3 axis.

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PUBLICATIONS RELATED TO THIS WORK

Publications

1. Lu JX, Kwan BC, Szeto CC: Update on the role of T cell subset in the pathogenesis of systemic lupus erythematosus. J Chin Clin Med 2009; 4: 400-409.
2. Lu JX, Szeto CC: Gene expression using the PALM system. Methods Mol Biol 2011; 755: 47-56.
3. Lu JX, Kwan BC, Lai FM, Choi PC, Tam LS, Li EK, Chow KM, Wang G, Li PK, Szeto CC: Gene expression of tweak/fn14 and ip-10/cxcr3 in glomerulus and tubulointerstitium of patients with lupus nephritis. Nephrology (Carlton) 2011;16:426-432.
4. Lu JX, Kwan BC, Lai FM, Choi PC, Tam LS, Li EK, Chow KM, Wang G, Li PK, Szeto CC: The gene expression of ngal and tlr9 in glomerulus and tubulo-interstitium of patients with lupus nephritis. Advances in Bioscience and Biotechnology 2011;2:33-39.
5. Lu JX, Tam LS, Lai FM, Kwan BC, Choi PC, Li EK, Chow KM, Li PK, Szeto CC. Repeat renal biopsy in lupus nephritis: a change in histological pattern is common. Am J Nephrol 2011; 34: 220-225.

Conference abstracts

1. Lu JX, Kwan BC, Lai FM, Choi PC, Tam LS, Li EK, Chow KM, Wang G, Li PK, Szeto CC: Gene expression of TWEAK/Fn14 and IP-10/CXCR3 in glomerulus and tubulointerstitium of patients with lupus nephritis. World Congress of Nephrology on April 8-12, 2011 in Vancouver, Canada.
2. Lu JX, Kwan BC, Lai FM, Choi PC, Tam LS, Li EK, Chow KM, Wang G, Li PK, Szeto CC: The gene expression of NGAL and TLR9 in glomerulus and tubulo-interstitium of patients with lupus nephritis. World Congress of Nephrology on April, 2011 in Vancouver, Canada.
3. Lu JX, Tam LS, Lai FM, Kwan BC, Choi PC, Li EK, Chow KM, Li PK, Szeto CC. Repeat renal biopsy in lupus nephritis: a change in histological pattern is common. 43rd Annual Meeting of American Society of Nephrology on November, 2010 in Denver, CO.

APPENDICES

Appendix I: Scoring sheet for lupus disease activity and chronic damage.

DATA COLLECTION SHEET

Date : _____
Name : _____
Sex : _____
Age : _____
HKID : _____

SLEDAI

Circle the SLEDAI Score if descriptor present at the time of the visit or in the preceding 10 days.

Descriptor	SLEDAI Score
Seizure	8
Psychosis	8
Organic brain syndrome	8
Visual disturbance	8
Cranial nerve disorder	8
Lupus headache	8
Cerebrovascular accident(s)	8
Vasculitis	8
Arthritis	4
Myositis	4
New rash	2
Alopecia	2
Mucosal ulcers	2
Pleurisy	2
Pericarditis	2
Low complement	2
Increased DNA binding	2
Fever	1
Thrombocytopenia	1
Leukopenia	1
Proteinuria	4
Urinary casts	4
Hematuria	4
Pyuria	4
Total SELDAI Score :	

SLICC / ACR DAMAGE INDEX FOR SLE

Damage (non-reversible change, not related to active inflammation) occuring since onset of lupus and present for at least 6 months.
Repeat episodes mean at least 6 months apart to score 2. The same lesion cannot be scored twice.

Descriptor	Score
Ocular	
Any cataract ever	1
Retinal change OR Optic atrophy	1
Neuropsychiatric	
Cognitive impairment OR Major psychosis	1
Seizures requiring therapy for 6 months	1
Cerebral vascular accident ever (score 2 if >1)	
OR resection not for malignancy	1 / 2
Cranial or peripheral neuropathy	1
Transverse myelitis	1
Renal	
Estimated or measured GFR<50%	1
Proteinuria 24h, >=3.5g	1
OR Endstage renal disease	3
Pulmonary	
Pulmonary hypertension (right ventricular prominence, or loud P2)	1
Pulmonary fibrosis (physical and X-ray)	1
Shrinking lung (X-ray)	1
Pleural fibrosis (X-ray)	1
Pulmonary infraction (X-ray)	
OR resection not for malignancy	1
Cardiovascular	
Angina OR Coronary artery bypass	1
Myocardial infraction ever (score 2 if >1)	1 / 2
Cardiomyopathy (ventricular dysfunction)	1
Valvular disease	
(diastolic murmur, or a systolic murmur > 3/6)	1
Pericarditis x 6 months, OR Pericardiectomy	1
Total score :	

Descriptor	Score
Peripheral vascular	
Claudication x 6 months	1
Minor tissue loss (pulp space)	1
Significant tissue loss ever (eg, loss of digit/limb)	
(score 2 if > 1 site)	1 / 2
Venous thrombosis with swelling, ulceration,	
OR Venous stasis	1
Gastrointestinal	
Infraction of resection of bowel below duodenum,	
spleen, liver, or gall bladder ever, for whatever	
cause (score 2 if > 1 site)	1 / 2
Mesenteric insufficiency	1
Chronic peritonitis	1
Stricture / Upper gastrointestinal tract surgery ever	1
Pancreatitis	1
Musculoskeletal	
Muscle atrophy or weakness	1
Deforming or erosive arthritis	1
Osteoporosis with fracture or vertebral collapse	1
Avascular necrosis (score 2 if >1)	1 / 2
Osteomyelitis	1
Ruptured tendon	1
Skin	
Scarring chronic alopecia	1
Extensive scarring or panniculum other than	
scalp anf pupl space	1
Skin ulceration (not due to thrombosis) for >6 months	1
Premature gonadal failure	1
Diabetes regardless of treatment)	1
Malignancy (score 2 if >1 site)	1 / 2

Appendix II : World Health Organization (WHO) morphologic classification of lupus nephritis (modified in 1982).

I. Normal glomeruli

(a) No pathology

(b) Normal by light microscopy, but deposits by electron or immunofluorescence microscopy

II. Pure mesangial alternations (Mesangiopathy)

(a) Mesangial widening and/or mild hypercellularity (+)

(b) Moderate hypercellularity (++)

III. Focal segmental glomerulonephritis

(associated with mild or moderate mesangial alternations)

(a) 'Active' necrotizing lesions

(b) 'Active' and sclerosing lesions

(c) Sclerosing lesions

IV. Diffuse glomerulonephritis

(severe mesangial, endocapillary or mesangio-capillary proliferation and/or extensive subendothelial deposits)

(a) Without segmental lesions

(b) With 'active' necrotizing lesions

(c) With 'active' and sclerosing lesions

(d) With sclerosing lesions

V. Diffuse membranous glomerulonephritis

(a) Pure membranous glomerulonephritis

(b) Associated with lesions of Category II (a or b)

(c) Associated with lesions of Category III (a-c)

(d) Associated with lesions of Category IV (a-d)

VI. Advanced glomerular sclerosis

Appendix III : International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of lupus nephritis.

Class I Minimal mesangial lupus nephritis

Normal glomeruli by light microscopy, but mesangial immune deposits by immunofluorescence

Class II Mesangial proliferative lupus nephritis

Purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposits

May be a few isolated subepithelial or subendothelial deposits visible by immunofluorescence or electron microscopy, but not by light microscopy

Class III Focal lupus nephritis[a]

Active or inactive focal, segmental or global endo- or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations

Class III (A)	Active lesions: focal proliferative lupus nephritis
Class III (A/C)	Active and chronic lesions: focal proliferative and sclerosing lupus nephritis
Class III (C)	Chronic inactive lesions with glomerular scars: focal sclerosing lupus nephritis

Class IV Diffuse lupus nephritis[b]

Active or inactive diffuse, segmental or global endo- or extracapillary glomerulonephritis involving 50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental(IV-S) lupus nephritis when 50% of the involved glomeruli have segmental lesions, and diffuse global (IV-G) lupus nephritis when 50% of the involved glomeruli have global lesions. Segmental is defined as a glomerular lesion that involves less than half of the glomerular tuft. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation

Class IV-S (A)	Active lesions: diffuse segmental proliferative lupus nephritis
Class IV-G (A)	Active lesions: diffuse global proliferative lupus nephritis
Class IV-S (A/C)	Active and chronic lesions: diffuse segmental proliferative and sclerosing lupus nephritis
Class IV-G (A/C)	Active and chronic lesions: diffuse global proliferative and sclerosing lupus nephritis
Class IV-S (C)	Chronic inactive lesions with scars: diffuse segmental sclerosing lupus nephritis
Class IV-G (C)	Chronic inactive lesions with scars: diffuse global sclerosing lupus nephritis

Class V Membranous lupus nephritis

Global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations

Class V lupus nephritis may occur in combination with class III or IV in which case both will be diagnosed

Class V lupus nephritis show advanced sclerosis

Class VI Advanced sclerosis lupus nephritis

90% of glomeruli globally sclerosed without residual activity

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Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions.

[a] Indicate the proportion of glomeruli with active and with sclerotic lesions.

[b] Indicate the proportion of glomeruli with fibrinoid necrosis and/or cellular crescents.