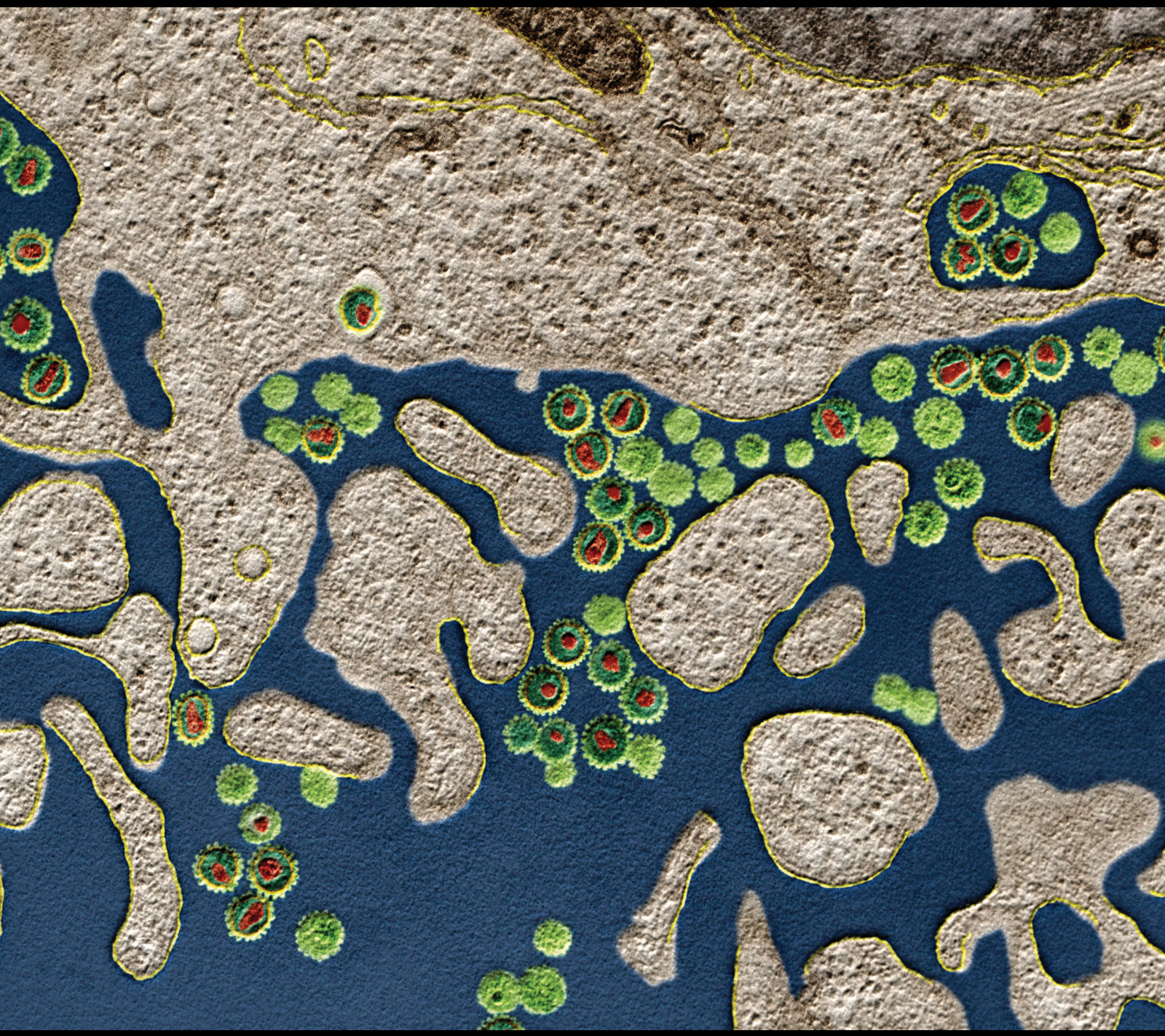
Journal of Immunology Research

Immunoregulation of Inflammation in Chronic Kidney Disease

Guest Editors: Osamu Takase, Kazuya Iwabuchi, and Richard J. Quigg



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Editorial Board

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Editorial

Immunoregulation of Inflammation in Chronic Kidney Disease

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Chronic kidney disease (CKD) is a commonly encountered condition in clinical practice. Up to 15% of the adult population worldwide is affected by CKD. Patients with CKD are at a high risk of developing hypertension and other cardiovascular diseases, with associated morbidity and mortality. Stage V is end-stage renal disease (ESRD), for which patients require renal replacement therapy with either dialysis or renal transplantation. A patient with ESRD on dialysis has a 50% chance of surviving three years. Overall, CKD is a disease with a tenfold increased incidence over three decades in which affected patients can lose 70% of their life span.

There are many underlying etiologies of CKD, including diabetes, hypertension, primary and secondary glomerulonephritis/vasculitis, and tubulointerstitial nephritis. Irrespective of initiator, there appears to be an important role for inflammation in CKD. In fact, patients with CKD are frequently treated nonspecifically with immunosuppressive and/or antihypertensive agents. Unfortunately, there have not been new treatment regimens for CKD induced by various underlying causes.

This special issue covers the broad topic of immunoregulation of inflammation in CKD. Four review articles and two research articles discuss the mechanism of inflammation in animal models of lupus nephritis and obstructive uropathy, as well as effects on resident renal cells. It is our hope that these contribute to a better understanding of immunoregulation of inflammation in CKD, which can stimulate development of better therapeutic approaches and provision of optimal care to patients.

In the first paper, "*Mediators of inflammation and their effect on resident renal cells: implications in lupus nephritis*," S. Yung et al. review immunoregulation in lupus nephritis, an important cause of CKD. They concentrate on interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), type I interferons (IFNs) and hyaluronan, as among the most important mediators in this disease. They provide background of the fundamental biology of IL-6, TNF-α, type I IFNs, and hyaluronan, followed by their roles in both experimental and human lupus nephritis.

In the second paper, "*LMW heparin prevents increased kidney expression of proinflammatory mediators in (NZB×NZW)F1 mice*," A. Hedberg et al. have studied effects of low molecular weight (LMW) heparin on lupus nephritis occurring in (NZB×NZW)F1 (B/W) mice. Like in human lupus nephritis, these mice progressively develop CKD. As this occurs, there is increased expression of a diversity of proinflammatory mediators. In their study, they show that LMW heparin specifically lowers CCR2, IL-1β, and TLR7 expression. This may be attributable to the ability of LMW heparin to enhance nucleosomal degradation and/or binding to glomerular sites as components of immune complexes.

In the third paper, "*Interactions between cytokines, congenital anomalies of kidney and urinary tract and chronic kidney disease*," A. C. S. Silva et al. reviewed the relative roles for cytokines and chemokines in the pathophysiology of congenital anomalies of the kidney and urinary tract and how they can affect progression of CKD. They include experimental and clinical evidence to show that urine measurements

of cytokines could prove useful as predictors of urinary tract obstruction and renal scarring.

In the fourth paper, "*MicroRNAs implicated in the immunopathogenesis of lupus nephritis*," C. B. Chafin and C. M. Reilly provide a review that addresses a current and important topic: the role of microRNAs in the pathogenesis of lupus nephritis. The authors collated a large amount of available data regarding the potential role of microRNAs as therapeutic targets and how these might underlie potential treatment strategies for this important disease.

In the fifth paper, "*Inflammatory chemokine expression via toll-like receptor 3 signaling in normal human mesangial cells*," H. Tanaka and T. Imaizumi summarize their experimental results regarding signaling pathways in human mesangial cells activated upon treatment with a synthetic analogue of viral dsRNA. The signaling pathways activated through TLR3 in mesangial cells may be proinflammatory. The relevance includes effects of viral and "pseudoviral" infections on existing CKD, as well as pathogenic mechanisms that may underlie primary glomerulonephritis.

In the sixth paper, "*Contrasting effects of systemic monocyte/macrophage and $CD4^+$ T cell depletion in a reversible ureteral obstruction mouse model of chronic kidney disease*," L. Chaves et al. show that depleting macrophage and $CD4^+$ T cells had distinct effects on manifestations of CKD in a reversible model of unilateral ureteral obstruction (rUUO). Based on these results, the authors concluded that modulation of immune cells during injury and repair altered the development of CKD in the rUUO model. Their rUUO model is unique, and findings in this study provide interesting clues to the mechanisms of CKD progression.

We are certain that the readers of this special issue will find several interesting points of discussion in the published papers. We hope these papers can stimulate further experimentation to dissect the immunoregulation of inflammation in CKD and allow development of new therapeutic strategies for CKD.

Acknowledgments

We would like to thank all authors for contributing their works for making this special issue, as well as the reviewers for evaluating all submitted papers.

Osamu Takase
Kazuya Iwabuchi
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Research Article

Contrasting Effects of Systemic Monocyte/Macrophage and CD4$^+$ T Cell Depletion in a Reversible Ureteral Obstruction Mouse Model of Chronic Kidney Disease

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Using a reversible UUO model (rUUO), we have demonstrated that C57BL/6 mice are susceptible to development of CKD after obstruction-mediated kidney injury while BALB/c mice are resistant. We hypothesized that selective systemic depletion of subpopulations of inflammatory cells during injury or repair might alter the development of CKD. To investigate the impact of modification of T_h-lymphocytes or macrophage responses on development of CKD after rUUO, we used an anti-CD4 antibody (GK1.5) or liposomal clodronate to systemically deplete CD4$^+$ T cells or monocyte/macrophages, respectively, prior to and throughout the rUUO protocol. Flow cytometry and immunohistochemistry confirmed depletion of target cell populations. C57BL/6 mice treated with the GK1.5 antibody to deplete CD4$^+$ T cells had higher BUN levels and delayed recovery from rUUO. Treatment of C57BL/6 mice with liposomal clodronate to deplete monocyte/macrophages led to a relative protection from CKD as assessed by BUN values. Our results demonstrate that modulation of the inflammatory response during injury and repair altered the susceptibility of C57BL/6 mice to development of CKD in our rUUO model.

1. Introduction

Between 10 and 16% of the adult population worldwide is affected by chronic kidney disease (CKD) [1]. From the periods of 1988–1994 to 2005–2010 the prevalence of CKD in the United States rose from 12.3 to 14.0 percent. The largest relative increase, from 25.4 to 40.8 percent, was seen in those with cardiovascular disease [2]. The life expectancy for a 50-year-old adult in the United States is 35.5 years; this decreases by 7.5 years in the presence of CKD [2].

From among a variety of possible rodent models of CKD, the unilateral ureteral obstruction (UUO) model has become widely used to evaluate features of renal injury [3–5]. Advantages of the UUO model include the fact that kidney injury and fibrosis occur over a time course of days to weeks and that the model can be used in mice of any strain. Typically in the UUO model, obstruction is achieved by irreversible ligation of the ureter. Importantly, functional consequences of kidney injury cannot be assessed using irreversible obstruction and findings must be interpreted in the context of ongoing injury from obstruction.

To model CKD in mice we generated a reliable reversible UUO model (rUUO) [6]. This model combines several key advantages for studying development and progression of CKD, including assessment of functional consequences of kidney injury using biomarkers such as blood urea nitrogen (BUN) measurements and the ability to study pathophysiology during kidney injury and during recovery from injury. Our previous work with this model showed a distinct genetic basis for development of CKD and suggested an immunogenetic mechanism. In our model C57BL/6 mice,

which generally produce a T helper cell type 1 immune response, progressed to CKD whereas BALB/c mice, with immune responses skewed to T helper type 2 responses, were resistant. Considering that, we hypothesize that one or more immune cell type may contribute to or be preventative of kidney disease development in our mouse model. To test that, in a separate set of experiments we systemically depleted $CD4^+$ or monocyte/macrophage cells using an anti-CD4 antibody or liposome encapsulated clodronate, respectively, and subjected the mice to rUUO.

2. Materials and Methods

2.1. Murine RUUO Model. We used a murine rUUO model as previously described [6]. For these studies, 6–8 week male C57BL/6 mice had six days of UUO followed by release of obstruction. To do this, a microvascular clip was placed on the right ureter (day −6) and then adjusted distally after 2 days (day −4) and again after 4 days (Day −2). The clip was removed 6 days after the initial UUO. For consistency, the time when the ureteral obstruction was reversed will be termed day 0. Then, on day 7 (i.e., 13 days after UUO), the left kidney was removed. Thus, the animal relied solely upon function of previously obstructed kidney, which was quantified by measurement of blood urea nitrogen (BUN) levels. The use of animals in these studies was approved by the University of Chicago Institutional Animal Care and Use Committee.

2.2. Cell Depletions. $CD4^+$ T cells were depleted in mice with monoclonal rat anti-mouse CD4 antibody GK1.5 (Fitch Monoclonal Antibody Facility, The University of Chicago). Briefly, intraperitoneal injection of 10 μg/g body weight (~200 μg) GK1.5 IgG or vehicle control was performed 1 day prior to and maintained during the rUUO protocol with three subsequent injections every 6 days (i.e., animals were injected on protocol days −7, −1, 5, and 11). $CD4^+$ cellular depletion was confirmed by flow cytometry.

Systemic monocyte/macrophage depletion was performed using clodronate liposomes (Encapsula NanoSciences). Empty liposomes in PBS were used as controls. Clodronate (200 μg) or vehicle (PBS control) liposomes were administrated to mice intravenously starting 6 days prior to UUO and continuing every 3-4 days until 10 days after release of obstruction (i.e., animals were injected on protocol days −12, −8, −5, −1, 3, 6, and 10).

2.3. Histology. Tissue harvest: right kidneys were surgically removed under anesthesia. The kidneys were bisected through a coronal section and processed as follows for routine light microscopic evaluation. Pieces of renal tissue no more than 1–1.5 mm thick were fixed in 4% paraformaldehyde in PBS for 24 h at 4°C. Following fixation, tissue samples were routinely processed and embedded in paraffin wax (TissuePrep II, Fisher Scientific). Five 3 μm tissue sections were cut onto Superfrost Plus slides (Fisher Scientific), dewaxed, and hydrated through a descending series of alcohols.

Immunohistochemical staining was conducted using a modified Vectastain Elite ABC kit (Vector Laboratories). Briefly, sections were deparaffinized and endogenous peroxidase activity blocked by incubation in hydrogen peroxide. Epitope retrieval was carried out by microwave treatment (10 min on high setting), blocked for endogenous biotin and nonspecific background staining, and incubated with rabbit anti-mouse F4/80 antibody (sc-25830; Santa Cruz) or control IgG. Sections were washed in Tween 20-buffered saline (TBS) and incubated with a biotinylated anti-rabbit antibody. After washing with TBS, the sections were incubated with an avidin-horseradish peroxidase complex. Sections were rewashed and developed by diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Procedures were carried out at room temperature unless otherwise noted.

Tissue sections were stained by periodic acid-Schiff (PAS) and Masson's Trichrome using standard methods. Stained slides were reviewed by a renal pathologist (AC) for semiquantitative assessment of interstitial fibrosis and tubular atrophy (IF/TA) using the following 0–3 point scoring system: $0 \leq 5\%$ (none), 1 = 6–25%, 2 = 26–50%, and $3 \geq 50\%$.

2.4. Flow Cytometry. Erythrocytes were removed from whole blood samples (50 μL) with 4 mL erythrocyte lysing reagent (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.5 M EDTA, pH 8) incubated at room temperature for 5 min. The process was repeated twice additionally, with the cells centrifuged at 250 g × 5 min prior to resuspension. Cells were then resuspended in 200 μL PBS and incubated with anti-CD4 (GK1.5) for 20 min on ice, washed with 1 mL PBS and centrifuged as above, resuspended in 200 μL PBS, and analyzed on a flow cytometer (FACScanto). Data were analyzed using FlowJo software (v. 10) and presented as percentage of peripheral blood mononuclear cells (PBMCs) that were $CD4^+$.

Single-cell suspensions from whole kidneys at the indicated time were isolated. Whole kidneys were minced into small pieces (<1 mm) in ice-cold 1× HBSS media and incubated with 1 mg/mL collagenase (type IA, Sigma) and 0.1 mg/mL deoxyribonuclease (DNase, type I, Sigma) at 37°C for 25 min with gentle shaking. The suspension was centrifuged at 250 g × 5 min and the pellet was resuspended in 2 mL of erythrocyte lysing reagent and incubated for 5 min at room temperature. The suspension was again centrifuged at 250 g × 5 min, and the supernatant was discarded. The cells were resuspended in 1-2 mL of ice-cold PBS and passed through a 40 μm cell strainer. Single-cell suspensions were incubated with anti-CD11b (M1/70) and F4/80 antibody (Cl : A3-1, Serotec). Cells were washed with ice-cold PBS (4 mL/wash) followed by centrifugation at 250 g × 5 min, resuspended in ice-cold PBS, and then analyzed on a flow cytometer (FACScanto). Data were analyzed using FlowJo software (v. 10).

2.5. Statistical Analyses. Numeric data were analyzed with Minitab software (v. 16.2.4). Data were confirmed to be normally distributed using Anderson-Darling tests. Comparisons between two groups of parametric data were made with two-sample t-testing. BUN and $CD4^+$ cell data from all

individual mice in the study are shown in the figures with means as horizontal lines. In the text, data are presented as means ± SEMs.

3. Results

3.1. Recovery from rUUO Is Delayed in Mice Depleted of CD4$^+$ T Cells. In control mice subjected to the rUUO protocol, the percentage of CD4$^+$ cells in total PBMCs declined (Figure 1). This was at least partially attributable to an expansion of non-CD4$^+$ cells in the PBMC pool (i.e., rather than a decline in absolute CD4$^+$ numbers). Anti-CD4 antibody treatment resulted in complete depletion of CD4$^+$ T cells prior to UUO, which lasted through 14 days following release of obstruction (Figure 1). Of necessity we were limited to the number of GK1.5 injections and chose to span the 18 days beginning prior to UUO. Thus, because the final injection was on day 12 after release of UUO, there was evidence for reconstitution of CD4$^+$ cells by day 21 after release of UUO (Figure 1).

Our hypothesis was that CD4$^+$ cells were involved in the pathophysiology of this rUUO model, either in the injury occurring during the period of UUO and/or after release. It was therefore surprising that BUN levels were significantly higher in CD4-depleted animals on day 14 compared to controls (Figure 2, 76.4 ± 4.8 versus 62.0 ± 1.9 mg/dL, resp.). As we typically see in this model of CKD, BUN levels are highest immediately after contralateral kidney removal and appear to equilibrate with time. Thus, as shown in Figure 2, BUN levels in control mice were lower on day 28 (53.8 ± 2.8 mg/dL). While BUN levels in CD4$^+$ cell-depleted mice remained higher than controls (62.3 ± 5.1 mg/dL), these were not statistically different than controls at this time.

3.2. Depletion of Monocyte/Macrophages with Clodronate Attenuates CKD in rUUO. To evaluate mononuclear phagocytic cell effects in our rUUO model of CKD, clodronate or PBS control liposomes were administrated to mice intravenously. Kidneys were evaluated by flow cytometry and immunohistochemistry at days 0 and 7 after release of obstruction, while BUN levels were evaluated on Days 14 and 28 after release of obstruction. On day 0, kidney F4/80$^+$CD11b$^+$ cells were reduced by more than 75% (Figures 3(a) and 3(b)). Immunohistochemical staining of kidney sections confirmed the depletion (Figure 3(c)). At day 7, there was still nearly 50% less F4/80$^+$CD11b$^+$ cells in clodronate-treated mice compared to controls (Figures 4(a) and 4(b)).

Depletion of mononuclear cells with clodronate led to a relative protection from CKD as assessed by BUN values (Figure 5). Thus, 14 days after release of obstruction, BUN values in control and clodronate-treated mice were 59.1 ± 2.1 and 48.6 ± 2.3 mg/dL. Semiquantitative assessment of interstitial fibrosis and tubular atrophy (IF/TA) based on PAS and Masson's Trichrome staining was performed by a renal pathologist blinded to the origin (treatment group) of slides using a 0–3 scale (as defined in the Methods). Vehicle-treated

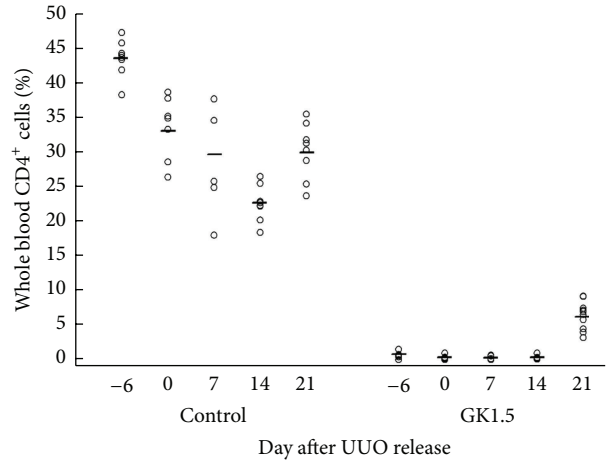

Figure 1: Flow cytometry of PBMCs confirming CD4$^+$ depletion in mice treated with GK1.5.

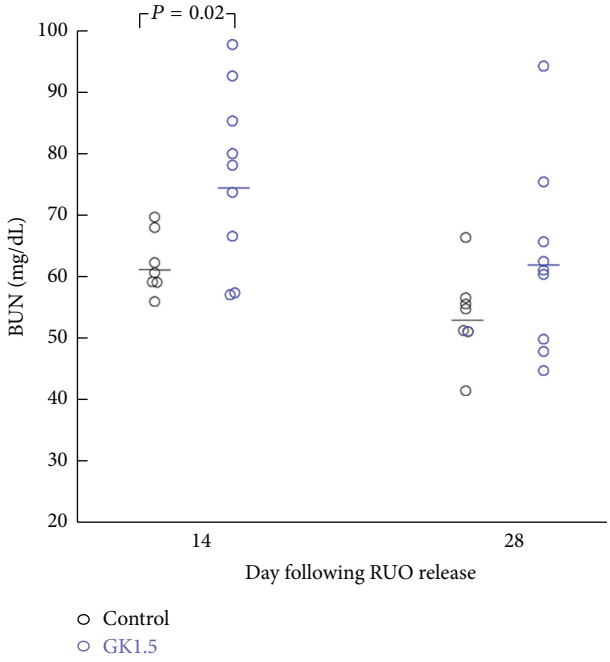

Figure 2: BUN measurements in CD4-depleted (GK1.5) and control mice on days 14 and 28 following release of UUO. On day 7, all animals had removal of the contralateral (unobstructed) kidney.

mice had a higher fibrosis score as compared to clodronate-treated mice (1.3 ± 0.30 versus 0.6 ± 0.24, mean ± SEM) at 14 days after release of obstruction (Figure 6(a)). IF/TA scores were consistent with the lower BUN levels in clodronate-treated mice after rUUO. Representative Masson's Trichrome stained sections (Figure 6(b)) illustrate the histological differences between the two treatment groups. Despite the fact that clodronate was not administered to animals after day 10, this relative protection was maintained through day 28. BUN values were 53.1 ± 3.1 and 44.8 ± 2.6 mg/dL in control and clodronate-treated mice, respectively (Figure 5).

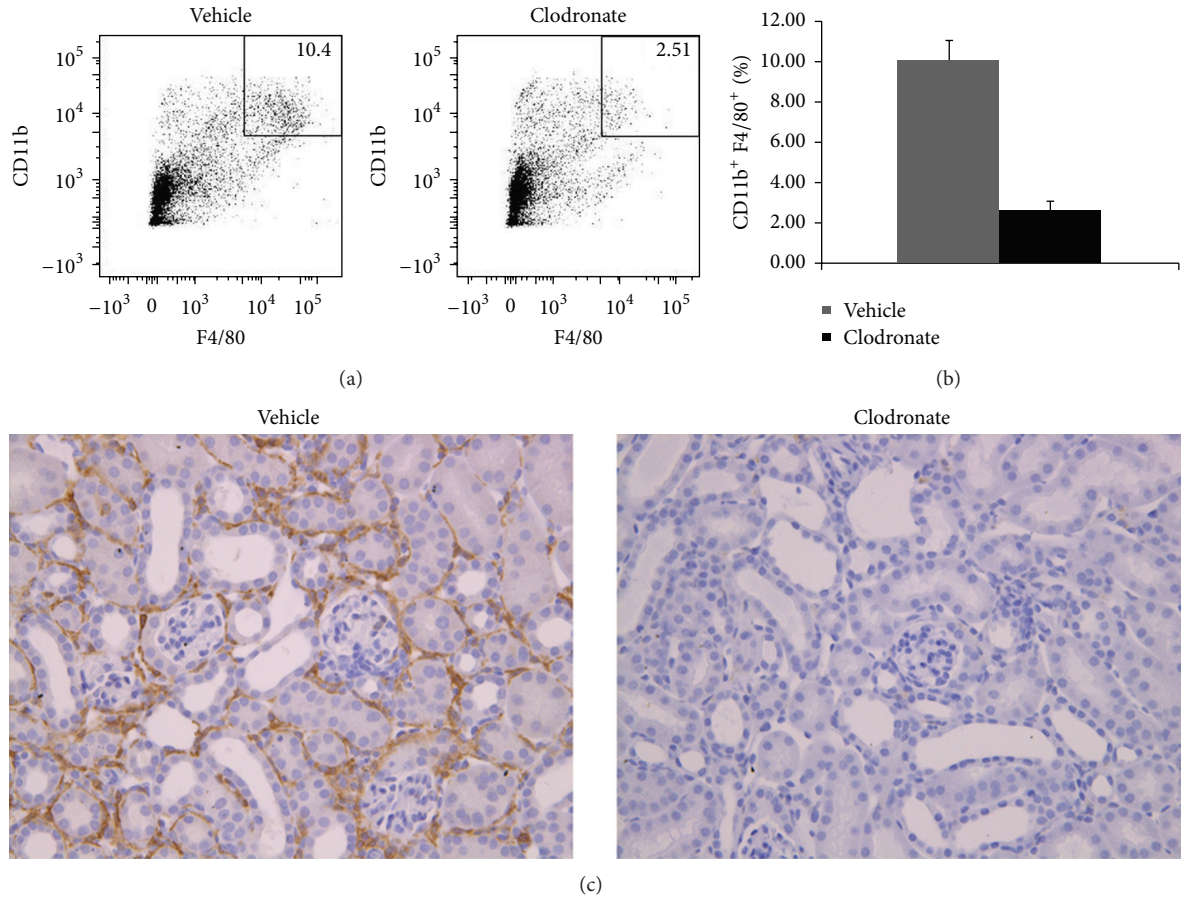

Figure 3: $CD11b^+$ and $F4/80^+$ cells in kidneys of clodronate-treated or control mice after 6 days of UUO (i.e., day 0 of the rUUO protocol). Representative flow cytometry is shown for $CD11b^+$ and $F4/80^+$ (a) with data from all kidneys shown graphically (b). Representative immunohistochemical staining for F4/80 is also shown (c). N = 5-6 per group.

4. Discussion

The functional development of CKD is characterized by histopathological features of renal parenchymal loss and replacement by fibrotic tissue. UUO is commonly used to induce renal fibrosis in rodents; as such, it is considered to be a viable model of human CKD [3–5]. Significant efforts have focused on characterizing responses to kidney injury, such as occurs in UUO, by inflammatory cells, growth factors, cytokines, matrix proteins, and other mediators [5, 7, 8]. Yet, in human renal diseases, periods of repair, including those induced by therapeutic maneuvers, often intervene. By allowing the study of responses related to both injury and repair, our model of rUUO provides considerable pathophysiological relevance.

Activation of profibrotic pathways, such as the transforming growth factor-β pathway, triggers profibrotic events including transcription of matrix protein genes and factors involved in matrix metabolism [9, 10]. Other mediators such as angiotensin II, connective tissue growth factor, and platelet derived growth factor have also been implicated in development of renal fibrosis and progression of kidney disease [11, 12]. Myofibroblasts are considered to be the primary source of the interstitial collagen contributing to fibrosis, including that seen in UUO models. The exact origin of the myofibroblast, from among local resident pericytes and/or fibroblasts, bone marrow-derived cells, and/or via epithelial- and endothelial-to-mesenchymal transition, continues to be debated [11, 13, 14]. In recent studies from Raghu Kalluri's group using the UUO model, the proportions of the latter three were approximately 50, 35, and 15%, respectively [15], while pericytes did not give rise to myofibroblasts. The latter is in distinction to work from Duffield's group showing a prominent role for pericyte-derived myofibroblasts [16, 17].

It is widely believed that $CD4^+$ cells affect the differentiation of monocytes into fibrocytes in chronic disease models [18]. In C57Bl/6 mice undergoing the 7-day UUO model, depletion of $CD4^+$ cells with GK1.5 led to ~20% reduction in collagen I deposition [19]. An additional study in C57Bl/6 mice of a 14-day UUO model utilizing the monoclonal anti-CD4 YTA3.1 for cell depletion found a similar 20% reduction in collagen deposition [20]. The authors further showed that RAG-/- mice developed significantly less collagen deposition in their 14-day UUO model and reconstitution of RAG-/- mice with $CD4^+$ cells, but not $CD8^+$ cells 14 days prior to UUO resulted in collagen deposition comparable to wild type

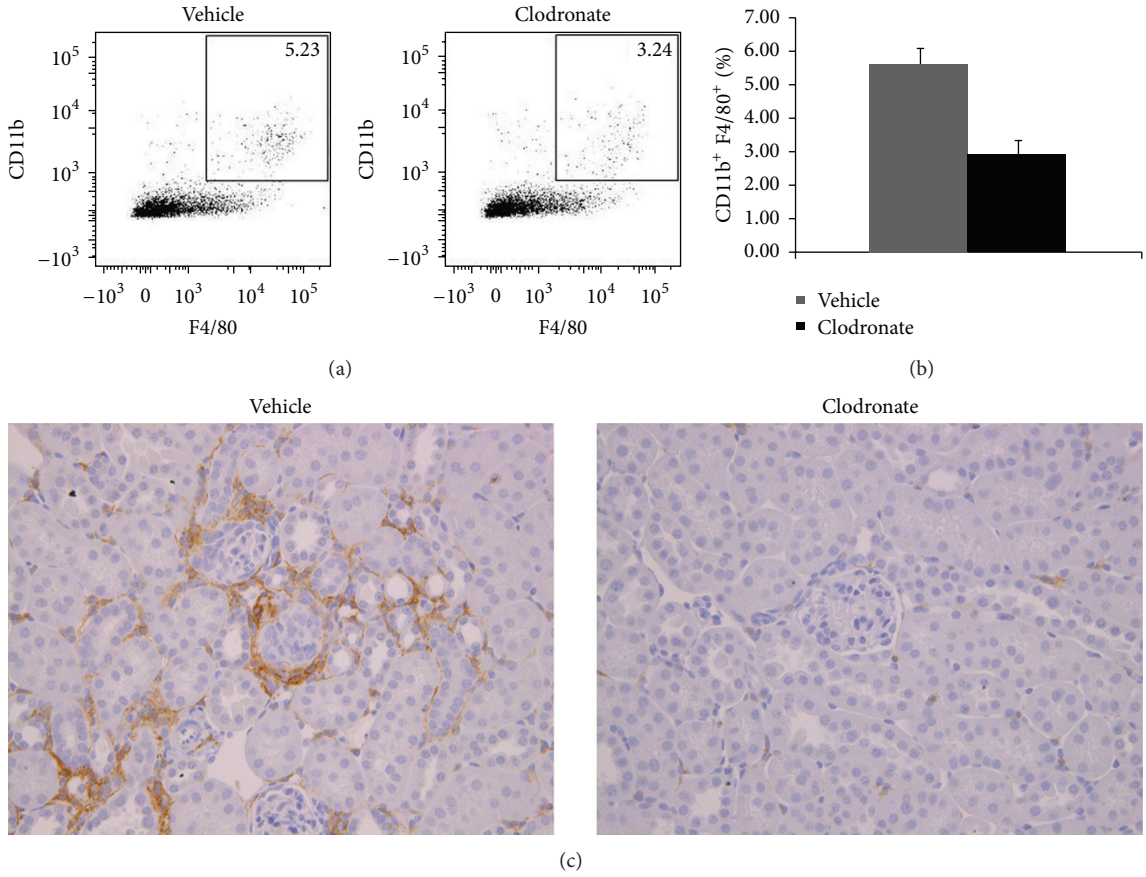

FIGURE 4: CD11b$^+$ and F4/80$^+$ cells in kidneys of clodronate-treated or vehicle-treated (control) mice 7 days after release of a 6 day UUO. Representative flow cytometry is shown for CD11b$^+$ and F4/80$^+$ (a) with data from all kidneys shown graphically (b). Representative immunohistochemical staining for F4/80 is also shown (c). $N = 5$ per group.

mice. In contrast, our data in the model of rUUO in C57Bl/6 suggest that CD4$^+$ T cells or a subpopulation thereof (e.g., T_h1 or T_{reg}) may have a protective role in the early stages of kidney disease development; however, any such benefit is overcome in the later stages of disease. Targeting a specific subpopulation in future studies may better define the role(s) of CD4$^+$ cells. In nonreversible UUO models others have described evidence suggesting a role for CD4$^+$ cells in the development of renal fibrosis. In recent studies in BALB/c mice, Liu et al. demonstrated that CD4$^+$ depletion caused a reduction in renal fibrosis due to ureteral obstruction [21]. In a nude mouse T cell reconstitution experiment they provided further evidence suggesting that the fibrosis can be attributed to T_h2 subsets. We previously demonstrated in our reversible obstruction model that BALB/c mice, genetically skewed to T_h2 responses, were resistant to the development of CKD [6]. The seemingly contradictory results may be explained by the absence of regulatory T cells in studies with nude mice. Perhaps the T_h2 T cell phenotype, through their production of IL4, promotes M2 macrophage skewing *in vivo*. M2 macrophages are thought to promote healing while limiting fibrosis and can serve as antigen presenting cells for the activation and propagation of T_h2 and T_{reg} cell responses [22]. Ureteral obstruction models are sterile, pathogen-free diseases. Interestingly, the cognate antigen of most regulatory T cells is a "self-antigen," which may underscore the necessity of this population to prevent fibrosis in the wild type mouse.

In a nonreversible model of ureteral obstruction, Kitamoto et al. utilized clodronate liposomes to evaluate renal phagocytes [23]. They obtained a similar level of F4 80$^+$ cell depletion and, interestingly, a reduction of renal fibrosis as measured by collagen II and smooth muscle actin deposits in the interstitium. These results appear to coincide with our function data showing a diminished extent of kidney damage as measured by BUN in clodronate-treated mice undergoing reversible ureteral obstruction. The same group followed up by selectively depleting either CD11b or CD11c expressing cells [24]. Systemic depletion of CD11c expressing cells had no effect on the development of renal fibrosis in their ureteral obstruction model. Alternatively, systemic depletion of CD11b expressing cells prior to obstruction resulted in a significant reduction of fibrosis markers in renal tissue. However, the role of either cell population in the recovery and further kidney function after obstruction reversal is still undefined. Further confounding is the nearly

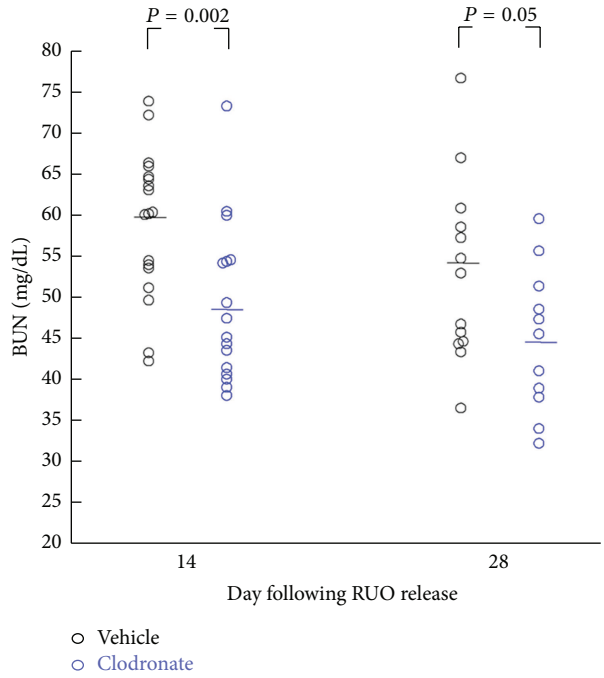

Figure 5: BUN measurements in clodronate-treated and vehicle-treated (control) mice on days 14 and 28 following release of UUO. On day 7, all animals had removal of the contralateral (unobstructed) kidney. $N = 12–19$ per group.

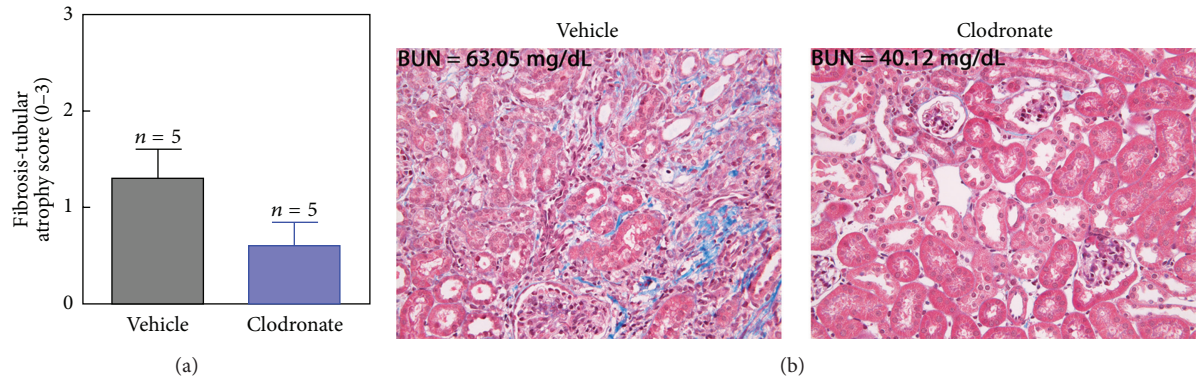

Figure 6: Interstitial fibrosis and tubular atrophy (IF/TA) scores at 14 days following release of UUO in vehicle- (black) and clodronate- (blue) treated mice (5 mice per treatment group, Y-error bars indicate SEM) (a). Representative Masson's Trichrome staining is shown with the BUN for the representative animals (b).

ubiquitous expression pattern of CD11b in the myeloid cell lineage. For example, Summers et al. provided evidence suggesting a role of mast cells, which also express CD11b, in initial renal fibrosis development due to ureteral obstruction [25].

Chronic kidney disease afflicts over 10% of the population over 20 years of age and more than 40% of the population age 65 and over. Understanding both the physiological and cellular processes that contribute is essential in order to mitigate or even prevent progression into end stage renal disease. By using our rUUO model of CKD in lieu of standard UUO models we can study the cellular mechanisms that lead to functionally significant kidney damage. Our future studies are designed to further examine these cellular responses including the likely possibility that there is interdependence in responses by different cell populations and subsets. Through this work we hope to elucidate the roles and mechanisms by which lymphocyte and mononuclear phagocyte subsets contribute to fibrosis and repair, for example, T_h2, T_{reg} or M1, M2 macrophages, respectively.

Acknowledgments

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Review Article

Mediators of Inflammation and Their Effect on Resident Renal Cells: Implications in Lupus Nephritis

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Lupus nephritis affects up to 70% of patients with systemic lupus erythematosus and is a major cause of morbidity and mortality. It is characterized by a breakdown of immune tolerance, production of autoantibodies, and deposition of immune complexes within the kidney parenchyma, resulting in local inflammation and subsequent organ damage. To date, numerous mediators of inflammation have been implicated in the development and progression of lupus nephritis, and these include cytokines, chemokines, and glycosaminoglycans. Of these, type I interferons (IFNs) can increase both gene and protein expression of cytokines and chemokines associated with lupus susceptibility, and interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and hyaluronan have been shown to elicit both pro- and anti-inflammatory effects on infiltrating and resident renal cells depending on the status of their microenvironment. Expression of IL-6, TNF-α, type I IFNs, and hyaluronan are increased in the kidneys of patients and mice with active lupus nephritis and have been shown to contribute to disease pathogenesis. There is also evidence that despite clinical remission, ongoing inflammatory processes may occur within the glomerular and tubulointerstitial compartments of the kidney, which further promote kidney injury. In this review, we provide an overview of the synthesis and putative roles of IL-6, TNF-α, IFN-α, and hyaluronan in the pathogenesis of lupus nephritis focusing on their effects on human mesangial cells and proximal renal tubular epithelial cells.

1. Introduction

Renal involvement (i.e. lupus nephritis) is a serious manifestation of systemic lupus erythematosus (SLE) that affects up to 70% of SLE patients and is a strong predictor of morbidity and mortality [1]. Depending on the severity of disease, up to 30% of lupus patients will progress to end-stage renal disease and will require dialysis to sustain life. Lupus nephritis is prevalent in non-Caucasian females especially those of child-bearing age and is characterized by a loss of immune tolerance, production of autoantibodies against nuclear antigens and immune-mediated kidney injury [1]. It is initiated by the deposition of immune complexes within the renal parenchyma leading to complement activation, mesangial expansion and induction of inflammatory and fibrotic processes, resulting in glomerulonephritis and progressive renal dysfunction.

There is evidence to suggest that anti-dsDNA antibodies contribute to the pathogenesis of lupus nephritis since many features of this disease can be reproduced in nonautoimmune mice after intraperitoneal administration of anti-dsDNA antibodies [2]. Anti-dsDNA antibodies are also essential to the diagnosis of SLE, and their levels correlate with disease activity [3, 4]. We and others have demonstrated that anti-dsDNA antibodies can bind to mesangial cells, endothelial cells, and proximal renal tubular epithelial cells to induce cell proliferation, apoptosis, and inflammatory and fibrotic processes [5–14]. The precise mechanisms through which anti-dsDNA antibodies are deposited in the renal parenchyma remain to be defined, but studies suggest that they can bind directly to cross-reactive antigens such as annexin II and α-actinin on the surface of mesangial cells or through nucleosomes bound to components of the glomerular basement membrane, where they induce downstream inflammatory processes [10, 15–17]. Histologically, glomerular lesions may range from no or mild mesangial proliferation to highly proliferative and crescentic glomerulonephritis, whereas tubulointerstitial lesions correlate with renal

Table 1: Mediators of inflammation that play important roles in the pathogenesis of lupus nephritis.

Inflammatory mediator	Putative roles in lupus nephritis
IL-6	(i) Activates B cells
	(ii) Induces glomerulonephritis
IFN-α	(i) Interferes with vascular repair by inducing endothelial progenitor cell apoptosis
	(ii) Induces renal dysfunction, glomerulonephritis, crescent formation, and tubulointerstitial nephritis
IFN-γ	Promotes macrophage recruitment into the kidney and the development of glomerulonephritis
TNF-α	(i) Regulates physiological and inflammatory immune responses
	(ii) Induces synthesis of IL-1β and IL-6 in mesangial cells and proximal renal tubular epithelial cells
	(iii) Elicits both proinflammatory and anti-inflammatory actions in lupus nephritis
Hyaluronan (HA)	(i) Forms HA cables that can prevent leukocyte adhesion to their receptors
	(ii) Induces chemokine secretion
	(iii) Possesses proinflammatory and anti-inflammatory properties

prognosis. Serum levels of IL-6, TNF-α, IFN-α, and hyaluronan (HA) are increased in patients with lupus nephritis [7, 18, 19]. There is accumulating evidence to demonstrate that their expression is increased in the renal parenchyma of patients and mice with active lupus nephritis, mediated in part through stimulation of resident renal cells with anti-dsDNA antibodies, which contribute to the development and progression of disease (Table 1) [7, 20–25]. Furthermore, their synthesis precedes inflammatory cell infiltration and renal injury. Mesangial cells are an important source of these inflammatory mediators during the early stage of lupus nephritis, but as disease progresses, infiltrating lymphocytes, macrophages, endothelial cells, and proximal renal tubular epithelial cells are activated by IL-6, TNF-α, IFN-α, and HA, which further drive the inflammatory processes in the kidney and highlight their prominent roles in the pathogenesis of lupus nephritis. This review will discuss the contributing roles of these inflammatory mediators and their synthesis in the pathogenesis of lupus nephritis, with particular focus on their effects in mesangial cells and proximal renal tubular epithelial cells. The contribution of lymphocytes and macrophages in amplifying inflammatory processes during lupus nephritis is outside the scope of this review and has been described elsewhere [26–29].

2. Interleukin-6 (IL-6)

IL-6 is a pleiotropic cytokine with an MW of 21 kDa that is secreted by both lymphoid and nonlymphoid cells such as B cells, T cells, monocytes, mesangial cells, proximal renal tubular epithelial cells, endothelial cells, and fibroblasts [10, 30–36]. It is a multifunctional cytokine essential for the differentiation and maturation of B cells, acute-phase protein production, and mesangial cell proliferation. IL-6 can target IFN-inducible genes such as *Ifi202* in murine fibroblasts and splenocytes through activation of STAT3, which results in the suppression of cell cycle progression and inhibition of apoptosis, thereby contributing to increased lupus susceptibility [22]. Serum and urinary IL-6 levels are increased in patients with lupus nephritis, especially in those with diffuse proliferative lupus nephritis, and correlate with nephritic flares [37]. In the normal kidney, IL-6 is localized to the mesangial area and within vascular walls. In patients with lupus nephritis, its expression is increased in mesangial cells, induced in podocytes, and is present in glomerular immune deposits and along the apical aspects of proximal renal tubular epithelial cells [20, 24, 33, 38].

The mechanisms through which IL-6 is locally produced in the kidney during pathogenesis of lupus nephritis have not been fully defined. We have recently demonstrated that human polyclonal anti-dsDNA antibodies bind to annexin II on the surface of human mesangial cells and are rapidly internalized to induce downstream inflammatory processes including increased transcription and translation of IL-6, mediated through increased activation of ERK and p38 MAPK [10]. We have also demonstrated that following binding and internalization, the subsequent cellular localization of anti-dsDNA antibodies can influence the amount of IL-6 secreted by mesangial cells. In this respect, induction of IL-6 secretion is more prominent in cells stimulated with anti-dsDNA antibodies with intranuclear localization compared to antibodies that are localized solely to the cytoplasm, and this mechanism of IL-6 induction occurs with autoantibodies derived from patients in remission and with relapse [10]. The importance of anti-dsDNA antibody-annexin II interaction in the induction of IL-6 secretion was corroborated in annexin II gene silencing studies [10]. Induction of IL-6 secretion by anti-dsDNA antibodies has also been observed in rat mesangial cells although the mechanism through which IL-6 was increased was not further investigated [39].

The severity of tubulointerstitial lesions is strongly associated with less favorable renal prognosis [40]. Although it was previously believed that glomerular injury provoked tubulointerstitial damage, there is compelling evidence to demonstrate that proximal renal tubular epithelial cells can directly contribute to the pathogenesis of lupus nephritis. Up to 70% of patients with lupus nephritis have discernible immune aggregates and IL-6 expression along the tubular basement membrane [33]. Tubulointerstitial expression of IL-6 correlates with IgG deposition, circulating levels of anti-dsDNA antibodies and tubular abnormalities such as inflammatory cell infiltration, tubular atrophy, and interstitial fibrosis in patients with diffuse proliferative lupus nephritis [33]. Proximal renal tubular epithelial cells constitute the predominant cell type in the tubulointerstitium and play a pivotal role in the immunopathogenesis of various renal parenchymal diseases, acting as an effector of immune-mediated inflammation. Exposure of HK-2 cells, an immortalized proximal renal tubular epithelial cell line [41], with

anti-dsDNA antibodies induced *de novo* synthesis of both gene and protein expression of IL-6 [33]. Depending on the disease status, induction of IL-6 secretion in these cells was mediated through distinct mechanisms. We demonstrated that during remission, induction of IL-6 secretion was mediated through the direct actions of anti-dsDNA antibodies or indirectly though the prior stimulation of IL-1β. In contrast, anti-dsDNA antibodies isolated from the same cohort of patients during relapse increased IL-6 secretion through prior induction of both IL-1β and TNF-α secretion, suggesting autoantibody heterogeneity within the same patient during remission and relapse [33]. The ability of anti-dsDNA antibodies obtained from remission patients to induce cytokine production in renal cells in most interesting since it would suggest persistence inflammation, albeit at a lower level to that observed during flare, within the glomerular and tubulointerstitial compartments of the kidney despite clinical quiescence. Given that autoreactive mature naïve B cells are detected in lupus patients during remission, which are precursors of antibody secreting plasma cells [42], it is plausible to suggest that this lymphocyte subset may contribute to persistent autoantibody production and inflammatory processes within the tubulointerstitium during the inactive phase of disease.

Inflammatory processes within the glomerular and tubulointerstitial compartments do not occur in isolation. We have demonstrated that mediators secreted by human mesangial cells and HK-2 cells upon stimulation with anti-dsDNA antibodies can induce IL-6 secretion in the other cell type, suggesting bidirectional communication between the glomerulus and tubulointerstitium. Furthermore, at an identical anti-dsDNA IgG concentration, HK-2 cells demonstrated a more prominent induction of IL-6 secretion compared to mesangial cells, thereby highlighting the importance of proximal renal tubular epithelial cells in the immunopathogenesis of lupus nephritis [33]. Consistent with our findings, immunoglobulins of the IgG subclass isolated from the sera of SLE patients induced IL-6 secretion in proximal renal tubular epithelial cells, which was accompanied by ERK activation [43].

Mycophenolic acid (MPA) is the active metabolite of mycophenolate mofetil [44], an immunosuppressive agent used in the treatment of patients with lupus nephritis [45–47]. MPA is a specific inhibitor of lymphocyte proliferation that noncompetitively inhibits inosine monophosphate dehydrogenase, a rate-limiting enzyme that plays a critical role in the *de novo* synthesis of guanosine nucleotides [44]. There is also accumulating evidence to demonstrate that MPA can have a direct effect on non-lymphoid cells and has been shown to inhibit cell proliferation and inflammatory processes in endothelial cells, smooth muscles cells, tubular epithelial cells, fibroblasts, and mesangial cells [9, 48–52]. MPA can suppress matrix protein synthesis in mesangial cells stimulated with exogenous TGF-β1 or anti-dsDNA antibodies [9, 53]. We have demonstrated that MPA can suppress anti-dsDNA antibody induction of IL-6 secretion in HK-2 cells, which was accompanied by a reduction in cell proliferation [Ng, Yung and Chan, unpublished data].

The importance of IL-6 in the pathogenesis of lupus nephritis has been highlighted by independent researchers who demonstrated that IL-6 can exacerbate glomerulonephritis and disease manifestations in NZB/W mice, whereas interruption of IL-6 signaling is associated with reduced circulating anti-dsDNA antibody levels, improved renal histology and function, decreased proteinuria, and increased survival in lupus-prone mice [21, 54–57]. Whether disease progression in patients with lupus nephritis can be suppressed by targeting IL-6 remains to be determined.

3. Tumour Necrosis Factor-α (TNF-α)

TNF-α is a prototype proinflammatory cytokine that is predominantly synthesized by activated macrophages and lymphocytes, and to a lesser extent by intrinsic renal cells. It is synthesized as a 26 kDa membrane-bound protein that is activated and released as a 17 kDa soluble cytokine by TNF-α-converting enzymes belonging to the ADAM (a disintegrin and metalloproteinase) family [58]. Low levels of TNF-α mRNA can be detected in lupus-prone mice prior to renal injury [59]. Renal expression and circulating levels of bioactive TNF-α are increased during clinical and experimental lupus nephritis and correlate with disease activity [19, 24, 60, 61]. We have previously demonstrated that the induction of TNF-α secretion by anti-dsDNA antibodies in mesangial cells and proximal renal tubular epithelial cells was an early event and contributed to increased IL-6 secretion [7, 33]. We further demonstrated that TNF-α can act synergistically with anti-dsDNA antibodies obtained from patients with active disease to amplify inflammatory responses, an observation not noted with autoantibodies isolated from patients in remission. Increased TNF-α secretion can induce apoptotic cell death in resident renal cells, a mechanism that may initiate organ-specific damage. TNF-α may also exert distinct effects on the kidney depending on the immunologic microenvironment during different stages of disease. Administration of TNF-α to predisease NZB/W mice delayed the onset of disease, whereas administration of low, but not high, doses of TNF-α to lupus-prone mice with active disease exacerbated renal injury [62]. In order to induce renal damage, researchers have suggested that TNF-α interacts with pathologic mediators present either locally or in the circulation that are not synthesized in pre-disease lupus-prone mice. Notable, these pathologic mediators have yet to be identified. Higher doses of TNF-α may exert a protective effect through the induction of tolerance [62]. In support of this hypothesis, Wallach et al. demonstrated that administration of sublethal doses of TNF-α to BALB/c mice resulted in tolerance following TNF-α rechallenge [63]. The beneficial role of TNF-α in the pathogenesis of disease is substantiated by findings that decreased synthesis of TNF-α in NZB/W mice is associated with the development of lupus nephritis [64]. In contrast to these findings, increased intrarenal TNF-α expression in NZB/W or MRL/lpr mice correlated with renal inflammation and disease activity, a finding not observed in their congenic littermate [59, 62]. It is possible that genetic predisposition may regulate the effect of TNF-α in the pathogenesis of lupus nephritis. So, how should

one conclude whether induction of TNF-α secretion by anti-dsDNA antibodies in resident renal cells is detrimental or protective? Given that anti-dsDNA antibody-mediated induction of TNF-α increased IL-1β and IL-6 secretions in mesangial and proximal renal tubular epithelial cells, it is plausible to suggest that under these experimental settings, TNF-α exerts a pro-inflammatory effect in the kidney. Induction of TNF-α expression in the kidney and cultured proximal renal tubular epithelial cells is mediated in part, through prior activation of p38 MAPK during progressive lupus nephritis [65].

Since TNF-α exerts dual effects on resident renal cells, administration of agents to block TNF-α in SLE patients should be approached with caution. Furthermore, their effectiveness in suppressing disease manifestations remains debatable. Aringer et al. reported that the treatment of patients with lupus nephritis with infliximab, a monoclonal antibody against TNF-α, for up to 10 weeks improved proteinuria but also transiently increased anti-dsDNA antibody production, a result of increased apoptotic bodies and thus autoantigens following TNF-α depletion [66, 67]. Longer treatment with infliximab was associated with adverse side effects that included fatal pneumonia and brain lymphoma although whether this was attributed to the use of infliximab or prior use of other immunosuppressive agents remains to be determined [66]. In patients with rheumatoid arthritis receiving anti-TNF-α therapy, side effects encountered included the development of drug-induced lupus-like syndromes, anti-dsDNA antibody production, and glomerulonephritis [68–70]. How can TNF-α mediate disease development? Experimental studies have suggested that TNF-α can inhibit type I IFN, a family of pro-inflammatory cytokines known to exert pathogenic roles in the development of lupus nephritis. When TNF-α is inhibited by anti-TNF agents, synthesis of type I IFN is no longer repressed, thereby permitting the exacerbation of inflammatory processes. Whether similar findings are observed in lupus patients remain to be determined. The actions of TNF-α in the pathogenesis of lupus nephritis have also been confounded by reports that anti-TNF-α treatment in NZB/W mice with active lupus nephritis induced by IFN-α protected the mice against renal damage and prolonged their survival by attenuating the kidney's response to glomerular immune complex deposition [71]. Blockade of TNF-α activity in patients or animals with lupus nephritis may be beneficial or otherwise depending on the dose, treatment duration, and status of disease when treatment is administered. Is it possible to suppress the pro-inflammatory properties of TNF-α, while retaining its anti-inflammatory properties?

4. Type I Interferons (IFNs)

The type I IFN family consists of IFN-α, IFN-β, IFN-ω, IFN-κ, and IFN-ε. These pleiotropic cytokines are key regulators of the innate and adaptive immunity, and their levels are increased during antiviral responses and autoimmune diseases [72]. Type I IFNs can promote cell proliferation and differentiation of monocytes and B cells into antigen-presenting cells or plasma cells, respectively. These cytokines mediate their inflammatory responses through their engagement with a common heterodimeric receptor composed of type I IFN receptor 1 and 2 subunits. There is evidence that IFN-α plays a critical role in the development of lupus nephritis. Serum levels of IFN-α and its expression in the glomeruli of lupus patients correlate with disease activity [73–76]. Studies have suggested that increased serum IFN-α bioactivity and polymorphism of interferon regulatory factor 5 gene, a transcription factor essential for IFN-α secretion, are associated with SLE and lupus nephritis susceptibility, respectively [77, 78]. Patients with viral infections or malignant tumors frequently develop SLE-like manifestations and anti-DNA antibodies following IFN-α treatment, thereby corroborating the importance of this cytokine in the development of lupus [79–81]. Although plasmacytoid dendritic cells are the primary source of type I IFNs in lupus patients, intrinsic renal cells such as mesangial cells and glomerular endothelial cells can also synthesize IFN-α following stimulation with viral components mediated through toll-like receptor dependent and independent pathways [82–84]. Synthesis of IFN-α by endothelial cells may contribute to the infiltration of inflammatory cells into the kidney parenchyma. Stimulation of mesangial and proximal renal tubular epithelial cells with anti-dsDNA does not induce IFN-α secretion (Yung and Chan, unpublished observation). Fairhurst et al. demonstrated that type I IFNs synthesized by resident renal cells in an experimental model of anti-GBM nephritis induced renal dysfunction, glomerulonephritis, crescent formation, and tubulointerstitial nephritis [85].

Exposure of NZB/W mice to IFN-α can accelerate pathogenic autoantibody production, proteinuria development, and glomerular IgG deposition and render these mice more resistantly to therapeutic intervention when compared to lupus-prone mice without IFN-α treatment [23, 86, 87]. Gene silencing of IFN-α/βR in NZB mice, which ablated the biological activities of IFN-α/β, suppressed splenomegaly, anti-dsDNA antibody production, and kidney pathology and improved survival compared to their wildtype littermates, thereby substantiating the pathogenic role of type I IFN in promoting SLE [88].

IFN-induced mRNA transcripts, otherwise known as IFN-signature, are increased in peripheral blood mononuclear cells isolated from SLE patients and may serve as a marker for more severe organ manifestations such as lupus nephritis [89, 90]. Increased IFN-α-inducible transcripts have been observed in the glomeruli of patients with lupus nephritis, which inversely correlated with many genes that promote renal fibrosis [91]. Intriguingly, this would suggest that the expression of IFN-inducible transcripts could either result in a milder form of renal injury or be protective against glomerular damage [91]. Whether the presence of this signature is a cause or consequence of disease remains to be fully defined.

5. Hyaluronan (HA)

HA is a large, negatively-charged, nonsulfated glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine [92]. It is synthesized on the inner surface of the plasma membrane by

HA synthases (HAS), and newly synthesized HA is either directed to the cell surface where it interacts with its receptor CD44 or is assembled into the extracellular matrix. Three HAS isoenzymes have been identified that share 55–70% homology and are termed HAS I, HAS II, and HAS III [93]. Under physiologic conditions, HA may possess up to 25,000 disaccharide units with a corresponding molecular mass of 10^6–10^7 Da [94]. HA contributes to basement membrane stability and sequestration of free radicals and plays critical roles in cell proliferation, differentiation, migration, and phenotypic changes.

In the normal kidney, HA is primarily expressed within the inner medulla interstitium where it contributes to the mechanical stability of tubules and blood vessels, and also in the concentration of urine [95]. In patients and mice with active lupus nephritis, HA expression extends into the renal cortex and has a periglomerular, crescentic, mesangial, and tubulointerstitial distribution [7, 96, 97]. We and others have demonstrated that cultured mesangial cells, glomerular endothelial cells, proximal renal tubular epithelial cells, and interstitial fibroblasts can synthesize HA [7, 98–103], and it is thus plausible to suggest that these cell types contribute to increased HA levels in the renal cortex in lupus patients. HA accumulation in the tubulointerstitium correlates with lymphocyte infiltration and renal damage mediated in part, through prior induction of TNF-α and IFN-γ [97]. There is mounting evidence to suggest that during chronic kidney inflammation, mesangial cells and proximal renal tubular epithelial cells synthesize HA that forms long cable-like structures that function as an adhesive matrix, which binds leukocytes and macrophages preventing them from interacting with adhesion molecules, thereby limiting glomerular and tubulointerstitial inflammation [104–106].

Human polyclonal anti-dsDNA antibodies can increase high molecular weight (HMW) HA synthesis and induce synthesis of low molecular weight (LMW) HA in mesangial and proximal renal tubular epithelial cells when compared to control cells, mediated in part through prior induction of IL-1β secretion, and increased HAS II gene expression [7, 107]. Independent researchers have demonstrated that the biological functions of HA is governed by its molecular weight. HMW HA possesses anti-inflammatory and antiangiogenic properties and can promote cell quiescence, whereas LMW HA is pro-inflammatory and can induce cytokine and chemokine secretion, activation of signalling pathways, cell proliferation, and angiogenesis [108–113]. The presence of LMW HA can arise through *de novo* synthesis during inflammation or through the depolymerisation of native HA following increased hyaluronidase activity or exposure to reactive oxygen species [110]. Pro-inflammatory cytokines can increase synthesis of both HMW and LMW HA in various cell types. Although we and others have shown an increase in intrarenal HA expression in patients and mice with active lupus nephritis, its MW was not investigated [7, 97, 114]. It is noteworthy that despite differences in the biological functions of HMW and LMW HA, reports detailing the presence of LMW HA in tissues undergoing inflammation and injury are scarce.

Exogenous LMW, but not HMW HA, can induce MCP-1 mRNA and protein secretion in proximal renal tubular epithelial cells [25]. Glomerular and tubulointerstitial expression of MCP-1 is increased in lupus-prone mice and precedes leukocyte infiltration, proteinuria, and renal damage [115]. The significance of MCP-1 in the pathogenesis of lupus nephritis was highlighted by Tesch et al. who observed that MRL/lpr mice deficient in MCP-1 demonstrated increased survival consequent to less severe renal histology and proteinuria compared to their wildtype littermates [116]. We have demonstrated that suppression of HA synthesis in NZBWF1/J mice was associated with an improvement in clinical parameters of disease and decreased intrarenal expression of IL-6 and TNF-α [96]. It would be most interesting to determine whether HA is a potential target for therapeutic intervention in the pathogenesis of lupus nephritis.

Serum HA levels are increased in patients with lupus nephritis compared to healthy controls and correlate with circulating anti-dsDNA antibody levels, suggesting that these autoantibodies contribute to increased HA synthesis [7]. It is noteworthy that an increase in the level of circulating HA is not specific to lupus but rather, a consequent of renal impairment and injury [117–119]. Elevated serum HA levels are associated with inflammation, malnutrition, and poor prognosis in patients with end-stage renal failure [120].

6. Conclusions

Although a plethora of inflammatory mediators have been implicated in the development and pathogenesis of lupus nephritis, our understanding of how they mediate renal injury has been confounded by their multifunctional roles. There is compelling evidence to demonstrate that resident renal cells can directly contribute to renal inflammation through their ability to secrete cytokines, chemokines, and glycosaminoglycans following their interaction with, but not limited to, anti-dsDNA antibodies and viral components.

Disease remission and prevention of irreversible renal damage are the ultimate goals of induction therapy but irrespective of achieving clinical remission, studies have demonstrated that low-grade inflammatory processes may persist within the renal parenchyma leading to further kidney injury. Further research into the mechanisms through which pro-inflammatory mediators are modulated during disease manifestations, how they interact with other inflammatory mediators, and the underlying mechanisms that dictate whether these molecules elicits pro- or anti-inflammatory responses will provide us with a better understanding of their roles in lupus nephritis and whether targeting these molecules can improve clinical outcome without affecting their physiological roles.

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Research Article

LMW Heparin Prevents Increased Kidney Expression of Proinflammatory Mediators in (NZBxNZW)F1 Mice

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We have previously demonstrated that continuous infusion of low molecular weight (LMW) heparin delays autoantibody production and development of lupus nephritis in (NZBxNZW)F1 (B/W) mice. In this study we investigated the effect of LMW heparin on renal cytokine and chemokine expression and on nucleosome-mediated activation of nucleosome-specific splenocytes. Total mRNA extracted from kidneys of heparin-treated or -untreated B/W mice was analysed by qPCR for the expression of several cytokines, chemokines, and Toll-like receptors. Splenocytes taken from B/W mice were stimulated with nucleosomes with or without the presence of heparin. Splenocyte cell proliferation as thymidine incorporation and the expression of costimulatory molecules and cell activation markers were measured. Heparin treatment of B/W mice reduced the *in vivo* expression of CCR2, IL1β, and TLR7 compared to untreated B/W mice. Nucleosome-induced cell proliferation of splenocytes was not influenced by heparin. The expression of CD80, CD86, CD69, CD25, CTLA-4, and TLR 2, 7, 8, and 9 was upregulated upon stimulation by nucleosomes, irrespective of whether heparin was added to the cell culture or not. In conclusion, treatment with heparin lowers the kidney expression of proinflammatory mediators in B/W mice but does not affect nucleosomal activation of splenocytes.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune syndrome characterized by inflammation and damage in several organs [1]. Lupus nephritis is one of the most severe manifestations of SLE, and autoantibodies against nuclear components such as dsDNA and nucleosomes are central in the development of the organ disease. These autoantibodies are found together with chromatin in electron dense structures (EDS) located in the mesangial matrix (MM) and glomerular basement membranes (GBM) of nephritic kidneys, as demonstrated in both murine [2–4] and human [5] forms of lupus nephritis. Several studies have demonstrated that the main autoantigen in lupus nephritis, assumingly serving as both inducer and target for the immune system, indeed is chromatin fragments or nucleosomes [6–9].

Studies have shown that SLE patients and lupus prone mice are assumed to suffer from impaired clearance of apoptotic debris [10, 11]. This may result in an increased load of extracellular chromatin and formation of immune complexes (ICs) [12–14]. Deposition of ICs within the MM and GBM is associated with renal expression of proinflammatory chemokines attracting leukocytes in SLE patients and murine models of lupus-like nephritis [15–17]. This will lead to increased influx of Fc receptor bearing effector cells activated by circulating ICs, which together will increase the ongoing inflammation and tissue destruction [18]. Interfering with activation of intrinsic kidney cells and effector cells may prevent or lower the expression of cytokines and chemokines.

We have previously demonstrated that lupus prone (NZBxNZW)F1 (B/W) mice receiving low molecular weight (LMW) heparin showed delayed anti-dsDNA antibody production compared to sham-treated control mice [19]. *In vitro* studies also showed that LMW heparin inhibited chromatin binding to components of GBM, and heparin increased enzymatic degradation of chromatin, as demonstrated using

Dnase1 and proteinase K enzymes [19]. The aim of this study was to investigate if LMW heparin treatment, by preventing binding of ICs to the GBM, had an effect on cytokine, chemokine and Toll-like receptor mRNA expression profiles during the development of lupus nephritis and if heparin could prevent nucleosomal activation of splenocytes.

2. Materials and Methods

2.1. Ethics Statement. The treatment and care of animals were conducted in accordance with the Norwegian Animal Experimental and Scientific Purposes Act of 1986. All experimental protocols were approved by the Norwegian Animal Research Authority (NARA).

2.2. Mice and Grouping of Mice. Female B/W and BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). The B/W mice were divided into 4 groups based on age, deposition of IgG in glomeruli, anti-dsDNA ab titers in sera taken at end point, proteinuria, and heparin treatment. Group 1 (4–10 w.o, $n = 17$) had no depositions of IgG within the kidneys and no detectable levels of anti-dsDNA antibodies in sera. Deposition of IgG was observed within the kidney of Group 2 (mesangial nephritis determined by mesangial deposits, 18–30 w.o, $n = 15$) and Group 3 (end-stage organ disease determined by GBM deposits and proteinuria, 23–36 w.o, proteinuric, $n = 18$) B/W mice with detectable levels of anti-dsDNA ab in sera. In the heparin-treated group (Group 4, $n = 5$) 1/5 mice were anti-dsDNA antibody negative, and 2/5 mice developed proteinuria [19]. Age-matched BALB/c mice was used as controls.

2.3. Isolation of Kidneys from B/W Mice. B/W mice at age 4 weeks old (w.o) until the development of severe proteinuria (23–40 w.o) and age-matched control BALB/C mice were sacrificed in groups of 3 as described previously [4]. The heparin-treated mice included five mice given a daily subcutaneous dose of 50 μg of Klexane (LMW heparin, Aventis Pharma AS) by osmotic pumps (Scanbur, Oslo, Norway) from the age of 12 weeks [19]. The osmotic pumps were primed and filled according to the manufacturer's instruction, implanted subcutaneously in the upper dorsal region, and replaced every ~30 days. Control mice received saline by saline-filled osmotic pumps. The heparin-treated mice were paired randomly with saline-treated control mice. The pairs of BW mice were sacrificed when the control mice developed full-blown lupus nephritis (31–39 w.o). The kidneys were isolated and processed for RNA isolation, immunohistochemistry (IHC) analysis, and immune electron microscopy (IEM) analysis, as described in [4].

2.4. Determination of Proteinuria and of Anti-dsDNA Antibodies by ELISA. Full-blown lupus nephritis was defined when proteinuria reached 4+, as determined by urine stix (Bayer Diagnostics, Bridgend, UK): 0-1+ (<1 g protein/liter urine) was regarded as physiological proteinuria; 2+ (≥1 g/liter to <3 g/liter) was regarded as mild proteinuria, and 3+ (≥3 g/liter to <20 g/liter) and 4+ (≥20 g/liter) was regarded as heavy proteinuria. Sera were collected and stored at −20°C until use. Serum antibodies against dsDNA were detected by ELISA as described in [20, 21]. Sera were diluted twofold from 1/100 to 1/6400 in PBS (0.02% Tween), and the 163c3 anti-dsDNA mAb (provided by T. N Marion, Memphis, TN, USA [22]) was included in each ELISA for assay validation and determination of cut-off value.

2.5. Immunohistochemistry. Detection of autoantibodies bound in glomeruli was performed on Zink-fixed kidneys embedded in paraffin. Four μm sections of the kidney samples were dewaxed with xylene, rehydrated in graded series of ethanol before blocking with 3% H_2O_2 to neutralize endogenous peroxidase. Sections were further blocked with 10% goat serum and 1% BSA in PBS before incubation with anti-mouse IgG antibodies conjugated with horseradish peroxidase (Sigma-Aldrich, St. Louis, MO, USA) diluted 1 : 100 in blocking solution. Washed sections were then incubated with chromogen DAB (Dako, Glostrup, Denmark) for detection of primary antibody. IHC using Polink-2 Plus HRP detection kits for tissue (Golden Bridge International, Inc, Mukilteo, WA, USA) was performed on frozen kidney sections. Antibodies against mice CCR2 and TLR7 were purchased from Abcam (Cambridge, UK), and IL1β was obtained from R&D systems (Minneapolis, USA).

2.6. Isolation of Splenocytes from B/W Mice. Spleens were collected and mashed through a 100 μm cell strainer with DMEM-10 (4.5 g/L glucose, 10% fetal bovine serum, 10000 U/mL penicillin, and 10 mg/mL streptomycin and L-glutamine) (Invitrogen, Carlsbad, CA, USA). Erythrocytes were lysed with ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA adjusted to pH 7.2–7.4), and splenocytes were washed and resuspended in DMEM-10.

2.7. Proliferation Assay. Splenocytes, at 10^5 cells/200 μL/well, were seeded out in a 96-well round bottom plate. The cells were incubated with different stimulators (all in triplicates): nucleosomes (10 μg/mL) prepared from the murine BALB/c 3T3 clone A31 fibroblast cell line (ATCC CCL-163) and characterized as previously described in [23], nucleosomes (10 μg/mL) together with LMW heparin (Enoxaparin, Klexane, Aventis Pharma AS, Oslo, Norway, 24 μg/mL) at a molar ratio of 1 : 100 (nucleosomes (determined as core nucleosome equivalents): heparin), LMW heparin (24 μg/mL), concanavalin A (con A) (2.5 μg/mL) (Sigma-Aldrich), and conA with LMW heparin (1 : 200) and HMGB1 (1 μg/mL) (Sigma-Aldrich). Tritiated thymidine (1 μCi/well) (Perkin Elmer, MA, USA) was added to the cell cultures 16 hours before harvesting at 20 h, 4 days, and 7 days. Cells were transferred to filter paper, each spot representing one well was isolated, and three mL of scintillation fluid (Ultima gold XR, Perkin Elmer) was added to each piece of filter paper. Counts per minute (cpm) were measured using a liquid scintillation analyzer (1900 TR, Packard Instruments). A proliferative response was defined as a stimulation index (SI) calculated as the mean cpm value for stimulated cells in triplicates divided with mean cpm value for medium stimulated cells at the same time point. Positive proliferation was regarded if SI was greater than 2,

provided that the cpm of antigen-stimulated cells was above 100 cpm.

2.8. Western Blot. Western blot was performed with SDS-NuPage-gels and blotting system according to the manufacturer (Invitrogen). Rabbit anti-mouse HMGB1 antibody (Sigma-Aldrich) was used for detection of HMGB1. Recombinant HMGB1 protein (Sigma-Aldrich) was used as control (31 kDa on SDS-PAGE).

2.9. RNA Isolation. Differently stimulated splenocytes (all in triplicate) were harvested for total RNA isolation at the same time points as for the proliferation assay. Cells were collected and washed in ice cold PBS before isolating RNA with Trizol reagent (200 μL) (Invitrogen) according to the manufacturer's protocol with minor modification. Chloroform (40 μL) was used for phase separation, and RNA was precipitated with 96% ethanol containing 0.3 M NaAc and 20 μg/mL glycogen. RNA was washed in 80% ethanol and dried before dissolving in Rnase-free water. The concentration and quality of extracted RNA were determined spectrophotometrically using NanoDrop (NanoDrop technologies, Wilmington, USA).

2.10. Gene Expression Analysis. Preparation of cDNA and quantitative PCR (qPCR) was performed exactly as described in [24]. The following TaqMan gene expression assays were used: Mm00446973_m1 for TBP as housekeeping gene, Mm01183378_m1 for CD69, Mm01340213_m1 for CD25, Mm00434256_m1 for IL2, Mm00515420_m1 for CD19, Mm00486849_m1 for CTLA-4, Mm01157262_m1 for TLR8, Mm00711659_m1 for CD80, Mm00444543_m1 for CD86, Mm00446590_m1 for TLR7, Mm00446193_m1 TLR9, Mm01210732_g1 for IL6, Mm00433859_m1 for CXCL1 (KC), Mm99999062_m1 for IL10, Mm99999061_mH for IL1β, Mm01168134_m1 for IFN-γ, Mm00443258_m1 for TNFα, Mn00441242_m1 for CCL2, Mn00438270_m1 for CCR2, Mn01308393_g1 for CCL7, Mn00444228_m1 for CCL20, Mn00436450_m1 for CXCL2, Mn00436451_g1 for CXCL5, and Mn00442346_m1 for TLR2. TaqMan Fast Universal PCR master mix (2X) and gene expression assays were all obtained from Applied Biosystems. Medium stimulated cells at each time point (20 hours, 4 days, and 7 days) served as reference, and changes in gene expression were calculated with the $\Delta\Delta$CT method shown as fold change.

2.11. Measurements of Cytokines in Cell Supernatants. Cytokine analyses were performed with ELISA MAX Standard Sets for mouse IL10 (BioLegend, San Diego, CA, USA) or mouse TNFα ELISA kit (Thermo scientific, Rockford, IL, USA). All assays were performed according to the manufacturer's instructions.

2.12. Statistical Analysis. Unpaired t-test was used to compare mean of two sets of measurements. Statistical comparisons of groups were made by one-way ANOVA followed by Bonferroni posttest. Statistical comparisons of treatment were analyzed using two-way ANOVA followed by Bonferroni posttest. All tests were performed using GraphPad Prism version 5.0.

3. Results

3.1. The Effect of LMW Heparin on In Vivo mRNA Expression Levels of Cytokines, Chemokines, Chemokine Receptor, and TLRs. To measure the effect of LMW heparin treatment on cytokine and chemokine expression individual TaqMan real time PCR assays (qPCR) on a selection of cytokine and chemokine genes were performed. The mRNA expression levels of CCL2, CCL7, CCL20, CXCL1, and CXCL2 were significantly upregulated in Group 3 B/W mice compared to Group 1 mice and were, although somewhat reduced, not significantly different in the heparin-treated mice (Figures 1(a)–1(f)). There were no significant increase of CCL2, CCL7, and CXCL1 mRNA expressions in age-matched BALB/c mice (Figures 1(f)–1(h)). CCR2, IL1β, IL10, TLR2, TLR7, TLR8, and TLR9 mRNA expressions were significantly increased in Group 3 mice (Figures 2(a)–2(g), resp.), and CCR2, IL1β and TLR7 mRNA expression levels were significantly lower in heparin-treated mice (Group 4) compared to nephritic mice (Group 3) (Figures 2(a), 2(b), and 2(e), resp.). Analysis of CCR2, IL1β, and TLR7 protein expression within the tissue verified these reduced gene expression levels observed in heparin-treated mice compared to untreated mice (Figure 3(a)). CCR2 expression was observed in tubular and glomerular areas of nephritic mice, whereas the expression in heparin-treated mice were confined to tubuli (Figure 3(a)). IL1β was observed in infiltrating cells that were reduced in heparin-treated mice (Figure 3(a)). TLR7 expression was observed on infiltrating cells, tubuli and glomeruli of untreated nephritic mice, and a reduced expression that was mainly observed in glomeruli and between tubuli of heparin-treated mice (Figure 3(a)). A Spearman correlation analysis (Table 1) on all parameters performed on age-matched pairs of nontreated and heparin-treated B/W mice demonstrated an inverse correlation of heparin treatment and the development of proteinuria and the gene expression of CCR2, IL1β, and IL10. The duration of anti-dsDNA antibody production (in weeks) correlated positively with the development of proteinuria and with expression of CCL2, CCR2, CCL20, TLR2, TLR7, CXCL1, and IL0 (Table 1). Anti-dsDNA ab production and successive deposition of immune complexes within the kidney during the disease increase the gene expression of cytokine and chemokines, while heparin treatment lowers the expression.

3.2. Splenocytes from Nephritic Mice Are Activated by Nucleosomes in Absence or Presence of Heparin. To analyse the effect of LMW heparin on cell proliferation, gene expression of cell activation markers, and proinflammatory cytokines, splenocytes isolated from prenephritic and nephritic B/W mice were stimulated with nucleosomes, either in absence or presence of heparin. Nucleosomes used in the present experiments contained HMGB1 (Figures 3(b) and 3(c)). The size of nucleosomes ranged from mononucleosomes to polynucleosomes (Figure 3(d)). The splenocytes from prenephritic mice did not respond to nucleosomes in any

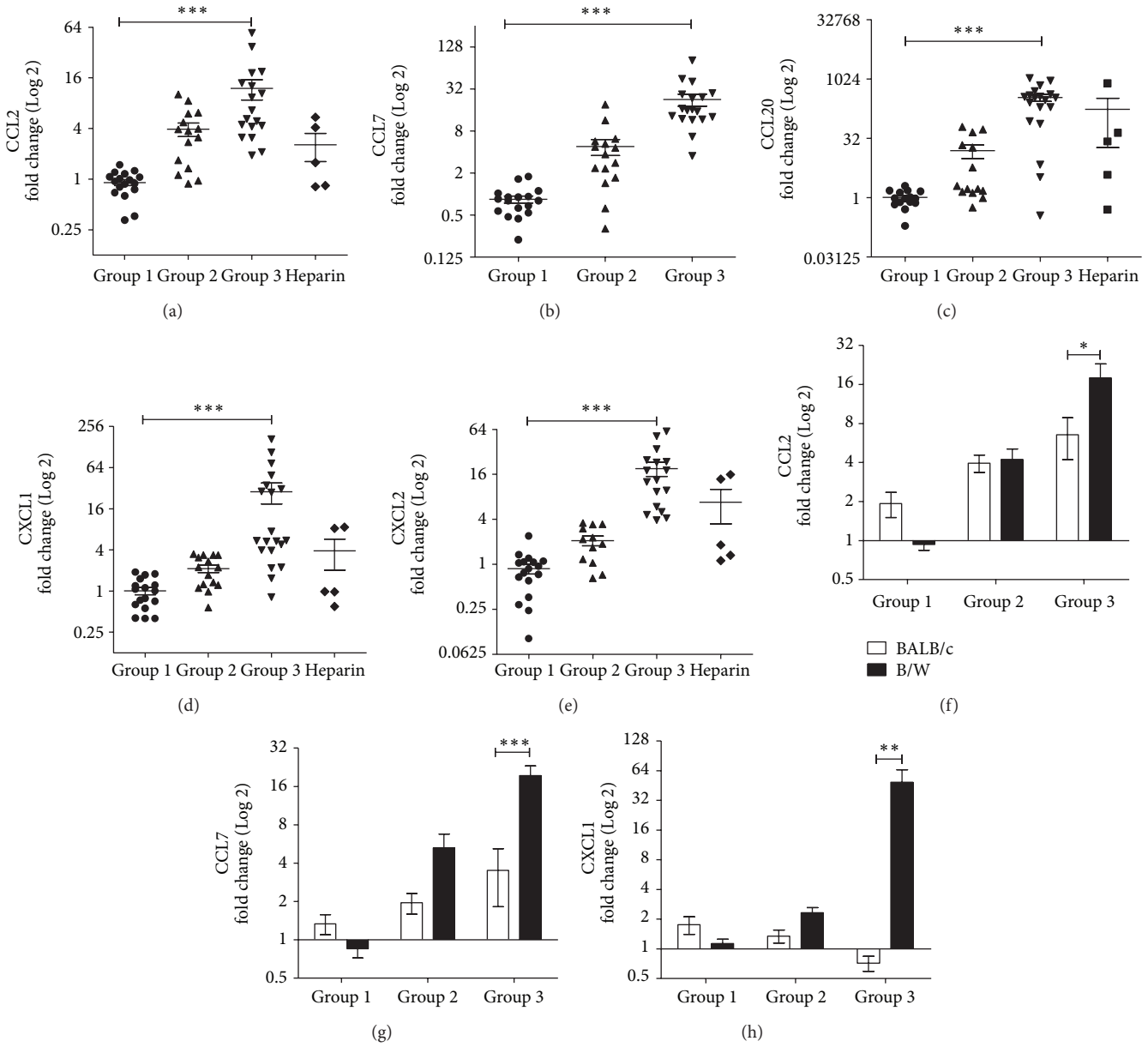

Figure 1: LMW heparin treatment does not affect chemokine mRNA expression within the kidneys of B/W mice. The mRNA expression of CCL2 (a), CCL7 (b), CCL20 (c), CXCL1 (d), and CXCL2 (e) was significantly increased in Group 3 mice and were not significantly reduced in heparin-treated mice. The mRNA expression of CCL2 (f), CCL7 (g), and CXCL1 (h) was also analyzed in age-matched BALB/c mice. Data is given as Log 2 of mean ± SEM of fold change values normalized against 4-week-old mice ($n = 3$). P values are calculated using one-way ANOVA followed by Bonferroni posttest. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Group 1: 4–10 w.o B/W mice ($n = 17$); Group 2: 18–30 w.o B/W mice with mesangial IC deposits without proteinuria ($n = 15$); Group 3: 23–36 w.o B/W mice with proteinuria ($n = 18$); Group 4: 31–39 w.o heparin-treated B/W mice ($n = 5$).

of the experiments (data not shown). However, stimulation of cells from prenephritic mice with conA resulted in a stimulation index similar to those obtained from nephritic mice with no significant reduction by heparin (Figures 3(e) and 3(f), resp.). The spontaneous proliferation in medium measured as cpm revealed that nephritic mice had a significantly higher proliferation at 20 hours which persisted over time compared to splenocytes from prenephritic mice (Figure 3(g)). Splenocytes from nephritic mice proliferated in response to nucleosomes, but the presence of LMW heparin did not affect this response (Figure 4). In three of the five mice, we observed a nucleosome-induced proliferation, while the kinetics differed between the mice (Figures 4(a)–4(c)). The presence of LMW heparin did not have any influence on the proliferation of splenocytes taken from these mice (Figures 4(a)–4(c)). Splenocytes from two nephritic mice did apparently not respond to nucleosomes (Figures 4(d) and 4(e)). Splenocytes from these mice demonstrated high initial

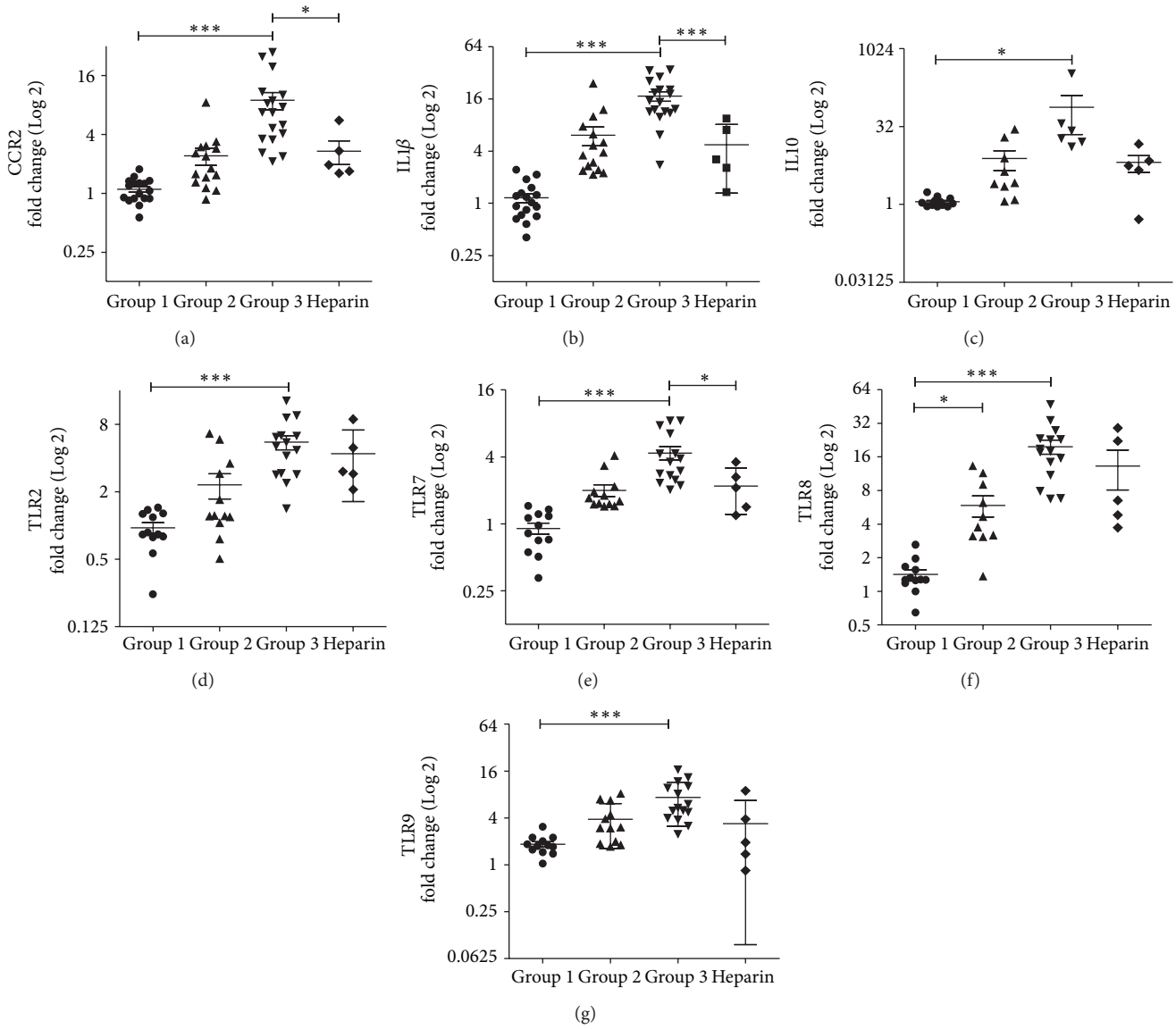

Figure 2: Heparin treatment affects the mRNA expression of CCR2, IL1β, and TLR7 in kidneys of B/W mice. The mRNA expression of CCR2 (a), IL1β (b), IL10 (c), TLR2 (d), and TLR7 (e) was increased in Group 3 mice compared to Group 1 mice. The mRNA expression of CCR2 (a), IL1β (b), and TLR7 (e) was significantly reduced in Heparin-treated mice compared to Group 3 mice. Data is given as Log 2 of mean ± SEM of fold change values normalized against 4-week-old mice ($n = 3$). P values are calculated using one-way ANOVA followed by Bonferroni posttest. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

levels of tritiated thymidine incorporation already at 20 h, indicating that they either were in a phase of proliferation when seeded into the wells, or they were fast responders giving responses before 20 h (Figures 4(d) and 4(e), (insets) and 4(f)). Stimulation with isolated HMGB1 or LMW heparin did not give a significant proliferative response in cells from any mouse tested.

Nucleosome-stimulated splenocytes from nephritic B/W mice, analysed by qPCR, showed transcriptional upregulation of genes encoding cell activation markers for antigen presenting cells: CD80, CD86 (Figures 5(a) and 5(b)), activated T cells: CD69, CTLA4, and IL2 (Figures 5(c)–5(e)), and the B cell marker CD19 (Figure 5(f)). The presence of LMW heparin in the cultures did not influence the increased transcription of these markers in response to stimulation with nucleosomes (Figures 5(a)–5(f)). Responses to nucleosome stimulation with or without heparin also included an increased transcription of the genes encoding the cytokines IL1β, IL6, IL10, IFN-γ, TNFα, the receptors TLR2, TLR7, TLR8, TLR9, and the chemokine CXCL1 (mouse IL8 analogue) (Table 2).

4. Discussion

Recent results have demonstrated that B/W mice treated with LMW heparin presented a significantly delayed and reduced

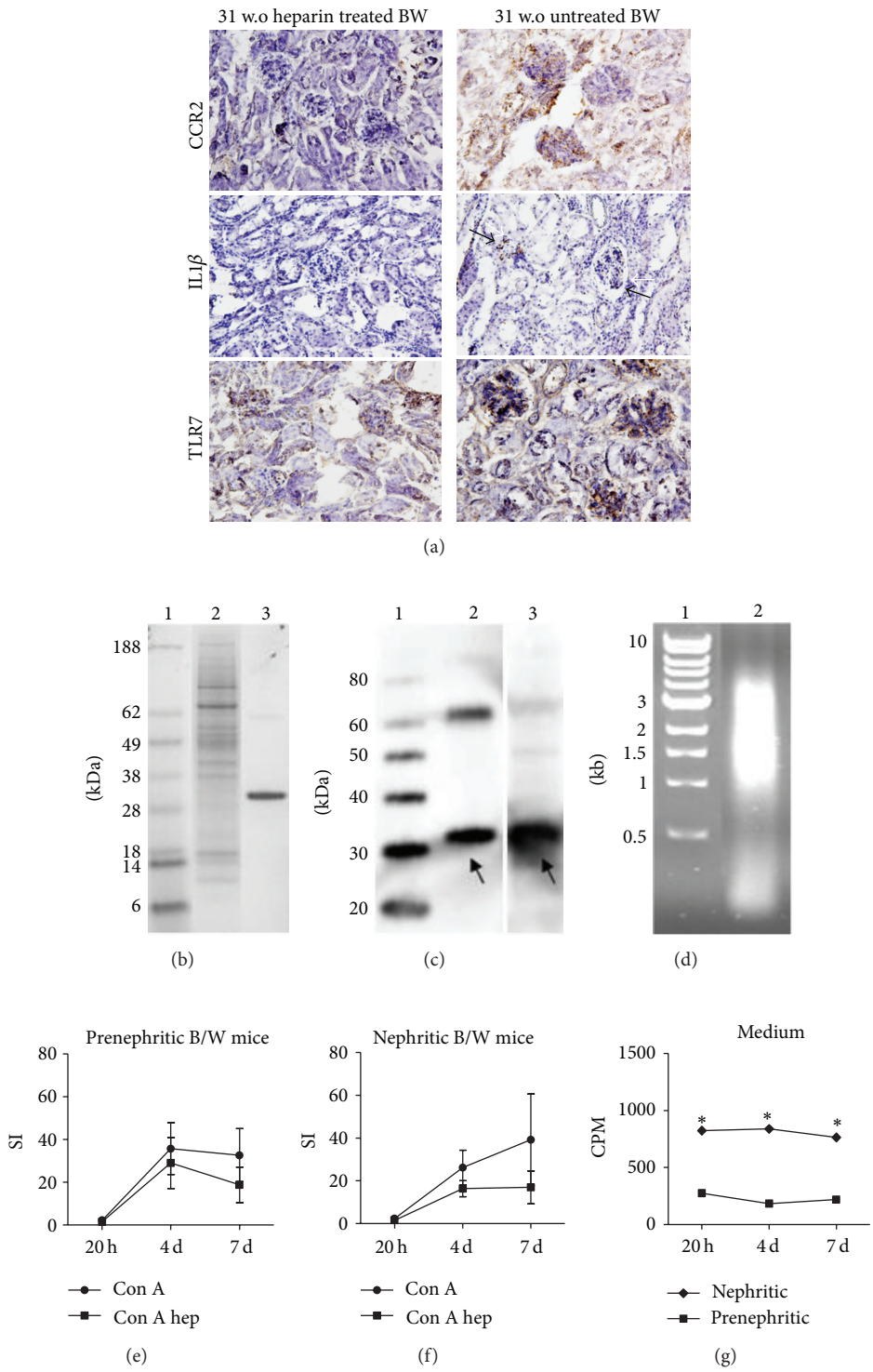

Figure 3: Characterization of nucleosomes used in this study and spontaneous splenocyte proliferation. Immunohistochemistry analysis detecting CCR2, IL1β, and TLR7 protein expression in kidneys of 31 w.o heparin or untreated B/W mice (a). SDS PAGE of nucleosomes and HMGB1 used in cell culture stimulations ((b), lane 1: MW standard, lane 2: nucleosomes (3 μg measured as DNA), and lane 3: HMGB1 (1 μg)). Western blot showing HMGB1 (arrows) in nucleosomes purified from A31 cell line ((c), lane 1: MW standard, lane 2: HMGB1 (0.1 μg), and lane 3: nucleosomes (2 μg measured as DNA)). Agarose gel electrophoresis of nucleosomes showing a distribution of nucleosome sizes ((d), lane 1: MW standard, lane 2: nucleosomes (3 μg measured as DNA)). Stimulation index (SI) of cells from prenephritic mice (e) and nephritic mice (f) stimulated with conA with or without heparin. Splenocytes from nephritic mice have a significantly higher proliferation (counts per minute, CPM) at 20 hours which persisted over time compared to splenocytes from prenephritic mice (g). Mean values and SD were calculated from triplicates from each cell culture. *Statistically significant ($P < 0.05$) compared to medium stimulated cells at the same time point. Scale = 100 μm.

Table 1: Spearman correlation matrix performed on gene expression in heparin and nontreated age-matched B/W mice.

	Age	Hep.	w. ab. Pos.	Prot.	CCL2	CCR2	CCL7	CCL20	CXCL1	CXCL2	TLR2	TLR7	IL1β	IL10
Age		0,000	0,363	0,463	0,517	0,369	0,591	**0,640**	**0,714**	**0,739**	0,443	0,394	0,271	0,074
Heparin	1,000		−0,601	**−0,655**	−0,453	**−0,731**	−0,383	−0,522	−0,313	−0,244	−0,453	−0,592	**−0,870**	**−0,801**
Weeks antibody positive	0,303	0,066		**0,694**	**0,677**	**0,874**	0,603	**0,812**	**0,652**	0,572	**0,739**	**0,855**	0,622	**0,745**
Proteinuria	0,178	*0,040*	*0,026*		**0,798**	**0,798**	**0,798**	**0,798**	**0,798**	**0,798**	**0,722**	**0,722**	**0,798**	**0,722**
CCL2	0,126	0,189	*0,032*	*0,006*		**0,745**	**0,964**	**0,830**	0,600	**0,636**	**0,903**	**0,806**	**0,661**	**0,624**
CCR2	0,294	*0,016*	*0,001*	*0,006*	*0,013*		**0,648**	**0,891**	0,527	0,515	**0,782**	**0,879**	**0,830**	**0,915**
CCL7	0,072	0,275	0,065	*0,006*	*<0,001*	*0,043*		**0,818**	**0,661**	**0,721**	**0,794**	**0,721**	0,552	0,515
CCL20	*0,046*	0,122	*0,004*	*0,006*	*0,003*	*0,001*	*0,004*		**0,685**	**0,733**	**0,830**	**0,915**	**0,697**	**0,770**
CXCL1	*0,020*	0,378	*0,041*	*0,006*	0,067	0,117	*0,038*	*0,029*		**0,964**	0,576	0,576	0,479	0,333
CXCL2	*0,015*	0,497	0,084	*0,006*	*0,048*	0,128	*0,019*	*0,016*	*<0,001*		0,588	0,588	0,442	0,333
TLR2	0,200	0,189	*0,015*	*0,018*	*0,000*	*0,008*	*0,006*	*0,003*	0,082	0,074		**0,927**	**0,733**	**0,721**
TLR7	0,260	0,071	*0,002*	*0,018*	*0,005*	*0,001*	*0,019*	*<0,001*	0,082	0,074	*<0,001*		**0,758**	**0,855**
IL1β	0,449	*0,001*	0,055	*0,006*	*0,038*	*0,003*	0,098	*0,025*	0,162	0,200	*0,016*	*0,011*		**0,855**
IL10	0,839	*0,005*	*0,013*	*0,018*	0,054	*<0,001*	0,128	*0,009*	0,347	0,347	*0,019*	*0,002*	*0,002*	

Table 2: Transcriptional levels of cytokines and Toll-like receptors in stimulated splenocytes.

Genes	Relative gene expression levels[a] in splenocytes from nephritic mice					
	Nucleosomes			Nucleosome lmw heparin		
	20 hours	4 days	7 days	20 hours	4 days	7 days
IL1β	15.87 ± 5.09	**77.78 ± 7.92***	**72.69 ± 29.08***	15.54 ± 4.62	**86.84 ± 13.09***	**80.86 ± 10.80***
IL6	1.80 ± 0.42	6.91 ± 2.46	**35.25 ± 8.08***	1.52 ± 0.57	6.57 ± 1.21	**37.25 ± 15.09***
IL10	1.29 ± 0.53	1.90 ± 0.25	**3.55 ± 0.12***	1.47 ± 0.48	1.79 ± 0.21	**4.45 ± 2.16***
IFN-γ	2, 35 ± 0.79	4, 10 ± 2.62	**10,99 ± 0.38***	3, 00 ± 1.18	6, 53 ± 4.57	**11,20 ± 4.03***
TNFα	**4.75 ± 0.32***	**5.45 ± 0.82***	**1.97 ± 0.34***	**5.44 ± 1.23***	**8.37 ± 1.24***	**2.04 ± 0.14***
CXCL1	**110.70 ± 47.16***	51.80 ± 11.37	**81.63 ± 46.31***	**91.75 ± 27.24***	45.92 ± 4.40	**91.75 ± 23.38***
TLR2	1.81 ± 0.75[b]	**10.94 ± 0.65***	**19.70 ± 4.40***	1.69 ± 0.48	**10.15 ± 1.39***	**19.02 ± 2.08***
TLR7	**2.09 ± 0.82***	**3.21 ± 0.57***	**2.30 ± 0.38***	1.47 ± 0.41	**2.04 ± 0.12***	**2.23 ± 1.10***
TLR8	**0.34 ± 0.12***	**1.64 ± 0.19***	**3.31 ± 0.09***	0.43 ± 0.23	**1.77 ± 0.31***	**2.61 ± 0.28***
TLR9	1.11 ± 0.23	2.01 ± 0.05	**4.08 ± 0.87***	1.98 ± 1.05	**2.76 ± 0.36***	**5.10 ± 1.79***

[a]Data is given as fold change compared to medium stimulated splenocytes at the same time point. *Statistically significant ($P < 0.05$) change in mRNA levels compared to medium stimulated cells. [b]Mean values and SD were calculated from triplicates.

anti-DNA antibody response *in vivo*. We also observed a significantly delayed development of lupus nephritis in the heparin-treated mice [19]. These results may theoretically be due to at least 2 different effects of LMW heparin. Heparin makes nucleosomes more sensitive to enzymatic degradation, and particularly to Dnase1 [19] similar to what has been described by, for example, Villeponteau [25]. This effect resulted in a nearby complete degradation of nucleosomal DNA *in vitro* [19]. LMW heparin also inhibited binding of nucleosomes to components of GBM, like laminins and collagen IV, possibly due to altered net charge and conformation of the nucleosomal structure induced by heparin [19]. These phenomenons have also been observed by van Bruggen et al. [26], although they provided a different explanation for reduced nucleosome binding to membranes.

When analysing cytokine and chemokine mRNA expression levels in the kidneys of treated and untreated mice, we demonstrated significantly reduced levels of CCR2, IL1β, and TLR7 in heparin-treated mice. CCR2 is mainly expressed by tubular cells in the murine kidney in addition to effector cells like macrophages. Reduced expression of CCR2 may either be because of less influx of macrophages or less expression by tubular cells. Here we also demonstrate an increased expression of CCR2 within the glomeruli of sick mice. The reduced mRNA levels of IL1β and TLR7 may indicate lower degree of influx of immune cells normally expressing them. Heparin has been shown to have an effect on adhesion molecules and cytokines, and can bind to chemokines [27]. In addition heparin can inhibit complement activation [28, 29]. Classical activation of the complement system also provides chemotaxis of granulocytes and macrophages through the split products of C3a-C5a [30]. LMW heparin has been shown to have an inhibitory effect on mesangial cell proliferation, signal transduction, and reduce apoptosis upon several

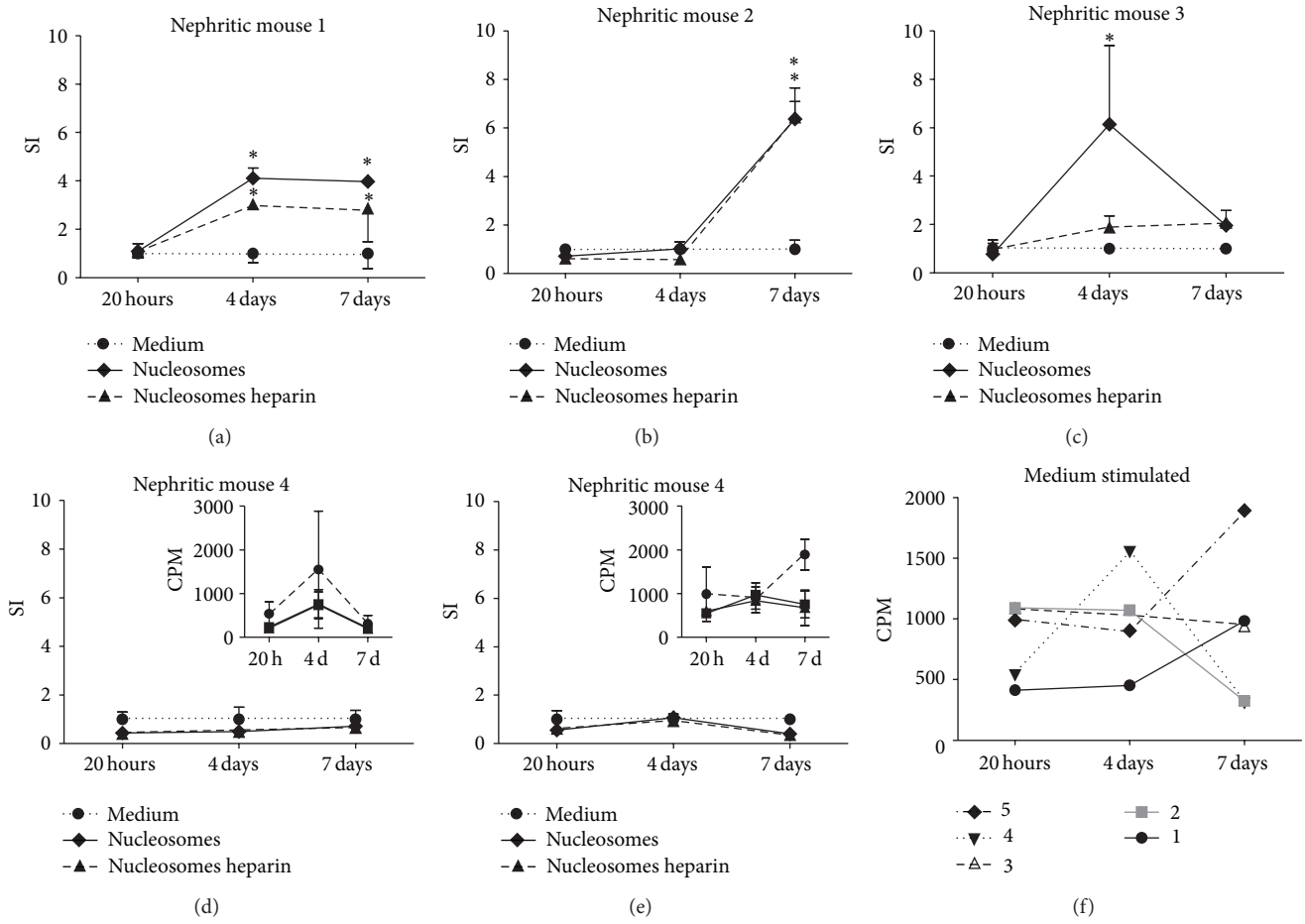

Figure 4: Splenocyte cell proliferation in response to nucleosomes. Splenocytes derived from three of the nephritic mice showed increased proliferation in response to nucleosomes; LMW heparin did not interfere with proliferation magnitude or kinetics ((a), (b), and (c), proliferation is shown as stimulation index (SI)). Apparently no proliferation was seen in cell cultures from two of the nephritic mice ((d), (e)), although responses in cpm indicate that the cells proliferate ((d), (e), inserted figures). Proliferations in medium for individual mice are shown as cpm (f). Mean values and SD were calculated from triplicates. *Statistically significant ($P < 0.05$) compared to medium stimulated cells at the same time point.

activation stimuli [31–33]. The effect of LMW heparin on activation of mesangial cells by nucleosome and nucleosome-containing immune complexes remains to be determined.

The ability of heparin to prevent binding of nucleosomes to membranes could theoretically also indicate that heparin could preclude binding and uptake of nucleosomes by APCs as well as binding of T-cell receptors to nucleosome-derived peptide-MHC class II complexes. This would eventually provide an explanation to the observed reduced autoimmune anti-dsDNA antibody response beyond pure degradation and loss of immunogenic nucleosomes [19]. In the present study, all nephritic mice produced antibodies to DNA, and splenocytes from 3 of them responded readily to nucleosomes *in vitro*. The reason why splenocytes from two of the five nephritic mice did not proliferate in response to nucleosomes is unclear but may be due to the fact that they seemed to be activated *in vivo* at the time they were cultured and unresponsive to further stimuli the next 7 days. This was demonstrated by high cpm values in medium-stimulated cultures already at the early phase of the cultures, and no increase in cpm was observed thereafter during the 7 days observation time. In cultures of splenocytes from Group 3 mice, T-cell activation markers together with upregulation of activation markers for APCs and B cells were observed. This was also accompanied by cytokine production reflecting a true innate immune response against nucleosomes. The cell proliferation in these cell cultures was the same when nucleosomes were presented in the presence of LMW heparin.

In the nucleosome-stimulated splenocyte cultures derived from nephritic mice, we also observed an increase in TLR mRNA expression levels in response to nucleosomes. TLR 7 and 8 are activated by ssRNA and g-rich oligonucleotides [34, 35], while TLR 9 is activated by CpG motifs on DNA usually found on bacterial DNA [36]. They exert important roles in induction of autoimmunity [37–39]. These receptors are located in intracellular compartments of APCs, and activation of them leads to upregulation of co-stimulatory molecules and cytokine secretion needed for

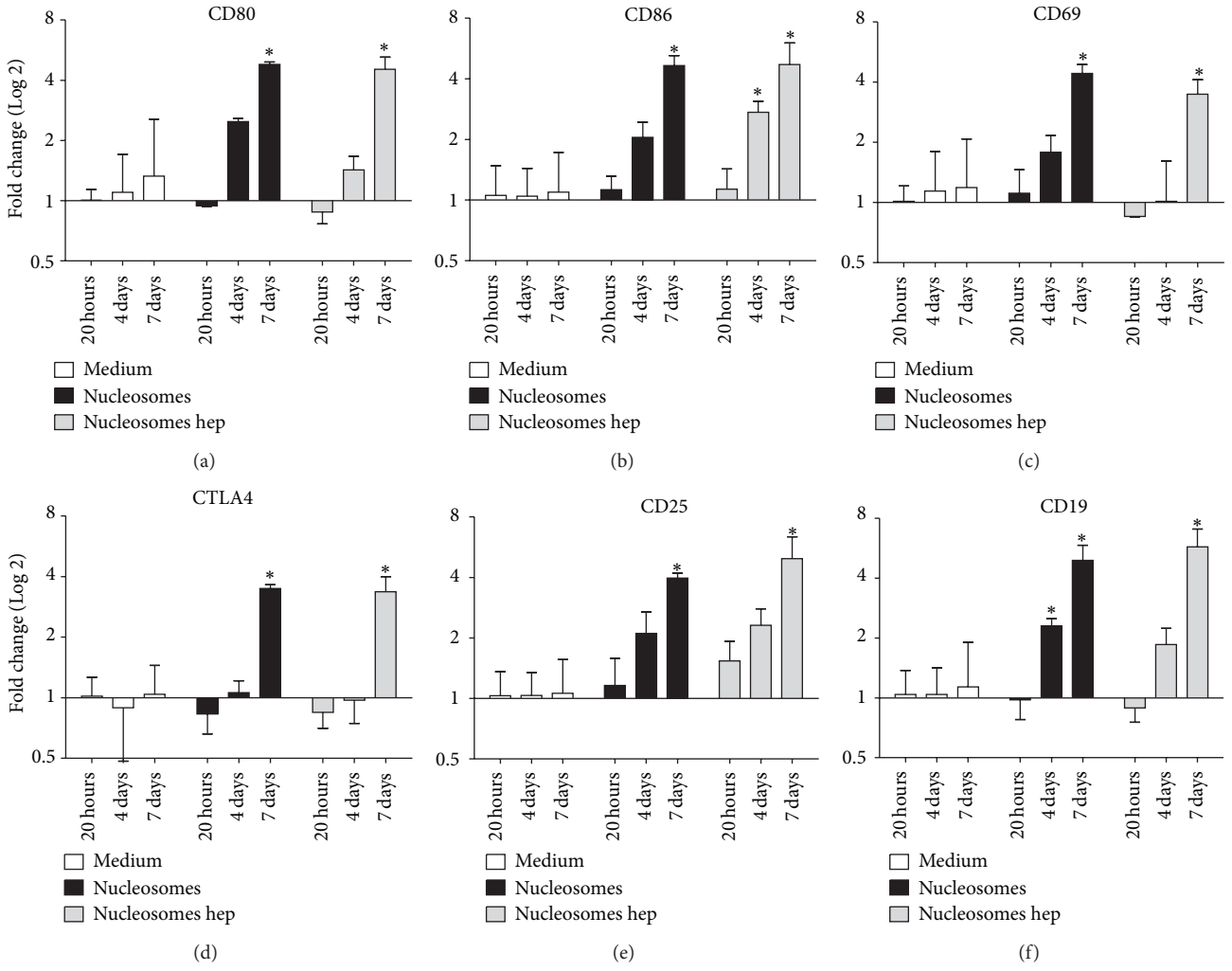

Figure 5: The mRNA levels of leukocyte activation markers in nucleosome- and nucleosome/heparin-stimulated splenocytes from a nephritic mouse. The mRNA levels of CD80 (a), CD86 (b), CD69 (c), CTLA-4 (d), CD25 (e), and CD19 (f) are all upregulated over time in response to stimuli with nucleosomes and nucleosomes/heparin. Expression levels for each gene, measured by qPCR, were normalized against the expression level of TBP. Data are given as Log 2 of mean ± SEM of fold change values normalized against medium stimulated cells and are representative for at least three independent analyses. Fold change is shown as mean of triplicates ± SD. *Statistically significant ($P < 0.05$) change in mRNA levels compared to medium stimulated cells at the same time point.

activation of T cells [40]. The upregulation of TLR 7, 8, and 9 seen in nucleosome-stimulated splenocytes indicates that nucleosomes activate APCs through interaction with these TLRs or that they activate cytokine production that will lead to their upregulation [41, 42]. Another TLR that has been implicated in inducing autoimmunity against nucleosomes is TLR2 which binds nuleosome-HMGB1 complexes [43]. This binding will result in activation of APCs with increased expression of costimulatory molecules required for activation of T cells [44]. The nucleosomes used in these experiments contained HMGB1 which explains the upregulation of TLR2 transcription [45]. Heparin can bind HMGB1 [46] which may dislocate HMGB1 from the nucleosomes and interfere with the binding and uptake by APCs through the TLR2 pathway. In our studies, we did not observe any difference when LMW heparin was added to the cultures. Stimulation with pure HMGB1 did not result in proliferative responses in splenocytes in agreement with previously reported results [43].

In this study we did not observe any inhibitory effects of LMW heparin on nucleosome-mediated activation of APCs or on proliferation of nucleosome specific T cells taken from the spleen. This indicates that even if LMW heparins bind nucleosomes and may change their net charge and conformation [19, 25, 47], it does not affect the uptake and presentation of nucleosomes to nucleosome specific T cells from mice with full-blown lupus nephritis. Thus, a more relevant explanation for the reduced anti-dsDNA antibody response in heparin-treated B/W mice would therefore be increased by enzyme-mediated elimination of the nucleosome as a central antigen [19]. Reduced load of nucleosomal antigens will lead to diminished activation of APCs, T cells, and B cells and will

consequently lead to reduced amount of autoantibodies. In context of the *in vitro* experimental results described in the present study, the concentration of nucleases and proteases available in the cell cultures may be too low to affect the elimination of nucleosomes, in contrast to the concentrations observed in sera [48]. In line with this, the reduced *in vivo* mRNA levels of CCR2, IL1β, and TLR7 indicate that reduced levels of nucleosomal antigens might lead to reduced activation of intrinsic cells and less influx of effector cells.

5. Conclusion

One of the beneficial effects of LMW heparin *in vivo* in B/W mice, demonstrating delayed development of autoimmunity to nucleosomes and lupus nephritis [19], relies on its ability to lower the inflammatory processes of immune complex deposition.

Authors' Contribution

Annica Hedberg, Premasany Kanapathippillai, and Kristin Andreassen Fenton contributed equally.

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Review Article

Interactions between Cytokines, Congenital Anomalies of Kidney and Urinary Tract and Chronic Kidney Disease

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Fetal hydronephrosis is the most common anomaly detected on antenatal ultrasound, affecting 1–5% of pregnancies. Postnatal investigation has the major aim in detecting infants with severe urinary tract obstruction and clinically significant urinary tract anomalies among the heterogeneous universe of patients. Congenital uropathies are frequent causes of pediatric chronic kidney disease (CKD). Imaging techniques clearly contribute to this purpose; however, sometimes, these exams are invasive, very expensive, and not sufficient to precisely define the best approach as well as the prognosis. Recently, biomarkers have become a focus of clinical research as potentially useful diagnostic tools in pediatric urological diseases. In this regard, recent studies suggest a role for cytokines and chemokines in the pathophysiology of CAKUT and for the progression to CKD. Some authors proposed that the evaluation of these inflammatory mediators might help the management of postnatal uropathies and the detection of patients with high risk to developed chronic kidney disease. Therefore, the aim of this paper is to revise general aspects of cytokines and the link between cytokines, CAKUT, and CKD by including experimental and clinical evidence.

1. Introduction

Fetal hydronephrosis is the most common anomaly detected on antenatal ultrasound, affecting 1–5% of pregnancies [1, 2]. Despite their high frequency of occurrence, there is little consensus on the management of infants with prenatal hydronephrosis (PNH) [3]. There have been a number of studies discussing the significance of fetal renal pelvic dilatation (RPD) as an indicator of urinary tract anomalies [4–7]. The degree of PNH varies from mild to severe, and intuitively, the degree of PNH should correlate with the severity of the underlying etiology [1, 2, 8]. More specifically, the risk of ureteropelvic junction obstruction (UPJO) increased significantly with greater degrees of PNH [9], but the risk of vesicoureteral reflux (VUR) was not significantly different among all severity groups. Most studies also have shown that a single postnatal US is unable to predict the presence or severity of VUR [6, 10, 11]. Consequently, postnatal management is heterogeneous, with some centers advocating detailed investigations including voiding cystourethrography (VCUG) in all cases and others indicating a less intensive approach [12–16]. Therefore, in spite of advances, the issue of postnatal diagnostic management of antenatal hydronephrosis remains a challenging problem [17, 18].

RPD can be an early sonographic sign of urinary tract obstruction or as a marker of other abnormalities such as renal duplication or VUR, which cannot be easily identified by US during pregnancy. Therefore, the patient is now presenting to the urologist or pediatric nephrologist before the baby is even born, with a presumptive diagnosis rather than

a symptom [19]. Consequently, infants diagnosed with PNH routinely undergo postnatal imaging evaluation. Classically, the prenatal diagnosis of hydronephrosis leads to postnatal investigations, including sonography, VCUG, and isotopic renography [17, 20]. Postnatal investigation has the major aim at detecting infants with severe urinary tract obstruction and clinically significant congenital anomalies of the kidney and urinary tract (CAKUT) among the heterogeneous universe of patients. Imaging techniques clearly contribute to this purpose. However, some of these exams are invasive and very expensive. Furthermore, sometimes imaging techniques are not sufficient to precisely define the indication of surgical approach as well as to determine the prognosis [21].

Biomarkers have recently become a focus of clinical research as potentially useful diagnostic tools in pediatric urological diseases [22]. Biomarkers are any tests that help distinguishing between two or more biological states and guide further clinical decision making [23]. In this regard, Muller et al. have reported that fetal serum ss2-microglobulin and cystatin C are good markers for postnatal renal function in bilateral renal hypoplasia and dysplasia [24]. More recently, Mersobian et al. [25] searched for specific proteins altered in UPJO by urinary proteome analysis and found a statistically significant difference in the expression of a number of urinary proteins and polypeptides between patients with UPJO and controls. These differences persisted at presentation and through time, although the profile of the candidate biomarkers varied according to the age of the patient. Further studies are needed to identify, among this group of proteins and polypeptides, which potential biomarker can help clinical decisions [25]. For instance, preliminary investigations looking at the urinary concentrations of transforming growth factor-beta (TGF-β) have suggested that this cytokine might be useful in detecting urinary tract obstruction and clinically relevant urinary tract anomalies among the heterogeneous universe of patients [26].

The obstructive nephropathy is not a simple result of mechanical impairment to urine flow but a complex syndrome resulting in alterations of both glomerular hemodynamics and tubular function caused by the interaction of a variety of vasoactive factors and cytokines that are activated in response to obstruction. The cytokines play a role in the development and progression of fibrotic and sclerotic changes in the obstructed kidney [27]. A large number of factors can initiate apoptosis, several of which may be related to obstructive nephropathy, such as hypoxia, ischemia, cytokines, growth factors, angiotensin II, endothelin-1, thromboxane, prostaglandins, and mechanical stretch [28–30]. However, it should be pointed that the biochemical, cellular, and molecular mechanisms of the obstructive uropathies are still largely unknown [28, 31]. The understanding of this process will certainly help in the management of fetal hydronephrosis and in the detection of patients at high risk for chronic kidney disease (CKD). In this regard, recent studies suggest a role for cytokines and chemokines in the pathophysiology of fetal hydronephrosis [28, 31, 32]. Indeed, we believe that the evaluation of these inflammatory mediators might help the management of CAKUT. The aim of this paper is to revise general aspects of cytokines and the link between cytokines, CAKUT, and CKD by including experimental and clinical studies. For this purpose, we have searched for articles at PubMed and Scopus by using the combination of words: "UPJO," "VUR," or "CAKUT" and "chemokines" or "cytokines." After this first step, we have selected the papers that evaluated cytokines as potential markers of clinical course, urinary tract obstruction, and/or CKD in pediatric patients. In that way, we have composed the list of papers presented in this review.

2. Cytokines: General Concepts and Characteristics

Cytokines are redundant secreted proteins with growth, differentiation, and activation functions that regulate and determine the nature of immune responses and control the immune cell trafficking and the cellular arrangement of immune organs. These mediators are involved in virtually every facet of immunity and inflammation, including innate immunity, antigen presentation, bone marrow differentiation, cellular recruitment and activation, and adhesion molecule expression. A cascade of responses is triggered in response to cytokines, and several cytokines acting together are required to express their optimal function. Numerous cytokines have proinflammatory properties such as interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), whereas others modulate the inflammatory response-like Interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) [33].

Chemokines constitute a large family of low molecular-weight cytokines whose main action is the recruitment and activation of leukocyte subsets in various models of inflammation—the word "chemokine" is a contraction of the terms "chemoattractant" and "cytokine" [34]. Tubular epithelial cells can be a rich source of inflammatory chemokines including regulated on activation, normal T expressed and secreted (CCL5/RANTES), monocyte chemotactic protein-1 (CCL2/MCP-1), Macrophage inflammatory protein 1 alfa (CCL3/MIP-1α), CX3CL1/fractalkine, and interleukin-8 (CKCL8/IL-8) [35]. Tubular epithelial cells are also targets for chemokines, since these cells respond to CCL2/MCP1 stimulation by releasing IL-6 and intracellular adhesion molecule-1 [36]. Messenger RNA for chemokine receptors can also be detected in podocytes and glomeruli [34].

3. Cytokines in Renal Diseases Related to CAKUT

A number of studies have shown the relation between renal diseases and cytokines production [28, 34, 37–40]. Indeed, the measurement of urinary, plasma, and renal tissue levels of cytokines has been used to monitor and diagnosis various urological and kidney diseases [34, 40, 41]. In this section, we reported studies that associated cytokines with relevant clinical consequences of CAKUT such as acute pyelonephritis, urinary tract obstruction, and renal scarring.

Acute pyelonephritis is most commonly observed in pediatric patients with CAKUT that resulted in urinary tract obstruction. However, predictive factors of renal scarring in patients with acute pyelonephritis remain unknown. In this regard, Sheu et al. [38, 39] evaluated serum and urinary levels of interleukin-1β (IL-1β), IL-6, and CXCL8/IL-8 in children with acute pyelonephritis. In the first study, these authors reported that the levels of IL1-β were significantly reduced in children with renal scarring, probably indicating a protective function for this cytokine [38]. IL-1β is primarily synthesized by cells of the mononuclear phagocyte lineage, but endothelial cells and neutrophils also produce this cytokine. The most important biological activity is its ability to activate T lymphocytes and to augment B-cell proliferation thus increasing immunoglobulin synthesis [33]. These effects might be responsible for a protection against renal scarring in patients with acute pyelonephritis. On the other hand, the same research group previously found that there is a significant elevation of serum and urinary levels of IL-6 and CXCL8/IL-8 in children with acute pyelonephritis when compared to children with lower urinary tract infection [39]. This finding supports the hypothesis that the release of IL-6 and of CXCL8/IL-8 from the urinary tract leads to systemic host responses [39], since IL-6 is a proinflammatory cytokine responsible for pyrexia and production of acute phase proteins [33], whereas CXCL8/IL-8 is a chemokine responsible for neutrophil infiltration into the urinary tract with an important role in acute inflammation [39]. In addition, gene polymorphisms of CXCL8/IL-8 seem to increase the susceptibility for acute pyelonephritis. For instance, the presence of the IL-8-251A allele in the genotype of children with urinary tract infection without vesicoureteral reflux has increased the risk of pyelonephritis [42].

In relation to renal scarring, TGF-β is a fibrogenic cytokine that stimulates extracellular matrix proteins deposition and scarring formation in kidney parenchyma. On the other hand, concerning immune system regulation, TGF-β exerts anti-inflammatory effects by inhibiting the proliferation of many different cell types [33]. Besides renal scarring, TGF-β also seems to be related to urinary tract obstruction. In this regard, Monga et al. [43] have studied 17 men with bladder outlet obstruction and 6 nonobstructed subjects and showed that, in the obstructed ones, the urinary levels of TGF- β were significantly higher than in non-obstructed.

4. Cytokines in CAKUT: Experimental Studies

Animal models have been frequently used to understand histopathological changes, mechanisms, and therapeutic approaches of obstructive nephropathies [41, 44–46]. The majority of the reported animal models utilized rats and mice, but rabbits, pigs, and sheep were also used [31].

Models of experimental postnatal unilateral ureteral obstruction have been developed in newborn rat pups that continue to exhibit active nephrogenesis in the postnatal period [31]. A partial unilateral ureteral obstruction was surgically created by entrapping the ureter in the animal psoas muscle, whereas the complete obstruction was produced by surgically clamping and occluding the ureter [31]. In rats, the major part of nephrogenesis occurs within 7 to 10 days after birth [47, 48]. Some models have used animals with congenital uropathies, while others have evaluated animals submitted to surgery after birth [47].

The induction of ureteral obstruction in newborn rats clearly interferes with ongoing nephrogenesis and this procedure usually leads to substantial renal damage [47]. This kind of experimental model mimics human ureteral obstruction at the second and third trimesters of pregnancy; however, significant renal damage is less common in infants [49]. The main features found in obstructive models are tubular cell apoptosis, mesenchymal myocyte transformation, and decreased glomerular endowment and glomerular injury [28, 48, 50]. The understanding of the pathophysiological mechanisms and the molecular events is important to define the moment of intervention [31]. Figure 1 shows the main mechanisms involved in models of obstructive uropathies.

Obstructed kidneys exhibited an elevation in angiotensin II activity, which, in turn, decreases renal blood and causes ischemia and kidney growth arrest. Although renal blood flow usually normalizes 6 weeks after the relief of temporary obstruction, renal growth remains altered, suggesting that other factors are responsible for growth impairment [47] such as the reduction in cell proliferation, the increase in cell apoptosis, and the progression of interstitial fibrosis [48].

Chevalier et al. [48] have studied neonatal rats submitted to unilateral ureteral obstruction or sham operation at one day of age, with relief five days later. In additional groups of neonatal rats, the operation was at 14 days, with relief at 19 days [48]. Three months following relief of unilateral ureteral obstruction during days 14 to 19, renal growth was decreased by 50%, compared to a 30% reduction following relief of unilateral ureteral obstruction during days 1 to 5. The number of glomeruli was reduced by approximately 50% regardless of the timing of obstruction, but glomerular size was reduced only in rats with unilateral ureteral obstruction from days 14 to 19 [48]. This study shows that, in the period immediately following nephrogenesis, the kidney is particularly susceptible to long-term injury from temporary unilateral obstruction. This suggests that a delay in relief of significant ureteral obstruction should be avoided if diagnosed in the perinatal or neonatal period [48]. The same group has also evaluated neonatal rats that underwent unilateral ureteral obstruction at one day of age whose obstruction was released at days 1, 2, 3, or 5 following the operation [51]. The growth of the obstructed kidney decreased linearly according to the duration of ureteral obstruction, while the contralateral kidney developed compensatory hypertrophy [51]. Indeed, contralateral renal hypertrophy should be considered as an important sign of advanced obstructive uropathy [52]. In summary, these animal models reveal that renal growth and function are impaired in proportion to the severity and duration of obstruction.

The microscopic alterations of obstructed kidneys are, initially, increased of tubular diameter secondary to tubular cell proliferation and dilatation. Next begins the apoptosis of tubular cell followed by the apoptosis of interstitial compartment [53]. There is a gradual, but continuous, apoptosis and

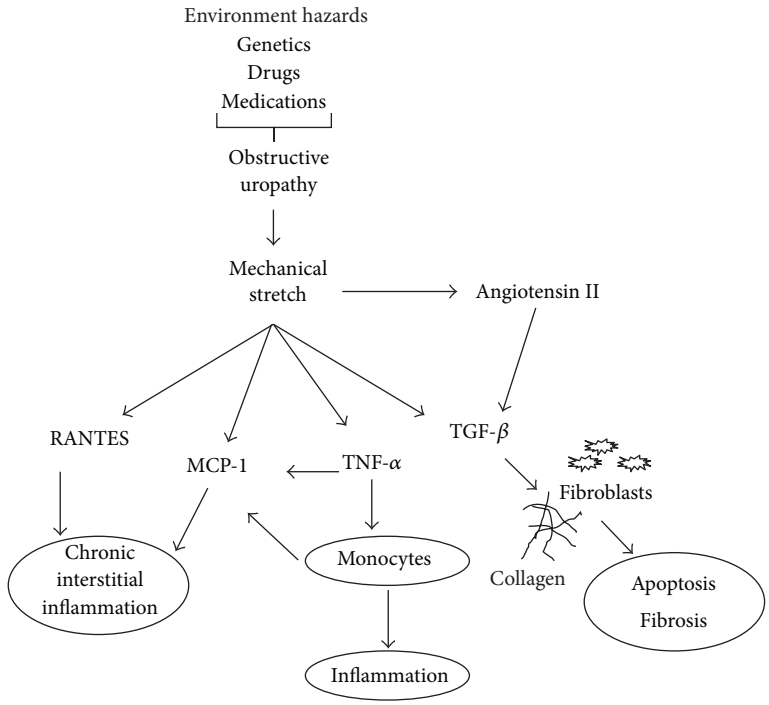

Figure 1: Potential mechanisms involved in obstructive uropathies.

proliferation of fibroblasts and inflammatory cells [53, 54]. Tubular cell apoptosis contributes to renal growth impairment [53], whereas proliferation of interstitial fibroblasts with myofibroblast transformation leads to excess deposition of the extracellular matrix and renal fibrosis [50]. Phenotypic transition of resident renal tubular cells, endothelial cells, and pericytes has also been implicated in this process.

A variety of intrarenal factors lead to progressive interstitial fibrosis, including growth factors and cytokines, such as angiotensin II, MCP-1, TGF-β, and adhesion molecules, which are produced by the hydronephrotic kidney [28]. Altered renal expression of growth factors and cytokines modulate cell death by apoptosis or phenotypic transition of glomerular, tubular, and vascular cells. Mediators of cellular injury include hypoxia, ischemia, and reactive oxygen species, while fibroblasts undergo myofibroblast transformation with increased deposition of extracellular matrix. On the other hand, a number of endogenous antifibrotic counter-regulatory molecules have been identified, opening the possibility of enhancing the kidney's own defenses against progressive fibrosis [28, 55].

Cytokines as TGF-β and TNF-α and chemokines like CCL2/MCP-1, CCL5/RANTES, macrophage inflammatory protein-2 (MIP-2), and γ-interferon-inducible protein (IP-10) have been evaluated in experimental hydronephrosis [27, 28, 31, 32].

TGF-β is highly involved in tubulointerstitial fibrosis. This cytokine increases matrix synthesis, collagen deposition, and tubular apoptosis, upregulates the integrin-matrix adhesion, and inhibits matrix degradation [32, 45, 56]. Resident renal tubular cells and interstitial cells may be responsible for TGF-β production; however, interstitial fibroblast cells seem to be the major source of TGF-β during the process of interstitial fibrosis [57]. In this regard, Mizuno et al. [58] found that the increased expression of TGF-β was correlated to fibrotic changes of interstitial regions in kidneys of mice subjected to unilateral ureteral obstruction. Accordingly, Seseke et al. [50] also detected the association between interstitial fibrosis and increased renal expression of TGF-β mRNA in an inbred strain of rats with congenital hydronephrosis. In addition, Zhou et al. [52] reported a marked elevation of renal TGF-β level in parallel to fibrotic changes of congenital and surgical ureteral obstruction in rats. Indeed, TGF-β expression increased significantly after completing nephrogenesis [47].

The role of TGF-β in obstructive nephropathies was also evidenced in other animal species. Seremetis and Maizels [56] have studied rabbit pups submitted to left partial ureteral constriction and human specimens of renal pelvis and ureter derived from cases of isolated renal obstruction managed by pyeloplasty and nephrectomy or of isolated vesicoureteral reflux managed by ureteral reimplantation. These authors have detected significantly higher expression of TGF-β mRNA in obstructed pelvis than in nonobstructed ones. This elevation in TGF-β mRNA expression was correlated to muscle hypertrophy and increased collagen deposition, both representing the process of renal pelvis remodeling in response to obstruction. The lower level of TGF-β mRNA expression may be a sign of less remodeling due to a steady state of obstruction. The expression of TGF-β mRNA emerges as a good predictor of early obstruction [56].

The molecular pathways for TGF-β receptor-mediated effects were also evaluated in experimental hydronephrosis [31]. In this context, Smad 3 is a protein responsible for

signaling downstream of the TGF-β receptors [59]. Sato et al. [60] have studied mice with genetic deletion of Smad3 and the wild type controls. The right proximal ureter was exposed and double ligated at 6–8 weeks of age. In the absence of Smad3, the formation of fibroblasts was blocked, clearly indicating a connection between fibrosis and TGF-β in obstructive uropathies [60]. In our point of view, animal models of CAKUT support the role of TGF-β as a potential biomarker for urinary tract obstruction. We also believe that translational studies should be done in order to establish the role of TGF-β in human CAKUT pathogenesis and to search for alternative pharmacological targets by inhibiting this cytokine.

TNF-α may play a role in initiating tubulointerstitial injury in obstructed kidney [28]. TNF-α stimulates the production of chemotactic factors by resident cells and upregulates CCL2/MCP-1 in human mesangial cells [28]. The increase of TNF-α at early stages of obstruction stimulates the production of chemoattractants for monocytes, which in turn contributes to leukocyte infiltration in obstructed kidneys [28]. Misseri et al. [61] have studied the expression of TNF-α mRNA in rats submitted to progressive degrees of left ureteral obstruction. Renal cortical TNF-α mRNA expression and protein production reached a peak at 3 days of ureteral obstruction. The TNF-α production, localized primarily to renal cortical cells, was not associated with significant inflammatory cell infiltrate [61]. Indeed, TNF-α might participate in initiating tubulointerstitial injury in the obstructed kidney by upregulating chemoattractants for monocytes and by producing leukocytes infiltration [32]. The data evaluating TNF-α are still very limited. However, considering that TNF-α has proinflammatory properties, it seems reasonable to investigate the role of this cytokine on the pathways linking tubulointerstitial injuries to CKD.

In relation to chemokines, Vielhauer et al. [62] found an increased expression of the CC chemokines, CCL2/MCP-1, and CCL5/RANTES, at sites of progressive tubulointerstitial damage in murine obstructive nephropathy model. It was also observed an interstitial infiltration of macrophages and T lymphocytes, which differentially expressed the CCR2 receptors. These data suggest that CCR2- and CCR5-positive monocytes and CCR5-positive lymphocytes are attracted by locally released CCL2/MCP-1 and CCL5/RANTES, resulting in chronic interstitial inflammation [62]. Indeed, CCL2/MCP-1 is an inflammatory chemokine that attracts and activates monocytes, T cells, and natural killer cells [33, 34]. In this regard, Stephan et al. [49] produced partial or complete ureteral obstruction in 28-day-old Wistar rats. These authors found that mRNA expression for CCL2/MCP-1 was moderately increased in partial ureteral obstruction, whereas kidneys without significant damage did not show any upregulation [49]. The study qualifies MCP-1 mRNA expression as a prognostic marker of partial ureteral obstruction [49]. On the other hand, Crisman et al. [63] detected the expression of CCL2/MCP-1, CCL5/RANTES, and IP-10 at 1 day of unilateral ureteral obstruction in mice, and, at 7 days, RANTES became the most abundant chemokine in the obstructed kidney [63]. Therefore, more studies still need to clearly define the role of CC chemokines in obstructive uropathies.

Other cytokines had also been associated to experimental models of CAKUT. For example, 75% of transgenic animals with overexpression of IL-9 developed congenital hydronephrosis, and the alteration was dependent on the presence of IL-4 and IL-13 [64]. In addition, Madsen [65] found significantly lower levels of IL-10 in renal parenchyma and urine of acute unilateral obstructed animals, while renal levels of IL-1β, IL-6, and TNF-α were increased to sham-operated animals.

The study of cytokines in hydronephrosis might provide new insights for the treatment or novel ways to blunt renal damage in obstructive uropathies. For instance, animals with right ureter obstruction treated with spironolactona exhibited less fibrosis than control group [46]. Since angiotensin II contributes at least in part to the increased expression of TNF-α mRNA in obstructed kidney [28], the use of angiotensin converting enzyme inhibitors emerges as an effective way in preventing renal fibrosis [44]. The use of statins also emerged as potential treatments. In this regard, the administration of atorvastatin ameliorated the tissue damaged of obstructed ureters in an experimental model [66]. The expression of TGF-β1 and of the proinflammatory cytokines IL-1β, IL-6, and TNF-α was decreased following atorvastain treatment [66]. Another rational approach to blunt renal fibrosis is to block growth factors effects. In this regard, Isaka et al. [57] showed that interstitial fibrosis could be blocked by TGF-β1 antisense oligodeoxynucleotides.

Additionally, the modulation of nitric oxide, epidermal growth factor (EGF), and hepatocyte growth factor seems to be a good strategy to treat obstructive nephropathy in the future [55, 58, 67]. In summary, there are very few studies on the role of immune markers as therapeutic targets in experimental CAKUT. However, the inhibition of proinflammatory and fibrogenic cytokines seems to be a reasonable strategy to preserve renal function.

5. Cytokines in CAKUT: Clinical Studies

It should be pointed that few data about the role of cytokines in CAKUT were provided by clinical studies and the majority of them evaluated ureteropelvic junction obstruction (UPJO) and vesicoureteral reflux (VUR).

5.1. Ureteropelvic Junction Obstruction. UPJO is the most common cause of severe hydronephrosis in children [68]. UPJO is unilateral in 90% of cases and may result from intrinsic narrowing at the junction between ureter and renal pelvis or extrinsic compression by an accessory lower pole artery of the kidney [21]. The degrees of hydronephrosis vary among patients with UPJO. The histological changes may vary from the absence of abnormalities to renal dysplasia with glomerulosclerosis and extensive interstitial fibrosis and tubular atrophy [69]. The UPJO area is consistently inflamed and has varying degrees of fibrosis and muscular hypertrophy [69].

Postnatal differentiation between obstructive and nonobstructive hydronephrosis is quite difficult. Several studies have been made in patients with UPJO in order to find out noninvasive biomarkers to allow the diagnosis and treatment of these patients. In this regard, cytokines and growth factors have been studied in UPJO [41]. The most relevant results were obtained with MCP-1, EGF, and TGF-β.

Healthy children presented high expression of EGF mRNA in renal tissue, whereas CCL2/MCP-1 mRNA was normally undetectable. On the other hand, in UPJO patients, CCL2/MCP-1 gene expression was strikingly increased at the tubulointerstitial level, while the EGF gene expression was markedly reduced. The interstitial mononuclear cell infiltrate in UPJO patients was strictly correlated with the degree of tubulointerstitial damage [70, 71]. Accordingly, the urinary concentrations of EGF were reduced in UPJO patients, whereas the CCL2/MCP-1 levels were increased [70, 72]. After surgical correction, there was a significant reduction in urinary levels of CCL2/MCP-1 accompanied by a marked increase in EGF concentration. Therefore, these two cytokines could be useful for the followup of obstructed patients [70]. In a prospective study, Madsen reported that urinary concentrations of EGF and of CCL2/MCP-1 were significantly increased in preoperative samples collected in UPJO patients before surgical procedure in comparison to urine from healthy children [65]. At this same study, the concentrations of CCL2/MCP-1, MIP-1α, IP-10, and RANTES were increased in urine from the obstructed kidney compared to urine from the contralateral nonobstructed kidney [65]. These urine samples were collected during the surgical procedure. One year after surgery, the concentrations of EGF, CCL2/MCP-1, MIP-1α, IP-10, and CCL5/RANTES were decreased to levels comparable to healthy controls [65, 73].

Taranta-Janusz compared obstructed PNH cases (who underwent surgery) with nonsurgically managed cases and with healthy subjects (control group). These authors found that urinary levels of CCL2/MCP-1 from voided urine before and after surgery and from the affected pelvis were significantly higher than nonsurgically managed cases as well than control group [74]. The authors also studied the level of osteopontin (OPN) and CCL5/RANTES in urine samples. Urinary levels of OPN were significantly higher in surgical cases than in nonsurgically managed patients [74]. Urinary levels of CCL5/RANTES were significantly higher in urine samples from affected pelvis collected during surgery than in voided urine before pyeloplasty [74]. Three months after surgery, the urinary levels of these three biomarkers did not return to control values [74].

Palmer et al. [75] have studied patients who undergoing pyeloplasty (UPJO patients), ureteral reimplantation (VUR patients), or circumcision/orchiopexy and measured urinary levels of TGF-β1 collected in bladder and pelvis. TGF-β1 concentrations were detected in all groups without significant differences in bladder samples. In contrast, the level of this cytokine was significantly elevated in the renal pelvis of children with UPJO when compared to the level obtained in the bladder of control group, of VUR group, and of UPJO patients [75]. More recently, Furness et al. [76] have measured urinary levels of TGF-β1 collected in the bladder and renal pelvis of patients with UPJO. Urinary levels of TGF-β1 in children with UPJO were 4-fold higher than in healthy controls, and samples obtained in renal pelvis had a 2-fold increase in cytokine concentrations when compared to bladder samples. In addition, if a cut-off point of 61 pg/mg creatinine was considered, a 92% of sensitivity was obtained for the urinary measurement of TGF-β1 in bladder [76]. The main concern of this study was the lack of correlation to patients with dilated nonobstructed uropathy conservatively managed. In a case-control study where 19 patients underwent pyeloplasty, Sager et al. found that when TGF-β1 levels were above 39.75 pg/mL, the patients have a 4.25-fold relative risk of having obstructive hydronephrosis compared with levels below 39.75 pg/mL [77].

El-Sherbiny et al. [78] have compared urinary TGF-β levels between obstructed and nonobstructed patients with grade 3 hydronephrosis. In obstructed patients, urinary concentrations of TGF-β measured in renal pelvis were 4-fold higher than the measurements in the bladder, which were, in turn, 3-fold higher than in healthy controls samples. There was also a trend in decreasing bladder TGF-β levels 3 months after surgical correction of obstruction. Furthermore, the measurement of urinary levels of TGF-β1 had 80% of sensibility and 82% of specificity for the recognition of obstruction [78]. At the same hospital in Egypt, Taha et al. [79] have evaluated 35 children with UPJO submitted to pyeloplasty who had grade 3 or higher hydronephrosis. These authors have found significantly elevated levels of TGF-β in UPJO group compared to healthy controls. The presence of high baseline urinary levels of TGF-β in younger children significantly increased the diagnostic accuracy of this measurement. In addition, there was a decrease of TGF-β concentration 1 month after of pyeloplasty that reached statistical significance 1 year after surgery [79]. The difference in the results obtained in both Egyptian studies might be due to time-point of the measurements: 3 versus 12 months after pyeloplasty.

Zieg et al. reported that urinary levels of TGF-β1 were significantly higher in patients with obstructive uropathies than in patients with nonobstructive hydronephrosis and healthy controls [80]. A positive correlation between urinary TGF-β1 levels and proteinuria was found in obstructive uropathies [80].

Older children normally have lower urinary levels of TGF-β1 in the bladder probably due to the reduction or the steady-state production of this cytokine in long-term obstruction [76, 78, 79]. In Canada, Almodhen et al. [26] have evaluated the role of TGF-β in the diagnosis and longitudinal followup of a homogeneous group of newborns with prenatal unilateral hydronephrosis. These authors showed that in the conservatively managed group the decrease in hydronephrosis grade through time was associated with a similar decrease in urinary concentrations of TGF-β1 [26]. This result indicates the utility of urinary measurement of TGF-β1 for monitoring patients with congenital hydronephrosis. In the surgical-treated group, urinary concentrations of TGF-β1 significantly decreased after pyeloplasty during a mean followup of 7 months. At a cut-off point of 17 pg/mmol of creatinine, the measurement of urinary TGF-β1 in the first 3 months of

Table 1: Studies on urinary cytokines in patients with UPJO.

Author	Year	Ref.	Age of patients	Cytokine	Study/control group (N)	Sensitivity	Specificity	Conclusions
Palmer et al.	1997	[75]	4.6 years (1 month to 11 years)	TGF-β_1	13/VUR (11) and healthy children (19)	—	—	Pelvic urinary TGF-β_1 levels are elevated compared to the control group
Furness et al.	1999	[76]	Median: 2.1 years	TGF-β_1	30/healthy children (19)	92%	—	Bladder urinary TGF-β_1 levels are significantly elevated
Grandaliano et al.	2000	[70]	1 months to 13 years	MCP-1; EGF	24/healthy children (15)	—	—	Bladder urinary EGF levels are reduced in UPJO, while MCP-1 levels are elevated
El-Sherbiny et al.	2002	[78]	5.2 ± 4.7 years	TGF-β_1	15/dilated non obstructed kidneys (11)	80%	82%	Elevated bladder urinary TGF-β_1 levels in obstructed kidneys decreased after surgery
Taha et al.	2007	[79]	Median: 5.9 years	TGF-β_1; EGF	35/healthy children (30)	100% (TGF-β_1)	80% (TGF-β_1)	Bladder urinary TGF-β_1 levels are significantly elevated, while no significant differences are detected in EGF levels
Almodhen et al.	2009	[26]	14 ± 6 months	TGF-β_1	42/—	82%	86%	Bladder urinary TGF-β_1 levels can predict the need for surgery
Sager et al.	2009	[77]	6.7 years ± 5.6	TGF-β_1	19/no renal pathology (19)	—	—	Bladder urinary TGF-β_1 levels in obstructed patients were higher than in controls, and renal pelvic urinary levels of TGF-β_1 in the hydronephrotic kidney were higher than preoperative bladder urine sample Postoperative TGF-β_1 concentration was significantly lower than preoperative
Bartoli et al.	2011	[72]	Functional UPJO: 55 (34) months; obstructive UPJO: 34 (28) months; underwent pyeloplasty group 80 (52) months; control 31 (23) months	MCP-1; EGF	76/30 healthy	—	—	Obstructive UPJO patients showed increased urinary levels of MCP-1 and decreased urine concentration of EGF. The urine EGF/urine MCP-1 and urine EGF/urine β2M ratios were significantly downregulated in untreated UPJO groups compared with control group, as well in the comparison between obstructive versus functional UPJO

Table 1: Continued.

Author	Year	Ref.	Age of patients	Cytokine	Study/control group (N)	Sensitivity	Specificity	Conclusions
Madsen, Madsen et al.	2013 2012	[65, 73]	8.1 (3.5–15) years at time of surgery	EGF, IP-10, MCP-1, MIP-1α, RANTES	28/13 healthy children	—	—	EGF and MCP-1 were significantly increased in preoperative UPJO samples. Concentration of MCP-1, MIP-1α, IP-10, and RANTES were increased in obstructed kidney and decreased one year after surgery
Taranta-Janusz et al.	2012	[74]	1.03 (0.08–14) years—surgical cases; 8 (0.75–17) years—conservative cases; 3 (0.33–16) years—control group	MCP-1, OPN, RANTES	15 surgical cases/21 conservative cases/19 control group			Only urinary MCP-1 has good diagnostic accuracy in identifying children with abnormal differential renal function (AUC 0.862) and in detecting kidney injury (AUC 0.704). MCP-1 levels from voided urine before and after surgery and from the affected pelvis were significantly higher than nonoperated patients and controls. Urinary levels of OPN were significantly higher in surgical cases than in nonoperated patients. Urinary RANTES was significantly higher in samples from affected pelvis during surgery than in voided urine before pyeloplasty. Three months after surgery, no significant changes were detected

Ref.: reference number.

life had 82% of sensibility and 86% of specificity in predicting surgery [26]. Besides different methodologies and timing of urine collection, TGF-β1 is the marker more investigated and promising in discriminating obstructive from nonobstructive CAKUT (Table 1).

5.2. Vesicoureteral Reflux. VUR is a congenital anomaly that increases the risk of repeated pyelonephritis and, consequently, can result in renal scarring, renin-mediated hypertension, and, in some cases, renal insufficiency [81, 82]. VUR is a heterogeneous condition that can be primary or associated with multicystic kidney, hypodysplastic kidneys, renal agenesia, and renal or ureteral ectopia. Kidneys with reflux nephropathy have disjointed glomeruli from proximal tubules, interstitial infiltration with chronic inflammatory cells, and periglomerular fibrosis. Dysplatsic feature is one of the characteristics of congenital reflux nephropathy. The main findings are areas of mesenchymal tissue containing primitive tubules [83].

Associations between gene polymorphisms of TNF-α, TGF-β and of VEGF with VUR were found [92–96]. Some of these polymorphisms were also associated to reflux nephropathy and progressive renal damage [94, 95]. Hussein et al. showed that specific variants in the promoter regions of the genes encoding TGFβ (−509T allele) and VEGF (−406CC genotype) were associated with an increased risk for the development of renal scarring [96]. These associations could help in understanding the mechanisms of reflux nephropathy and could allow the detection of patients at risk of CKD.

TNF-α and TGF-β are abundant in the smooth muscle cell of the ureter of VUR patients [97]. On the other hand, patients without VUR have higher expression of growth promoting factors like insulin growth factor-1 (IGF-1), nerve

Table 2: Studies on urinary cytokines in patients with vesicoureteral reflux (VUR).

Author	Year	Ref.	Age of patients	Cytokine	Study/control group (N)	Sensitivity	Specificity	Conclusions
Haraoka et al.	1996	[84]	Mean age 6.7 years	IL-8	32/—	—	—	Levels of IL-8 are elevated in patients with VUR or renal scarring
Ninan et al.	1999	[85]	5 months to 13.33 years	IL-6; TNF-α	17/healthy children (15)	—	—	Levels of IL-6 and TNF-α receptor-1 are elevated in reflux associated with renal damage
Wang et al.	2001	[86]	Mean age 14.6 years	IL-6	66/healthy children (28)			Levels of IL-6 are elevated in severe bilateral renal scarring
Galanakis et al.	2006	[87]	1 month to 2 years	IL-8	24/ITU+/VUR− (14); ITU−/VUR− (21)	88%	69%	Levels of IL-8 are elevated in VUR patients
Sabasiñska et al.	2008	[88]	6.23 ± 4.15 years	TGF-β_1	54/healthy children (27)	—	—	Highest urinary concentrations of TGF-β_1 are detected in grades IV and V reflux
Gokce et al.	2010	[89]	1 month 16 years	IL-6; IL-8	87/healthy children (27)	—	—	IL-6 levels are elevated in VUR and IL-8 levels in renal scarring
Merrikhi et al.	2012	[90]	ITU+/RVU+: 4.3 ± 2.9; ITU+/RVU−: 4 ± 2.6; control group: 4 ± 2.1	IL-8	28 (ITU+/VUR+); 28 (ITU+/VUR−); 28 healthy	71.4% (cutoff point: 3 pg/μmoL)	58.9% (cutoff point: 3 pg/μmoL)	IL-8 levels were significantly higher in patients with RVU. At the cutoff point of 3 pg/μmoL, IL-8 was accurate in detecting VUR
Tramma et al.	2012	[91]	71 ± 42.5 months	IL-6; IL-8	50/history of pyelonephritis (23 RS+/VUR+; 10 RS+/VUR−; 13 RS−/VUR−; 4RS−/VUR+)	—	—	Urinary levels of IL-8 were undetectable in all samples. There were no differences between urinary IL-6 levels in children with or without VUR. The levels of IL-6 were directly correlated with the grade of renal scars

Ref.: reference number.

growth factor (NGF), and vascular endothelial growth factor (VEGF) than those with VUR [97]. In this regard, Chertin et al. [83] have showed that the reduced production of EGF associated with high expression of CCL2/MCP-1 might cause an overproduction of proinflammatory and profibrotic cytokines that trigger apoptosis, ultimately leading to tubular atrophy and renal dysfunction in reflux nephropathy [83].

The inflammatory process in VUR is ongoing despite the occurrence or not of urinary tract infection (UTI). The elevated urinary level of CXCL8/IL-8 in children with reflux and without UTI might contribute to reflux nephropathy [84, 87]. Haraoka et al. [84] have found a significant difference between urinary levels of IL-8 in children with and without renal scarring and in patients with and without VUR. Merrikhi et al. [90] also showed significantly higher levels of IL-8 in patients with RVU than in those without RVU. This finding suggests that urinary IL-8 measurements could be useful to detect VUR patients with more pronounced renal damage and who need strict followup [84]. Galanakis et al. [87] proposed the use of IL-8 as a biomarker for the diagnosis of VUR. A cut-off concentration of 5 pg/μmol has a sensitivity of 88% and a specificity of 69% [87]. Our research group

has recently reported a correlation between high urinary levels of IL-8/CXCL8 and reduced glomerular filtration rate in CAKUT patients, suggesting that this chemokine might be associated to renal scarring and CKD [98].

IL-6 may also be involved in the pathogenesis of reflux nephropathy. IL-6 induces B and T cells activation and differentiation during inflammation [33]. Ninan et al. [85] have detected a significant elevation of urinary IL-6 levels in patients with VUR. In addition, Wang et al. [86] have found that urinary IL-6 was significantly higher in children with severe bilateral renal scarring than in those with mild scarring and normal controls. Gokce et al. [89] have related high urinary levels of IL-6 with the presence of VUR and increased IL-8 concentrations with renal scarring. Concerning serum measurements of cytokines, Jutley et al. [99] have detected significant elevation of IL-6 and TNF-α in patients with reflux nephropathy when compared to those without reflux nephropathy or to healthy controls.

Since the main histological alteration in reflux nephropathy is renal fibrosis, Sabasiñska et al. [88] have measured urinary levels of TGF-β in patients with VUR. These authors have found that urinary concentrations of TGF-β were increased in high-grade reflux and in bilateral cases [88]. Our research group studied the urinary concentrations of TGF-β, IL-6, and TNF-α in three different groups: idiopathic RPD, urinary tract anomalies, and dysplastic kidneys. TGF-β levels tended to be higher in the hypodysplastic kidney group compared to idiopathic RPD, while very similar values for IL-6 and TNF-α were found in these groups. On the other hand, urinary levels of TGF-β were significantly higher in patients with reduced dimercaptosuccinic acid (DMSA) uptake on technetium-99 m DMSA scintigraphy (AUC 0.67 [95%CI, 0.56–0.79]) [100]. A cut-off value of 2 pg/mL for TGF-β1 showed a sensitivity of 82.8% [95% CI, 64.2–94.1] and a specificity of 47.9% [95% CI, 35.9–60.1] for identifying those patients with reduced DMSA uptake [100]. Our findings also support the general idea that TGF-β has a role in renal fibrogenic processes.

Studies about renal scarring and VUR pathogenic process are still scarce making any powerful analysis very difficult. On the other hand, based on the available data, we consider that the proinflammatory cytokines (IL-6 and TNF-α), the chemokine, CXCL8/IL-8, and the fibrogenic cytokine, TGF-β, should be more intensively evaluated as potential biomarkers for renal scarring and for the emergence of CKD in reflux nephropathy (Table 2).

6. Concluding Remarks

CAKUT accounts for a great fraction of CKD in children [101]. Genetic, inflammatory, fibrogenic, environmental, and epigenetic factors responsible for these lesions are largely unidentified, and attention has been focused on minimizing obstructive renal injury and optimizing long-term outcomes to avoid or, at least, delay the progression of CKD. The renal response to urinary tract obstruction is complex and involves a wide array of interacting molecules in an early timing, being surgical *in utero* interventions performed when renal lesions were already irreversible [102].

New diagnostic approaches to and alternative therapies for CAKUT are clearly necessary. In this context, research into biomarkers has reached great importance. Clinical and experimental lines of evidence leave no doubt about the role of inflammation in renal diseases. Understanding the effects of cytokines on the onset and progression of renal injury is thus paramount, as new prognostic markers and maybe as alternative therapeutic targets.

Therefore, urine measurements of cytokines seemed to be useful in CAKUT as predictors of urinary tract obstruction and renal scarring. The chemokine CCL2/MCP-1 and the cytokine TGF-β have been frequently associated with urinary tract obstruction in patients with UPJO, whereas high urinary levels of IL-6 and of CXCL8/IL-8 were found in many patients with VUR and correlated to renal scarring and to renal function deterioration.

Yet, in spite of great advances in our knowledge about the pathophysiological mechanism linking the cytokines to CAKUT and CKD, much remains to be elucidated.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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Review Article

MicroRNAs Implicated in the Immunopathogenesis of Lupus Nephritis

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the deposition of immune complexes due to widespread loss of immune tolerance to nuclear self-antigens. Deposition in the renal glomeruli results in the development of lupus nephritis (LN), the leading cause of morbidity and mortality in SLE. In addition to the well-recognized genetic susceptibility to SLE, disease pathogenesis is influenced by epigenetic regulators such as microRNAs (miRNAs). miRNAs are small, noncoding RNAs that bind to the 3′ untranslated region of target mRNAs resulting in posttranscriptional gene modulation. miRNAs play an important and dynamic role in the activation of innate immune cells and are critical in regulating the adaptive immune response. Immune stimulation and the resulting cytokine milieu alter miRNA expression while miRNAs themselves modify cellular responses to stimulation. Here we examine dysregulated miRNAs implicated in LN pathogenesis from human SLE patients and murine lupus models. The effects of LN-associated miRNAs in the kidney, peripheral blood mononuclear cells, macrophages, mesangial cells, dendritic cells, and splenocytes are discussed. As the role of miRNAs in immunopathogenesis becomes delineated, it is likely that specific miRNAs may serve as targets for therapeutic intervention in the treatment of LN and other pathologies.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the loss of immune tolerance to nuclear self-antigens. The deposition of autoantibodies along the glomerular basement membrane results in immune complex-mediated glomerulonephritis [1]. Mesangial cells, the primary immunoregulatory cells in the renal glomerulus, become activated due to the deposition of ICs. This recruits macrophages, B cells, T cells, and dendritic cells (DCs) to the kidney. Activated macrophages, mesangial cells, and DCs induce the maturation and activation of infiltrating T cells, which further activate macrophages and increase the B cell response [2]. Lupus nephritis (LN) is the major cause of morbidity and mortality in patients with SLE, affecting up to 70% of SLE patients [3]. Histological features include increased numbers of mesangial cells, overproduction of extracellular matrix, and inflammatory cell infiltration, which can lead to the development of sclerosis and fibrosis [4]. Depending on the severity of disease, 10–30% of LN patients will progress to end-stage renal failure [5].

It has been shown that genetic predisposition coupled with known and unknown environmental factors contributes to the development of SLE [6, 7]. Epigenetic defects have also been shown to play an important role in LN pathogenesis [8–10]. Epigenetics, which includes microRNA (miRNA) regulation, refers to stable and heritable changes in gene expression that alter the phenotype but not the underlying DNA sequence itself. miRNAs are small, non-coding RNAs that endogenously regulate gene expression by partially binding to the 3′ untranslated region (UTR) of target mRNAs [11–14]. miRNAs contribute to diverse physiological and pathophysiological functions including cell developmental timing, cell cycle control, apoptosis, and carcinogenesis [15, 16]. Hematopoiesis is fine-tuned by miRNAs at virtually every step [17]. In the last decade, increasing evidence has shown that miRNAs are critical not only for the regulation of

immune cell development but also for modifying innate and adaptive immune responses [18].

A computational analysis performed on 72 lupus susceptibility genes in humans and mice revealed that most lupus susceptibility genes contain numerous target sites for over 140 conserved miRNAs. Three miRNAs (miR-181, miR-186, and miR-590-3p) are predicted to target over 50% of all lupus susceptibility genes [19]. Several studies have suggested that miRNAs play a role in the pathogenesis of LN by altering proinflammatory mediator production, innate immune cell responses, lymphocyte function, and Toll-like receptor (TLR) and NFκB signaling pathways [20–24]. For example, miRNAs can induce the expression of proinflammatory cytokines, dictating the magnitude and duration of the immune response [25, 26]. miRNA dysregulation can result from genetic variation, hormonal influences, environmental triggers, or even the proinflammatory environment itself [27]. Lipopolysaccharide (LPS) induces the expression of miRNAs and activates transcription factors that further regulate miRNA expression [28, 29]. LPS has been shown to induce the expression of several miRNAs including miR-9, miR-132, miR-146, miR-155, and miR-let-7a (let-7a) [30–33]. Dysregulated miRNA expression may represent an underlying trigger that induces multifactorial diseases such as SLE.

As pathogenic miRNAs are identified in LN pathogenesis, treatment strategies aimed at altering miRNA expression or signaling pathways may be employed to ameliorate disease pathogenesis in patients with SLE. Determining a patient's miRNA expression profile from the blood or urine may allow the use of targeted therapies to specifically modulate abnormal miRNA expression patterns in individuals suffering from lupus. It has been well documented that lupus patients respond to immunosuppressive agents with varying degrees of efficacy [34]. This has presented a major challenge in selecting the most effective treatment option. Determining how particular therapeutics alter pathogenic miRNAs may ultimately provide a viable screening tool for specific, targeted therapy in SLE. In this review, we summarize the current data on miRNAs in the major immune cells as related to LN pathogenesis and examine the future directions in miRNA-based therapy for SLE.

2. miRNAs Broadly Implicated in Inflammatory Diseases

2.1. miR-21. miR-21 is induced upon inflammatory stimulation and is a key component of TLR, NFκB, and signal transducer and activator of transcription (STAT) signaling pathways [35–37]. The 3′ UTR of *programmed cell death 4* (*PDCD4)* is a direct target of miR-21. PDCD4 is a proinflammatory protein that promotes NFκB activation and suppresses production of the anti-inflammatory cytokine IL-10. Overexpression of miR-21 in LPS-stimulated murine macrophages blocked NFκB activity, decreased PDCD4 production, and promoted the production of anti-inflammatory IL-10. PDCD4-deficient mice are protected from LPS-induced death, presumably by an IL-10-mediated reduction in NFκB activation [38].

miR-21 has been implicated in the immunopathogenesis of numerous inflammatory diseases [39]. Using an *in vitro* model of diabetes, Kato et al. showed that miR-21 overexpression in glucose-stimulated mesangial cells prevented cell proliferation by downregulating *tumor suppressor phosphatase and tensin homolog* (*PTEN*), whose 3′ UTR contains a binding site for miR-21 [40, 41]. miR-21 expression is induced by STAT3, a transcription factor activated by IL-6. miR-21 inhibition of PTEN leads to increased NFκB activation that is required to maintain the transformed state. miR-21, PTEN, NFκB, IL-6, and STAT3 are dynamic players in the positive feedback loop linking inflammation to cancer [35].

2.2. miR-146a. miR-146a may contribute to lineage determination in T cells as it is one of the only miRNAs that is differentially expressed in highly purified subsets of murine Th1 and Th2 cells [42]. Lu et al. demonstrated that miR-146a knockout mice develop a fatal immune-mediated disease similar to Foxp3 knockout mice that are devoid of functional Treg cells [43]. Although miR-146a knockout mice have increased Treg cells, their suppressive function is impaired. Treg cells without miR-146a (or Foxp3) acquire the ability to produce proinflammatory cytokines such as interferon-γ (IFN-γ). miR-146a deficiency in Treg cells caused an increase in STAT1 production, a key transcription factor required for Th1 effector cell differentiation. Because miR-146a regulates Treg cell suppressor function, the authors suggest that miR-146a maintains an optimal threshold of cytokine receptor-dependent activation of transcription factors that are necessary to suppress Th1 responses [43].

Since miR-146a has been reported to be an important negative regulator of acute responses during the activation of innate immunity, it has been suggested to play a regulatory role in the pathogenesis of SLE. miR-146a is induced by TLR activation (via LPS stimulation) and by proinflammatory mediators including tumor necrosis factor-α (TNF-α) and IFN-α [30]. miR-146a negatively regulates type I IFN production and myeloid differentiation factor 88 (MyD88) pathway activation induced by TLR stimulation [44]. Upon LPS-stimulated induction, miR-146a directly decreases TRAF6 and IRAK1 production, two signal transducers in the NFκB activation pathway whose 3′ UTRs contain multiple miR-146a target sequences [30, 45]. Therefore, miR-146a reduces or terminates the inflammatory response through a negative-feedback loop by downregulating *TRAF6* and *IRAK1*.

Due to its importance in the control of inflammation, several studies have sought to determine if miR-146a-based therapy can improve disease outcome in lupus-prone animal models or human patients. It was recently shown that treatment with the anti-inflammatory drug calcitriol alters the expression of miR-146a in SLE patients. Sera miR-146a expression, which is downregulated in patients with SLE, was significantly increased in patients after treatment with calcitriol for 6 months [46, 47]. These findings suggest that the immunomodulatory effects exerted by calcitriol in patients with SLE may be due, in part, to alterations in miR-146a expression. In addition, sera levels of miR-146a may be used to monitor treatment response.

2.3. miR-155. Like miR-146a, miR-155 is vital to proper immune system functioning; it is highly expressed in Treg cells and is induced upon activation of T effector cells and myeloid cells [48, 49]. miR-155 is induced in macrophages in response to both bacterial and viral antigens, functions in the hematopoietic compartment to promote the development of inflammatory T cells, and is required for DC production of Th17-promoting cytokines [28, 50]. By developing miR-155-deficient mice, Rodriguez et al. found that miR-155 is required for the proper functioning of DCs, B cells, and T cells [51]. The DCs of miR-155-deficient mice were unable to effectively activate T cells, indicating a defect in antigen presentation or costimulatory function. As they aged, the lungs of miR-155-deficient mice showed increased airway remodeling due to the increased numbers of lymphocytes in bronchoalveolar lavage fluids. The authors noted that these changes are similar to the lung fibrosis that often complicates systemic autoimmune processes with lung involvement [51]. Zhou et al. examined the regulatory role of miR-155 in the regulation of plasmacytoid dendritic cell (pDC) activation and type I IFN production [52]. They found that miR-155 is upregulated upon TLR stimulation, providing another example of the link between stimulation, miRNA expression, and cellular activation. These studies show that miR-155 is broadly implicated in LN pathogenesis and dysregulated miR-155 expression may play various roles in pathophysiology by altering immune cell function [52].

3. LN-associated miRNAs in Tissues

3.1. Renal Tissue. miRNA expression profiles of renal tissue have gained much attention since Dai et al. provided a broad analysis of differentially expressed miRNAs in LN kidney biopsies [53]. They identified 36 upregulated and 30 downregulated miRNAs in LN renal tissue compared to healthy controls. Their previous studies had identified 16 differentially expressed miRNAs in the peripheral blood mononuclear cells (PBMCs) of SLE patients, none of which constituted any of the 66 miRNAs identified in SLE kidney biopsies. These studies suggest that miRNA expression patterns are cell and organ specific [53, 54].

Lupus rodent models have revealed miRNAs implicated in LN pathogenesis (Table 1). In the anti-Thy1.1 rodent model of glomerulonephritis, TGF-β and other cytokines and growth factors promote mesangial cell proliferation and activation, leading to mesangial proliferative glomerulonephritis [55]. Denby et al. identified 2 miRNAs (miR-21 and miR-214) that are induced upon transforming growth factor-β (TGF-β) stimulation *in vitro* and characterized them further using the anti-Thy1.1 rat model [56]. TGF-β-induced overexpression of miR-21 and miR-214 in tubular epithelial cells caused epithelial-mesenchymal transition- (EMT-) like changes characterized by decreased *E-cadherin* expression and increased α-smooth muscle actin (α-*SMA*) and *collagen type I* expression. These changes are characteristic of proliferating cells and tissue remodeling [57]. Blocking TGF-β downstream signaling in rat epithelial cells decreased the expression of miR-21 and miR-214 and prevented TGF-β-induced EMT by increasing *E-cadherin* expression and decreasing α-*SMA* and *collagen type I* expression. These results suggest that TGF-B-induced miR-21 and miR-214 expression may contribute to extracellular matrix production and mesangial proliferative glomerulonephritis [56].

miRNAs that contribute to inflammation in chronic kidney disease (CKD) were recently examined in the B6.MRLc1 model, a congenic strain carrying a region of chromosome 1 derived from MRL/MpJ mice that develop IC-mediated glomerulonephritis [58]. miR-146a expression was found to be significantly increased in B6.MRLc1 kidneys compared to healthy controls. B6.MRLc1 mice that expressed high levels of miR-146a expression showed severe glomerular and interstitial lesions including cell infiltration, tubular atrophy, and interstitial fibrosis. The lesions had increased macrophage and T cell infiltration as well as increased expression of cell-specific mRNAs associated with the development of renal lesions (*CD68* and *S100a4* for macrophages and fibroblasts, resp.) [59]. miR-146a expression was also positively correlated with *IL-1β*, *IL-10*, and *CXCL* expression. Because miR-146a is increased in the kidneys of B6.MRLc1 mice and continues to increase as they age, this model may be predisposed to increased miR-146a expression that initiates and perpetuates renal inflammation [59].

Research by Lu et al. confirmed that miR-146a is upregulated in glomerular tissue from LN patients and found that miR-146a is not overexpressed in LN tubulointerstitial tissue [60]. miR-638 expression, on the other hand, was underexpressed in glomerular tissue but higher in tubulointerstitial tissue. Glomerular expression of miR-146a positively correlated with both estimated glomerular filtration rate (GFR) and histological activity index, determined from the sum of semiquantitative scores of inflammation parameters [61]. Increased tubulointerstitial expression of miR-638 was positively correlated with proteinuria and SLE Disease Activity Index (SLEDAI) score. While the correlation between changes in miRNA expression and clinical disease severity suggests that these miRNAs may play a role in the pathogenesis of LN, it is currently unknown whether changes in miR-638 expression are pathogenic or an epiphenomenon [60].

3.2. PBMCs. While miR-146a was not initially reported to be decreased in SLE PBMCs, other miRNA expression screenings have revealed that miR-146a is significantly decreased in SLE patients and is inversely correlated with SLEDAI and IFN-α/β scores in SLE patients [46, 54, 62]. Furthermore, *in vitro* studies by Tang et al. revealed that overexpression of miR-146a reduced type I IFN induction in PBMCs [62]. They found that miR-146a negatively regulates both type I IFN production and TLR-stimulated downstream pathway activation by targeting the 3′ UTR of *interferon regulatory factor-5* (*IRF5*) and *STAT1*, key components in the type I IFN signaling cascade. The authors concluded that miR-146a deficiency is one of the causal factors in the abnormal activation of the type I IFN pathway in SLE [62].

A follow-up study identified a functional variant in the promoter of miR-146a that is associated with SLE disease risk; the promoter mutation resulted in decreased binding to the transcription factor ETS-1. Intriguingly, genomewide association studies have identified an association between

Table 1: miRNAs implicated in LN pathogenesis.

Cell or tissue type	miR ID(s)	Origin	Strain	Expression	Results	Mechanism(s)	Reference
Renal	21 and 214	R	WKY (anti-Thy1.1)	↑	Expression is induced by TGF-β in tubular epithelial cells *in vitro* and in renal tissue *in vivo*	Unknown	[56]
					Overexpression in tubular epithelial cells *in vitro* decreased *E-cadherin* expression and increased *collagen type I* and *α-SMA* expression	Unknown	[56]
	146a	M	B6.MRLc1	↑	Increased expression positively correlated with *IL-1β*, *IL-10*, and *CXCL* expression, severe glomerular and interstitial lesions, and T cell and macrophage infiltration	Unknown	[59]
		H	—	↑	Glomerular expression positively correlated with estimated GFR and histological activity index	Unknown	[60]
	638	H	—	↑	Tubulointerstitial expression positively correlated with proteinuria and disease activity score	Unknown	[60]
PBMCs	21	H	—	↑	Strongly correlated with disease activity and activated T cells	Unknown	[23]
					Inhibition *in vitro* reversed the activated T cell phenotype by increasing *PDCD4* expression	The 3′ UTR of *PDCD4* is a target of miR-21	[23]
	125a	H	—	↓	Underexpression contributes to the elevated expression of *RANTES* (*CCL5*) in SLE, increasing T cell recruitment to inflammatory tissues	The 3′ UTR of the *RANTES* upstream regulator *KFL13* is a target of miR-125a, indirectly increasing *RANTES* expression	[65]
	145	H	—	↓	Decreased expression increased *STAT1* expression in SLE patients	The 3′ UTR of *STAT1* is a target of miR-145	[64]
	146a	H	—	↓	Inversely correlated with disease activity and IFN-α/β scores	Unknown	[62]
					Overexpression reduced the induction and downstream effects of type I IFN	The 3′ UTR of *IRF5* and *STAT1* are targets of miR-146a, reducing the induction of type I IFN	[62]
					Promoter variant associated with SLE disease risk	SLE-associated SNP (rs57095329) decreases miR-146a expression levels	[63]
	155	H	—	↓	Positively correlated with GFR, CRP, and other renal function parameters; inversely correlated with proteinuria and SLEDAI	Unknown	[46]
					Positively correlated with GFR, CRP, and other renal function parameters	Unknown	[46]
	224	H	—	↑	Increased expression accelerated T cell activation-induced cell death by suppressing *API5* expression in SLE patients	The 3′ UTR of *API5* is a target of miR-224	[64]
Mesangial cells	Let-7a	M	NZB/W	↑	Increased expression throughout the lifetime of NZB/W lupus mice; overexpression increased *IL-6* expression and IL-6 production *in vitro*	The 3′ UTR of *IL-6* is a target of let-7a; the exact mechanism of let-7a is unknown	[32]

Table 1: Continued.

Cell or tissue type	miR ID(s)	Origin	Strain	Expression	Results	Mechanism(s)	Reference
Dendritic cells	155	H	—	↑	Induced by TLR stimulation after miR-155*; overexpression of miR-155 in normal pDCs significantly decreased *IFN-α*, *IFN-β*, and *TNF-α* expression	The 3′ UTR of the type I IFN regulator *TAB2* is a target of miR-155, indirectly decreasing IFN-*α* and IFN-*β*	[52]
	155*	H	—	↑	Induced by TLR stimulation before miR-155; overexpression of miR-155* in normal pDCs significantly increased *IFN-α*, *IFN-β*, and *TNF-α* expression	The 3′ UTR of the negative IFN regulator *IRAKM* is a target of miR-155*, indirectly increasing IFN-*α* and IFN-*β*	[52]
Splenocytes	15a	M	NZB/W	↑	Increased expression after disease was accelerated by IFN administration; differentially expressed in B cell subsets	Unknown	[69]
	21	M	B6.Sle123	↑	Inhibition increased *PDCD4* expression in T cells and reversed splenomegaly, improving overall disease outcome	The 3′ UTR of *PDCD4* is a target of miR-21	[70]
		M and H	MRL-*lpr*	↑	Downregulated *DNMT1* expression in T cells	The 3′ UTR of the *DNMT1* upstream regulator *RASGRP1* is a target of miR-21, indirectly downregulating *DNMT1*	[71]
	126	H	—	↑	Overexpression contributes to T cell autoreactivity by decreasing *DNMT1* expression	The 3′ UTR of *DNMT1* is a target of miR-126	[72]
					Overexpression in healthy donors was sufficient for T cell autoreactivity and B cell hyperstimulation, while inhibition in SLE patients resulted in T and B cell inactivation	Unknown	[72]
	142-3p and 142-5p	H	—	↓	Underexpressed in SLE $CD4^+$ T cells	Dysregulated DNA and histone methylation of the miR-142 promoter	[73]
					Underexpression in $CD4^+$ T cells increased production of CD84, IL-10, and SAP	The 3′ UTR of *CD84* and *IL-10* are targets of miR-142-3p; the 3′ UTR of *SAP* is a target of miR-142-5p	[73]
					Inhibition in healthy donor $CD4^+$ T cells caused T cell overactivation and B cell hyperstimulation, while overexpression in SLE $CD4^+$ T cells had the opposite effect	Although CD84 and SAP stimulate T-B cell interactions, the exact mechanism of miR-142 is unknown	[73]
	146a	M	MRL/*lpr*	↑	Increased expression associated with disease development	Unknown	[74]
	148a	M and H	MRL/*lpr*	↑	Downregulated *DNMT1* expression in T cells	The protein coding region of the *DNMT1* transcript is a target of miR-148a	[71]
					Induced overexpression of autoimmune-associated, methylation-sensitive genes in $CD4^+$ T cells including *CD70* and *LFA-1*	Inhibition of *DNMT1* results in DNA hypomethylation and the overexpression of methylation-sensitive genes	[71]
	155	M	MRL/*lpr*, NZB/W	↑	Increased expression associated with disease development	Unknown	[74]

Abbreviations: H: human; M: mouse; R: rat.
↑: increased expression; ↓: decreased expression.
α-SMA: *α*-smooth muscle actin; API: apoptosis inhibitory protein; CRP: C-reactive protein; DNMT: DNA methyltransferase; GFR: glomerular filtration rate; IFN: interferon; IL: interleukin; IRAK: IL-1 receptor-associated kinase, IRF: interferon regulatory factor; NZB/W: New Zealand Black/White; PBMCs: peripheral blood mononuclear cells; pDC: plasmacytoid dendritic cell; SNP: single-nucleotide polymorphism; PDCD: programmed cell death; SLE: systemic lupus erythematosus; SLEDAI: SLE Disease Associated Index; STAT: signal transducer and activator of transcription; TGF-*β*: transforming growth factor-*β*; TTP: tristetraprolin.

SLE risk and a functional variant of *ETS1*. The researchers observed additive effects of the risk alleles of miR-146a and *ETS1*, which suggests that individuals with 2 or more of these alleles are at a greater risk of developing SLE than those carrying only one allele [63].

Stagakis et al. identified 27 differentially expressed miRNAs in the PBMCs of SLE patients, 2 of which corresponded with the miRNAs identified by Dai et al. and 19 of which correlated with disease activity [23, 54]. Of these disease-correlated miRNAs, eight were differentially expressed in T cells and 4 in B cells. Upregulation of miR-21 strongly correlated with disease activity and activated T cells; inhibition of miR-21 reversed the activated T cell phenotype by increasing *PDCD4* expression [23]. Another recent study found 7 abnormally expressed miRNAs (miR-145, miR-224, miR-150, miR-483-5p, miR-513-5p, miR-516a-5p, and miR-629) in SLE T cells compared to healthy controls. In a larger follow-up study, underexpression of miR-145 was confirmed and increased levels of *STAT1*, a target of miR-145, were observed in SLE T cells compared to healthy controls. Overexpression of miR-224 and decreased expression levels of its target, *apoptosis inhibitory protein 5* (*API5*), were also confirmed. T cells transfected with miR-224 *in vitro* were more susceptible to activation-induced apoptosis, indicating that SLE T cells overexpressing miR-224 may have an intrinsic defect that causes accelerated cell activation-induced apoptosis [64].

An additional study examining PBMC miRNAs found that decreased miR-125a expression in SLE patients contributed to increased KLF13 production by T cells. miR-125a has binding sites in the 3′ UTR of *KLF13*, which belongs to the family of transcription factors that regulates the expression of the inflammatory chemokine *RANTES* (*CCL5*) in T cells. Increased *RANTES* expression is associated with persistent or recurrent organ inflammation due to its recruitment of T cells to inflammatory tissues. Increasing miR-125a levels in T cells from SLE patients *ex vivo* alleviated elevated *RANTES* expression. This study confirmed that underexpression of miR-125a contributes to the elevated expression of *RANTES* in SLE, increasing T cell recruitment to inflammatory tissues [65].

4. LN-associated miRNAs in Innate Immune Cells

The innate immune response provides the initial defense against infection by external pathogens and is predominantly mediated by macrophages, DCs, and neutrophils. The presence of pathogens is commonly detected by macrophage and DC TLRs that bind conserved microbial products, triggering downstream signaling pathways to initiate inflammatory responses [66]. Through TLR activation, ICs from lupus patients induce pDCs to secrete type I IFN [67]. Activated DCs induce maturation and activation of infiltrating T cells, which further activates macrophages and increases the B cell response. The innate immune response, in particular DCs, promotes the activation of the adaptive immune system [68]. Since miRNAs are critical for modifying innate and adaptive immune responses, dysregulated miRNA expression may represent an underlying cause to LN pathogenesis (Table 1).

4.1. Macrophages/Mesangial Cells. miRNA expression is directly and indirectly altered after TLR activation and regulates macrophage signaling pathways that lead to the secretion of proinflammatory cytokines [28, 31, 75]. Let-7a and miR-147 are directly induced upon LPS stimulation due to NFκB binding sites in their promoter regions, which induces the expression of proinflammatory cytokines including TNF-α and IL-6 [29, 75]. TNF-α, a critical cytokine involved in the response to LPS stimulation, increases miR-155 expression via JNK pathway activation, further increasing TNF-α production [28, 76, 77]. Inhibition of JNK blocks the induction of miR-155, demonstrating that upregulated miR-155 expression in LPS-stimulated macrophages is indirectly due to JNK pathway activation [31, 75]. These well-defined positive feedback loops demonstrate that stimulation-dependent miRNA expression induces cytokine production that further activates cells, which continues to alter miRNA expression.

Mesangial cells, the primary immunoregulatory cells resident to the renal glomerulus, possess phagocytic and contractile properties. Regulatory mechanisms of mesangial cells include a complex array of factors that control cell proliferation, survival, apoptosis, and GFR. Mesangial cells from LN patients and lupus-prone mice have a heightened response to inflammatory stimulation [78, 79]. Mesangial cells from NZB/W mice have been shown to produce significantly more chemokines in response to LPS stimulation than controls [80]. Kato et al. demonstrated the involvement of miRNAs in mesangial cell activation [41]. They determined that TGF-β activates Akt in glomerular mesangial cells by inducing miR-215a and miR-217, revealing a role for miRNAs in kidney disorders. We recently found that let-7a expression was significantly increased in the mesangial cells of prediseased and actively diseased New Zealand Black/White (NZB/W) mice compared to age-matched New Zealand White (NZW) mice. Using *in vitro* techniques, we demonstrated that let-7a has a key role in regulating IL-6. Overexpression of let-7a increased IL-6 production in stimulated mesangial cells compared to nontransfected controls. Increased let-7a expression in the prediseased and diseased state may contribute to the increase in IL-6 production in young and old NZB/W mice. These data suggest that increased let-7a expression may predispose lupus mice to increased inflammatory mediator production with immune stimulation [32].

4.2. Dendritic Cells. Another significant immune cell that contributes to immunity in complex ways is the dendritic cell. DCs are widely considered to be critical for activating T cell responses, promoting B cell antibody production, and secreting cytokines in response to infections [81]. In these ways they may direct autoimmunity and tolerance by serving as the primary antigen presenting cells (APCs) to initiate T cell autoimmunity, promoting B cell autoantibody production, and secreting proinflammatory cytokines. Altered function of DCs is known to play a major role in the development of autoimmunity [82]. A recent study examining the functional characteristics of DCs in lupus patients found a significant increase in the percentage of cytokine-producing DCs in addition to an increase in the amount of cytokine per cell in SLE patients compared with healthy subjects [83]. pDCs are

a specialized subset of DCs that are very active in IFN-α production, which promotes B cell differentiation into antibody-producing plasma cells (among many other functions). LN patients have been shown to have increased numbers of pDCs in the kidney as well as sustained IFN-α production [84].

The importance of TLR-induced miRNA expression in the regulation of pDC activation and type I IFN production has been examined. miR-155 and miR-155* (the complementary passenger strand in the miRNA duplex) were found to be the most strongly induced miRNAs in pDCs and were also differentially induced over time. The investigators found that miR-155* is induced before miR-155 and has biological activity. miR-155* induction after TLR stimulation increases IFN-α production by targeting *IRAKM*, which negatively regulates the TLR pathways by preventing the dissociation of IRAK1 and IRAK4 from MyD88 and the formation of IRAK1/TRAF6 complexes. The continual increase in miR-155 expression resulted in a reduction in IFN-α due to the targeting of *TAB2* by miR-155. TAB2 regulates type I IFN production in pDCs upon TLR stimulation. Taken together, these results suggest that there is cooperative involvement of both strands of the miRNA duplex in pDC activation [52].

5. LN-associated miRNAs in Adaptive Immune Cells

miRNAs were shown to be essential for altering the adaptive immune response in studies that conditionally depleted the enzyme Dicer from T or B cells. Dicer cleaves pre-miRNAs into double-stranded RNA products (duplexes) once they reach the cytoplasm [85]. T cell Dicer depletion indicated that miRNAs regulate diverse aspects of T cell biology, including basic cellular processes such as proliferation and survival as well as cell lineage decisions and cytokine production during T helper cell differentiation [86]. Dicer depletion in B cells resulted in the complete developmental block of B cells in the pro- to pre-B cell transition, affecting antibody diversity. These results indicate that miRNAs are critical for modifying adaptive immune responses and that irregular miRNA expression may represent an underlying cause to LN pathogenesis (Table 1) [87].

5.1. Splenocytes. Although many miRNAs are expressed in T cell subsets, one study found 7 miRNAs (miR-16, miR-21, miR-142-3p, miR-142-5p, miR-150, miR-15b, and let-7f) account for almost 60% of all T cell miRNAs. These miRNAs (except for miR-21) were downregulated in effector T cells compared to naïve cells. Memory T cell expression was similar to the expression seen in naïve T cells. miR-21 expression was higher in effector and memory T cells compared to naïve T cells, indicating that miRNAs are differentially expressed in hematopoietic lineages. These results suggest that miRNAs may contribute dynamically to cell differentiation and the maintenance of cell identity [88].

It has recently been demonstrated that murine lupus models share a common disease-associated miRNA expression pattern despite strain differences in lupus susceptibility loci and clinical manifestation. In the MRL/*lpr* model, miR-146a was associated with disease development due to increased expression in splenocytes from 3-4-month-old mice compared to 1-month-old mice. miR-155 was found to be associated with disease development in both the MRL/*lpr* and the NZB/W models [74]. An additional study investigated the relationship between IFN-accelerated disease, miRNAs, and B cell subsets in NZB/W mice due to the acceleration of disease by type I IFN in this model. Splenic and plasma miR-15a levels were elevated in diseased mice compared to prediseased mice. Increased autoantibody levels were significantly correlated with increased miR-15a expression. The immunosuppressive B cell subset (B-10) was reduced following IFN treatment, yet it had the highest miR-15a expression that increased with disease development. miR-15a expression in the pathogenic B cell subset (B-2) only increased upon disease onset. Although it is currently unknown whether changes in miR-15a expression are pathogenic or an epiphenomenon, these results suggest that miR-15a is implicated in the development of SLE in NZB/W mice by directing the balance of splenic B cell subsets [69].

Pathogenic miRNAs have also been examined in the lymphocytes of B6.Sle123 mice. These mice spontaneously develop autoimmune disease characterized by autoantibodies, splenomegaly, and IC-mediated glomerulonephritis. They also have elevated ratios of $CD4^+$ to $CD8^+$ T cells. The expression of miR-21, which is upregulated in SLE T cells and has been shown to regulate apoptosis and cell proliferation pathways in part by targeting *PDCD4*, was found to be upregulated in B6.Sle123 splenocytes [23, 70]. Short-term inhibition of miR-21 *in vivo* resulted in an approximate 20% decrease in *PDCD4* expression in naïve $CD4^+$ T cells compared to T cells from control mice. Long-term inhibition of miR-21 *in vivo* significantly reduced splenomegaly in B6.Sle123 mice compared to the controls. In addition, the number of Fas receptor-expressing splenic B cells and the $CD4^+$ to $CD8^+$ T cell ratio were reduced, which suggests that miR-21 inhibition skews the T cell ratio towards that of the non-autoimmune strain [70].

The overexpression of miR-148a has also been investigated in $CD4^+$ T cells from patients with lupus as well as lupus-prone mice. Due to the importance of DNA methylation abnormalities in SLE pathogenesis, Pan et al. examined the roles of mir-21 and miR-148a in aberrant $CD4^+$ T cell DNA hypomethylation [71]. miR-21 and miR-148a downregulated *DNA methyltransferase 1 (DNMT1)* expression *in vitro* and *in vivo*, decreasing DNMT1 production in T cells. Downregulation of *DNMT1* in $CD4^+$ T cells contributes to lupus autoreactivity by inducing T cell DNA hypomethylation; this results in the overexpression of autoimmunity-associated genes including *lymphocyte function-associated antigen 1* (*LFA-1* or *CD11a*) and *CD70* [89]. While a putative miR-148a binding site has been predicted in the 3′ UTR of *DNMT1*, there are no predicted binding sites for miR-21. The researchers discovered that miR-21 indirectly downregulated *DNMT1* expression by targeting its upstream regulator in the Ras-MAPK pathway, *RASGRP1*. Intriguingly, miR-148a directly downregulated *DNMT1* expression by targeting the protein coding region of its transcript. In addition, miR-21

and miR-148a induced the overexpression of methylation-sensitive, autoimmune-associated genes in $CD4^+$ T cells including *CD70* and *LFA-1*. Furthermore, the investigators found that the effects were reversed when inhibitors of either miR-21 or miR-148a were transfected into $CD4^+$ T cells isolated from SLE patients, implying that hypomethylation in $CD4^+$ T cells can potentially be alleviated by inhibiting these miRNAs [71].

Another posttranscriptional modifier of *DNMT1*, miR-126, was found to be overexpressed in $CD4^+$ T cells from SLE patients [72]. Its degree of overexpression negatively correlated with DNMT1 protein levels. In addition, the expression of miR-142-3p and miR-142-5p was reduced to less than half in SLE $CD4^+$ T cells compared to $CD4^+$ T cells from healthy controls. miR-126, miR-142-3p, and miR-142-5p are predicted to target genes associated with SLE, which implicates their aberrant expression in $CD4^+$ T cells in LN pathogenesis. Overexpression of miR-126 in primary $CD4^+$ T cells from SLE patients contributed to T cell autoreactivity by targeting *DNMT1*, while inhibition in SLE patients resulted in T and B cell inactivation. Overexpression of miR-126 in primary $CD4^+$ T cells from healthy donors resulted in the demethylation and upregulation of autoimmunity-associated genes including *CD11a* and *CD70*, inducing T cell and B cell hyperactivity. These results demonstrate that overexpression of miR-126 can aberrantly induce splenocyte activity towards that of an autoimmune phenotype [72].

Decreased expression of miR-142-3p and miR-142-5p in SLE $CD4^+$ T cells was confirmed in studies by Ding et al. [73]. *CD84* and *IL-10* are predicted targets of miR-142-3p, while *signaling lymphocytic activation molecule-associated protein* (*SAP*) is a potential target of miR-142-5p. When miR-142-3p was inhibited in $CD4^+$ T cells from healthy donors, protein levels of CD84 and IL-10 increased. SAP protein production was decreased after inhibition of mir-142-5p. Inhibition in healthy donor $CD4^+$ T cells caused T cell overactivation and B cell hyperstimulation. These results were reversed after transfection of the corresponding miRNA mimic. Overexpression in SLE $CD4^+$ T cells decreased CD40L, inducible T cell costimulator (ICOS), IL-4, IL-10, and IL-21 protein levels, reduced T cell proliferation, and reduced IgG production compared to controls. These results indicate that reduced expression of miR-142-3p and miR-142-5p in $CD4^+$ T cells of SLE patients contributes to T cell hyperactivity and B cell hyperstimulation [73].

6. Future Directions in LN Treatment

miRNAs are being recognized as potential therapeutic targets in the treatment of LN and other diseases as increasing numbers are identified as specific disease-modifying agents and not merely disease correlates. Recent studies have shown that exogenously increasing let-7a, a well-known tumor suppressor that is downregulated in many types of cancer, is effective in treating tumorigenesis by decreasing cell migration, invasion, and proliferation *in vitro* and *in vivo* [90–94]. Intranasal let-7 administration reduced lung tumor formation in a murine model of lung cancer [95]. Tumorigenesis was suppressed in murine gastric cancer cells *in vivo* by overexpression of let-7a, which decreased cell proliferation by causing G_1 arrest [96].

Lupus therapeutics have recently been recognized for their ability to alter miRNA expression levels [97, 98]. Once disease-associated miRNA expression is determined in patients with SLE, tailored therapies can be designed using immunosuppressant treatments that alter pathologic miRNAs. Examining miRNA expression profiles during the course of immunosuppressant therapy may more accurately assess treatment responsiveness. Since lupus susceptibility genes contain target sites for various miRNAs, future treatments may target multiple disease-associated miRNAs that synergistically contribute to LN pathogenesis. Additionally, pathogenic miRNA expression may be used to assess treatment feasibility. This will allow the use of targeted therapies to specifically modulate abnormal miRNA expression patterns in individuals suffering from lupus.

Circulating miRNAs have been used as diagnostic markers in the treatment and diagnosis of certain cancers [99, 100]. Since the discovery of dysregulated miRNA expression in the serum and urine of SLE patients, the interest in using miRNAs as noninvasive biomarkers has increased [54, 97]. One of the many advantages of using miRNAs as disease biomarkers is the availability of highly sensitive PCR detection methods and their low complexity compared with protein biomarkers [101]. In addition, pathogenic miRNAs may be able to detect early SLE disease onset before clinical, pathological findings arise. Assessing miRNA expression in different tissues may alter our organ-specific and systemic understanding of SLE. For example, detecting alterations in urinary miRNA expression may offer valuable information regarding changes in the glomerular microenvironment, while pathogenic alterations in PBMCs may reveal the global state of the SLE patient.

6.1. Therapeutic Modulation of miRNAs. Because of the vast and critical roles miRNAs perform in fundamental immune processes (and due to their dysregulated expression in many pathological conditions), they have become an increasingly attractive target for therapeutic modulation. While the endogenous delivery of miRNAs has had limited testing *in vivo*, the risk of altering unintentional targets remains high as a single miRNA can have multiple gene targets and these targets can have profound effects on a variety of miRNAs [102–104]. The solution to this potential problem may lie in targeting miRNAs broadly associated with SLE such as miR-146a [42, 60, 62, 74, 105]. Pan et al. therapeutically altered miR-146a levels using virus-like particles (VLPs) containing miR-146a, which were delivered via tail vein injection to lupus-prone BXSB mice [106]. After administration of the miR146a-containing VLPs, high levels of miR-146a were detected in PBMCs, lung, spleen, and kidney tissues from BXSB and control mice. miR-146a therapy significantly reduced autoantibody, IFN-α, IL-1β, IL-6, and total IgG production. Widespread restoration of miR-146a by VLPs was effective in ameliorating SLE progression in lupus-prone mice, providing a potential novel therapy for SLE treatment [106].

While the initial findings from studies that systemically increase miRNA levels are promising, a more effective treatment may utilize targeted delivery systems. A novel approach

to manipulating mesangial miRNA expression alone could be employed by targeting the mesangial cell surface marker that is unique to the kidney glomeruli: Integrin $\alpha 8$ [107]. Scindia et al. revealed that this molecule can be used to effectively target immunoliposomes to mesangial cells by tail vein injection [108]. pDCs also possess a unique cellular marker: plasmacytoid dendritic cell antigen (PDCA) [109]. In this way, pDCs may be specifically targeted instead of all splenocytes or PBMCs, considering that a miRNA may not be differentially expressed in other cell types found in the spleen or peripheral blood. For example, while miR-146a is decreased in PBMCs, it is upregulated in murine Th1 cells compared to naïve T cells and Th2 cells [62, 110].

6.2. Tailored Therapy Based on the Patient's miRNA Profile. Although glucocorticoids are the first-line treatment for a wide range of autoimmune diseases, up to 30% of patients with SLE are steroid resistant, demonstrating persistent tissue inflammation despite treatment with high doses of steroids [111, 112]. Disease-associated miRNAs may become unique biomarkers that help determine the course of the patient's immunosuppressant therapy. The use of miRNAs as selection markers for disease treatment is underway in the treatment of ovarian cancer. Researchers found that let-7a expression was predictive of a patient's outcome after chemotherapy; let-7a expression differed substantially between the patients who did or did not respond to chemotherapy containing platinum and paclitaxel. The survival of patients with low let-7a expression was higher when they received platinum and paclitaxel in combination; patients with high let-7a expression did not have improved survival after adding paclitaxel to platinum-based therapy [113].

If disease-associated miRNAs are targeted, the treatment of SLE could be greatly improved. Steroid-resistant patients, amongst others, may benefit from tailored immunotherapy. Revealing miRNAs with therapeutic potential may provide insight in treating other inflammatory diseases as well. Polikepahad et al. showed that the inhibition of let-7 miRNAs in an experimental model of asthma *in vivo* profoundly inhibited the production of allergic cytokines and the disease phenotype, indicating that let-7a may be a potential therapeutic target in other diseases as well [114].

7. Conclusion

miRNAs are now recognized as key regulators of gene expression. A single miRNA, or even multiple miRNAs, may contribute to cell development and immunoregulation in diverse ways. Increasing evidence has shown that miRNAs are not only critical for the regulation of immune cell development but also for modifying innate and adaptive immune responses. Evidence suggests that miRNAs are involved in LN pathogenesis by altering innate immune cell responsiveness, lymphocyte function, proinflammatory mediator production, and TLR and NFκB signaling pathways.

Increasing evidence indicates that dysregulated miRNA expression in specific cell types contributes to LN immunopathogenesis. While it is becoming clear that miRNAs modulate components of inflammatory signaling cascades, it is not fully understood how miRNAs are regulated by different cell types in SLE. Overall, the possibility of altering miRNA expression in order to ameliorate disease remains promising. Studies that alter pathogenic miRNAs have shown that miRNA-based therapies have the potential for becoming therapeutic tools for the treatment of SLE and other diseases. As we learn more about the intricacies of miRNAs and epigenetics, targets for drug development will continue to emerge.

Abbreviations

H:	Humans
M:	Mice
R:	Rats
↑:	Increased expression
↓:	Decreased expression
α-SMA:	α-Smooth muscle actin
API:	Apoptosis inhibitory protein
CRP:	C-Reactive protein
DNMT:	DNA methyltransferase
GFR:	Glomerular filtration rate
IFN:	Interferon
IL:	Interleukin
IRAK:	IL-1 receptor-associated kinase
IRF:	Interferon regulatory factor
NZB/W:	New Zealand Black/White
PBMCs:	Peripheral blood mononuclear cells
pDC:	Plasmacytoid dendritic cell
SNP:	Single-nucleotide polymorphism
PDCD:	Programmed cell death
SLE:	Systemic lupus erythematosus
SLEDAI:	SLE Disease Associated Index
STAT:	Signal transducer and activator of transcription
TGF-β:	Transforming growth factor-β
TTP:	Tristetraprolin.

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Review Article

Inflammatory Chemokine Expression via Toll-Like Receptor 3 Signaling in Normal Human Mesangial Cells

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The innate and adaptive immune systems have been reported to play an important role in the pathogenesis of glomerular diseases. Since viral infections may trigger the development of inflammatory renal disease or the worsening of preexisting renal disease, recent studies have focused on the involvement of toll-like receptors (TLRs) and their signaling pathways in the inflammatory processes of glomerular cells. Viral double-stranded RNA (dsRNA) can activate not only TLR3 located within intracellular endosomes but also retinoic-acid-inducible-gene-I- (RIG-I-) like helicase receptors located within the cytosol. RIG-I and melanoma differentiation-associated gene 5 (MDA5) are members of the RNA helicase family in the cytosol, and both act as pathogen recognition receptors. The activation of TLRs and their downstream immune responses can be induced by both infectious pathogens and noninfectious stimuli such as endogenous ligands, and this mechanism may be involved in the pathogenesis of autoimmune renal diseases. However, there are few data on the interaction between TLR3, MDA5, and RIG-I in autoimmune glomerular diseases. Based on our recent experimental studies using cultured normal human mesangial cells (MCs), we found that novel TLR3-mediated signaling pathways in MCs may be involved in the pathogenesis of glomerular diseases. In the present paper, we summarize our recent findings.

1. Introduction

The innate and adaptive immune systems have been reported to play an important role in the pathogenesis of glomerular diseases. Since viral infections may trigger the development of inflammatory renal disease or the worsening of preexisting renal disease, recent studies have focused on the involvement of toll-like receptors (TLRs) and their signaling pathways in the inflammatory processes of glomerular cells [1]. The activation of TLRs and their downstream immune responses can be induced by both infectious pathogens and noninfectious stimuli such as endogenous ligands, and this mechanism may be possibly involved in the pathogenesis of autoimmune renal diseases [1–3]. Viral double-stranded RNA (dsRNA) can activate both TLR3 located within intracellular endosomes and retinoic acid-inducible gene-I (RIG-I)-like helicase receptors located within the cytosol [4]. RIG-I and melanoma differentiation-associated gene 5 (MDA5) are members of the RNA helicase family in the cytosol, and both act as pathogen recognition receptors [5]. Therefore, RIG-I and MDA5 may also be involved in the pathogenesis of autoimmune renal diseases [6–14]. Recent studies have revealed the expressions of TLRs in resident renal cells, suggesting the involvement of the expression of TLRs and activation of their downstream signaling pathway in the pathogenesis of glomerular diseases [1–3]. Once presumptive antigenic ligands bind to TLRs, the activation of transcriptional factors, such as interferon regulatory factors (IRFs) and nuclear factor kappa B (NF-κB) is induced through intracellular signaling cascade activation. This activation results in the release of adhesion molecules, cytokines, and chemokines, which play a pivotal role in innate and adaptive immune responses [1–3].

Since glomerular mesangial cells (MCs) have been reported to produce a wide variety of proinflammatory

molecules that play an important role in immune and inflammatory reactions in the kidney [15], MCs itself are now thought to play a pivotal role in the pathogenesis of renal diseases [16]. Indeed, the activation of mesangial TLR3 induced by polyinosinic-polycytidylic acid (poly IC), a synthetic analogue of viral dsRNA, upregulated the expression of functional molecules such as interleukin (IL)-6 [15], CC chemokine ligand (CCL) 2 (or monocyte-chemoattractant protein-1) [6], CCL5 (or regulated on activation, normal T-cell expression and secretion) [17], matrix metalloproteinase 9, plasminogen activator inhibitor type 1, and tissue plasminogen activator in human MCs. These findings suggest that viral RNA can influence, at least in part, the generation and degradation of the extracellular matrix in the mesangium in ways other than through direct viral stimulation and that glomerulosclerosis might subsequently develop [8, 18], although this theory remains speculative. However, the precise role of the interaction between TLR3, MDA5, and RIG-I in mesangial inflammation in human glomerular diseases remains to be elucidated.

In our previous studies, we had observed high levels of RIG-I expression in the glomeruli and urinary sediments of patients with lupus nephritis [7, 19]. RIG-I overexpression may be a pathological feature of lupus nephritis. Further, we recently observed intense glomerular expression of human myxovirus resistance protein 1, a type-I-interferon- (IFN-) dependent transcript, in biopsy specimens from patients with lupus nephritis, whereas negative staining occurred in specimens from patients with IgA nephropathy or purpura nephritis. Interestingly, it has been reported that the implication of "psuedoviral" immunity as a novel disease concept of lupus nephritis, that is, self-nucleic acid particles resembling viral particles activates viral nucleic sensors and subsequent type I IFNs production [20]. The nucleic acid-specific TLR3, TLR7, TLR8, and TLR9 cover a spectrum of different endogenous ligands as well as viral RNA formats, and the activation of these TLRs signaling cascades result in inducing type I IFNs release [20]. Also, increased type I IFNs produced by resident renal cells have been associated with lupus nephritis, that is, type I IFNs directory promote and modulate pathogenesis of murine lupus nephritis [21]. Thus, recent studies support, at least in part, the theory of innate immune system activation in the pathogenesis of lupus nephritis. To examine this theory, we recently performed several experiments using cultured normal human MCs treated with poly IC and/or a poly IC/cationic lipid complex and found the involvement of novel TLR3-mediated signaling pathways that upregulate inflammatory chemokine expression during mesangial inflammation in human MCs [10–14, 22]. Poly IC has been widely used to mimic viral infection in various cell types including MCs. Accordingly, we used poly IC in our recent experiments. The treatment with poly IC is a model of cells exposed to viral dsRNA released from dying cells, whereas treatment with poly IC/cationic lipid complex is a model of cytosolic viral dsRNA recognition [13, 22].

In the present paper, we summarize our recent findings of mesangial inflammation via TLR3-mediated signaling pathways. Details of our experimental procedures and results obtained were published previously in our recent papers [10–14, 22]. We believe that these novel pathways may be involved in the pathogenesis of human autoimmune glomerular diseases.

2. TLR3 and RIG-I in Human MCs

To evaluate the potential role of RIG-I in response to viral dsRNA in human MCs, we treated the cells with poly IC, an authentic viral dsRNA. In this experiment, the cells were simply treated with poly IC and not transfected using the poly/cationic lipid complex. Stimulation with poly IC resulted in increased expression of both RIG-I mRNA and protein in a concentration-dependent and time-dependent manner accompanied by CCL5 expression [11]. Furthermore, treatment with RIG-I small interfering RNAs (siRNA) significantly lowered poly IC-induced CCL5 expression. In contrast, the poly IC-induced expression of CCL2 mRNA was not affected by RIG-I siRNA (Figure 1). Interestingly, the poly IC-induced RIG-I expression was suppressed in response to treatment with siRNA against TLR3. In addition, TLR3 siRNA downregulated the poly IC-induced expressions of TLR3 and interferon (IFN)-β, whereas RIG-I siRNA did not affect the expression of either TLR3 or IFN-β. Interestingly, poly IC treatment did not induce IFN-α or IFN-γ in this experiment [11]. Thus, IFN-β siRNA was used to examine the role of IFN-β as a potential mediator of poly IC-induced RIG-I expression. As a result, the poly IC-induced expressions of IFN-β and RIG-I were markedly inhibited in cells transfected with IFN-β siRNA. Pretreatment of the cells with a blocking antibody against the type I IFN receptor also reduced the poly IC-induced expression of RIG-I. On the contrary, the expression of both RIG-I and CCL5 was induced after transfection of the cells with IFN-β expression plasmid [11]. Moreover, pretreatment of the cells with dexamethasone reduced the poly IC-induced expression of both RIG-I and IFN-β, whereas this treatment had no effect on IFN-β-induced RIG-I expression [11].

Our results suggested that the expression of CCL5 was selectively regulated by RIG-I expression in human MCs because poly IC-induced CCL5 expression was inhibited in response to RIG-I knockdown, whereas CCL2 expression was not affected by RIG-I siRNA treatment. It has been reported that viral dsRNA activates human and murine MCs to produce IL-6 and CCL2 via TLR3 [6, 17]. Further, viral nucleic acids occur in formats other than dsRNA, such as 5′-triphosphate RNA (3P-RNA) can activate murine MCs via TLR3-independent RIG-I pathways, suggesting complexed 3P-RNA and dsRNA trigger antiviral responses via both TLR3-dependent and independent pathways in MCs, which may promote glomerulonephritis, although the role of RIG-I in innate pathogen recognition can vary between cell types and species [23]. A recent report suggested that RIG-I, but not TLR3, mediated the secretion of type I IFN in poly IC/cationic lipid complex-treated cultured murine glomerular endothelial cells [9]. The cross talk between glomerular endothelial cells and MCs may be an important aspect of glomerular inflammation, and the RIG-I/CCL5 pathway in MCs may contribute to glomerular inflammation, although

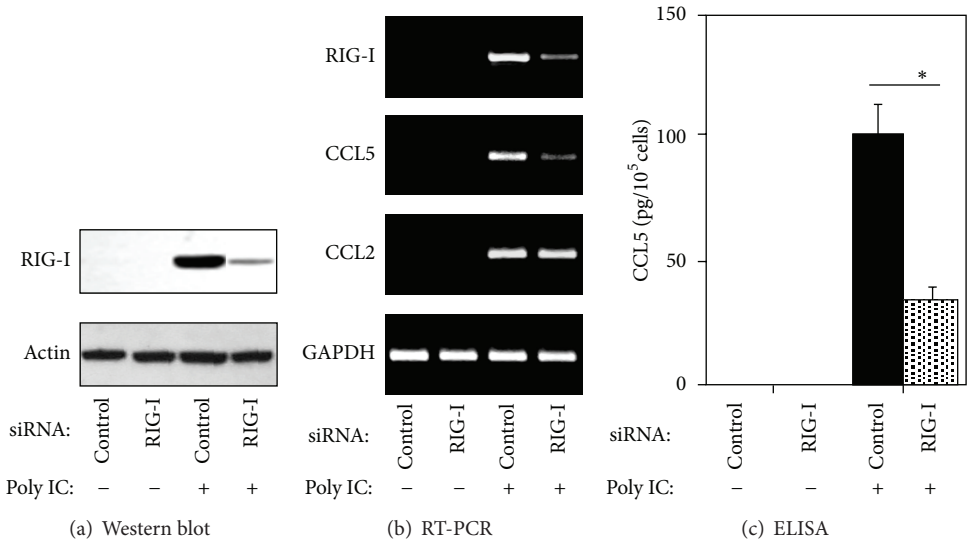

Figure 1: Knockdown of RIG-I reduces the poly IC-induced expression of CCL5 in human MCs. The cells were transfected with siRNA against RIG-I or control siRNA and then stimulated with 20 μg/mL of poly IC (Imaizumi et al. NDT 2010). (a) After 24 h of poly IC treatment, the cells were lysed, and western blotting for CCL5 was performed. (b) The cells were incubated for 16 h with poly IC, RNA was extracted, and RT-PCR was performed for RIG-I, CCL5, and CCL2. (c) The culture medium was collected after 24 h, and the concentration of CCL5 was determined by ELISA ($n = 3$, $^*P < 0.01$).

the implication of RIG-I may vary between species. Our recent study showed that RIG-I may function downstream to TLR3 in the signaling cascade activated by poly IC-induced expression of CCL5 in human MCs [11]. In addition, the inhibitory effect of dexamethasone against CCL5 expression may depend on the suppression of IFN-β production, but not on the IFN-β-induced RIG-I expression. In this signaling pathway in MCs, TLR3 and newly synthesized IFN-β are involved in poly IC-induced RIG-I expression. Since dexamethasone had no effect on IFN-β-induced RIG-I expression, the inhibitory effect of dexamethasone may depend on the suppression of IFN-β production [10–12].

3. TLR3, MDA5, and RIG-I in MCs

MDA5 and RIG-I were recently shown to function as pathogen recognition receptors of viral dsRNA in the cytostome, and both receptors may play an important role in innate immune reactions [4, 5]. Although the expression of MDA has been documented in human MCs [13] as well as murine MCs [15], the detailed implications of MDA5 expression in human MCs have not yet been clarified. Since C-X-C motif chemokine 10 (CXCL10, also known as IFN-γ-induced protein 10), a chemokine with chemotactic activity for leukocytes with CXCR3, is thought to be involved in the pathogenesis of glomerular diseases [23], we examined the effect of poly IC and the role of MDA5 in CXCL10 expression in cultured human MCs [13, 14]. Poly IC, either simply applied to the cells or transfected as a complex with a cationic lipid, induced MDA5 expression in a concentration-dependent and time-dependent manner. TLR3, localized in the endosomes, is thought to serve as a receptor for nontransfected poly IC, whereas RIG-I and MDA5, localized in the cytoplasm, are thought to serve as receptors for transfected poly IC in this experiment. Transfection of the cells with siRNA against TLR3 suppressed the poly IC-induced expression of MDA5 mRNA and protein, while siRNA against TLR3 did not suppress poly IC/cationic lipid complex-induced MDA5 expression. On the other hand, the siRNA against RIG-I clearly inhibited the MDA5 expression induced by the poly IC/cationic lipid complex, whereas MDA5 knockdown had no effect on RIG-I expression induced by poly IC or the poly IC/cationic lipid complex. Thus, MDA5 may be located downstream of RIG-I in this signaling pathway in cultured human MCs [13]. Interestingly, these results are inconsistent with those of an earlier report of MDA5 expression in murine MCs [15]. The molecular mechanisms of pathogen recognition may vary among species [23], but this issue remains to be elucidated in future studies [12]. Since we observed that IFN-β, not IFN-α, is a key mediator of MDA5 expression in MCs, as suggested by our recent examinations of poly IC-induced TLR3 signaling pathways in MCs [11, 12], induction of IFN-β mRNA was confirmed in the cells treated with poly IC as well as those transfected with the poly IC/cationic lipid complex. In the present experiment, TLR3 knockdown suppressed IFN-β induction in the poly IC-treated cells, while RIG-I knockdown suppressed that induction in the cells transfected with poly IC/cationic lipid. Transfection of the cells with IFN-β siRNA markedly inhibited production of MDA5 and CXCL10 induced by poly IC treatment or poly IC/cationic lipid transfection. On the other hand, MDA5 was markedly induced by transfection with an IFN-β expression plasmid. This finding suggests that newly synthesized IFN-β mediates poly IC-induced MDA5 expression. In the present study, we observed that IFN-β is induced either by poly IC or a poly IC/cationic lipid complex and that *de novo*

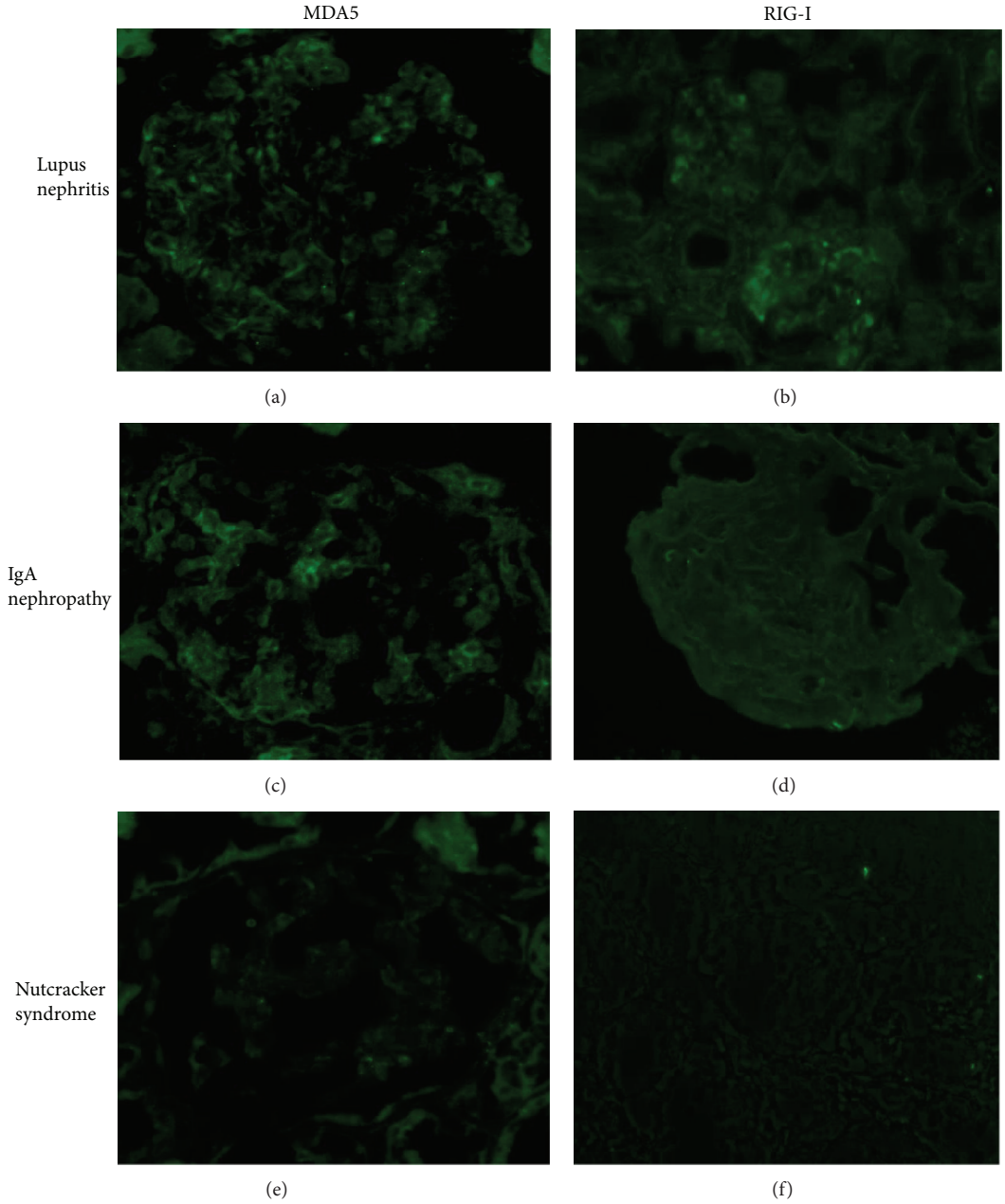

Figure 2: Immunoreactivity of MDA5 and RIG-I in biopsy specimens (Imaizumi et al. Tohoku J Exp Med 2012). Stored kidney specimens in good condition obtained from cases of diffuse proliferative lupus nephritis, proteinuric IgA nephropathy, minimal change nephrotic syndrome, and nutcracker syndrome were used for immunofluorescent study of MDA5 and RIG-I expression. After blocking by incubation with 1% goat serum, the slides were incubated with an anti-MDA5 antibody (1 : 100) or an anti-RIG-I antibody (1 : 1000). Intense MDA5 immunoreactivity was detected in MCs of the specimens from diffuse proliferative lupus nephritis and proteinuric IgA nephropathy, while the expression in nonimmune complex-mediated renal diseases was undetectable. Interestingly, RIG-I immunoreactivity was only in diffuse proliferative lupus nephritis.

synthesized IFN-β may mediate the expression of MDA5 [13]. Apart from its antiviral property, IFN-β is thought to be an important mediator in virus-associated glomerulonephritis and immune complex-mediated glomerulonephritis exacerbated by viral infections [24]. Interestingly, RIG-I was involved in IFN-β expression induced by the poly IC/cationic lipid complex, but not in the MDA5 expression induced by IFN-β. Expression of CXCL10 in resting cells was faint and was markedly upregulated by treatment of cells with poly IC or by transfection of cells with poly IC/cationic lipid complex transfection. Knockdown of MDA5 resulted in partial inhibition of CXCL10 induction by poly IC or poly IC/cationic lipid complex [13]. Taking together, the involvement of the TLR3/IFN-β/MDA5/CXCL10 and

the RIG-I/IFN-β/MDA5/CXCL10 pathways and possible interaction between these signaling cascades may play an important role in immune and inflammatory reactions against both viral and "pseudoviral" infections [20] in human MCs, although these observations remain preliminary.

Further, we observed mesangial MDA5 immunoreactivity in biopsy specimens from patients with severe lupus nephritis and proteinuric IgA nephropathy (urinary protein excretion/urinary creatinine >1.0) but no MDA5 expression in patients with noninflammatory renal diseases (Figure 2). Interestingly, there was no mesangial expression of RIG-I in the specimens from patients with IgA nephropathy despite the positive MDA5 staining. These observations suggested that the expression of MDA5 in severe lupus nephritis is associated with signaling pathway activation via RIG-I [7], whereas MDA5 expression in IgA nephropathy may be RIG-I-independent. The differential roles of MDA5 and RIG-I in severe lupus nephritis and proteinuric IgA nephropathy may predict the specific molecular mechanisms of these glomerulonephritis forms. In this context, we previously observed that mesangial expression of RIG-I was induced by IFN-γ, and which may promote inflammatory process in the pathogenesis of lupus nephritis [10]. This issue should be further investigated in future studies.

4. Interaction between Interferon-Stimulated Gene (ISG) 56, MDA5, and RIG-I

It has been reported that the IFN-stimulated gene 56 (ISG56) regulates cellular function and can be induced by IFN, dsRNA, or viruses in most cell types [25]. Since ISG56 expression in mouse MCs has been reported [26], we recently examined whether ISG 56 expression is involved in TLR3 signaling, which induces CCL5 [11] and CXCL10 [13] in human MCs. When the cells were treated with poly IC, ISG56 mRNA and protein were markedly increased in a concentration-dependent and time-dependent manner. The induction of ISG56 mRNA and protein by this treatment was inhibited by siRNA against TLR3 or IFN-β. On the contrary, overexpression of IFN-β by IFN-β plasmid transfection resulted in significant induction of ISG56. The transfection of cells with siRNA against ISG56 had no effect on IFN-β expression but significantly decreased the expressions of MDA5, RIG-I, CXCL10, and CCL5 mRNA and protein [14]. Knockdown of ISG56 did not affect cell viability. Since IFN-β, but neither IFN-α nor IFN-γ, was induced by poly IC treatment in MCs under the conditions used here [11, 12], we conclude that IFN-β is a major player in the TLR3 signaling within MCs [11–14]. As a result, we confirmed that newly synthesized IFN-β is involved in poly IC-induced ISG56 expression [14].

We previously showed that TLR3 signaling in human MCs induces CCL5 and CXCL10 via the TLR3/IFN-β/RIG-I/CCL5 [11] and TLR3/IFN-β/MDA5/CXCL10 [13] axes, respectively. Further, this study suggests that ISG56 regulates the expression of MDA5/CXCL10 and RIG-I/CCL5 pathways in the downstream of TLR3/IFN-β [14]. Increased expression of RIG-I [7, 13] and MDA5 [13] has been observed in renal biopsy specimens from patients with proliferative lupus nephritis, while only MDA immunoreactivity was observed in biopsy specimens from patients with proteinuric IgA nephropathy. The differential roles of RIG-I and MDA5 in proliferative lupus nephritis and proteinuric IgA nephropathy may predict specific molecular mechanisms for these diseases [13]. Taking together, ISG56 may also be involved in inflammatory renal diseases, although this theory remains speculative. Further detailed studies are needed to resolve this issue. Proposed inflammatory pathways via TLR3 signaling in MCs are shown in Figure 3.

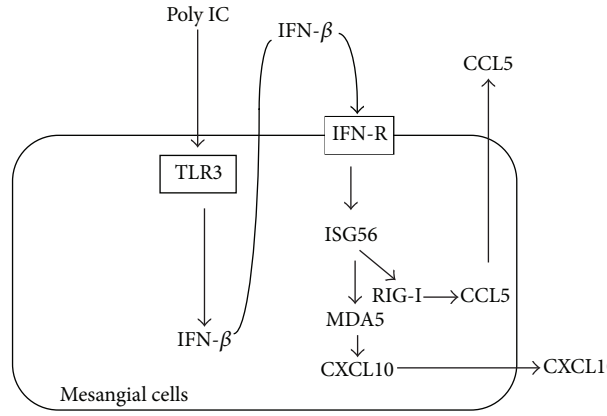

Figure 3: Proposed RIG-I- and MDA5-mediated signaling pathways via TLR3 activation induced by poly IC in human mesangial cells. (NDT 2010, Tohoku J Exp Med 2012, Am J Nephrol 2013).

5. Conclusion

We believe that involvement of the novel TLR3/IFN-β/RIG-I/CCL5 and TLR3/IFN-β/MDA5/CXCL10 signaling pathways in MCs may contribute to mesangial inflammation. Cross talk between these signaling pathways may be involved in the pathogenesis of human glomerulonephritis including lupus nephritis and in the aggravation of glomerulonephritis due to both viral and "pseudoviral" infections. Since the inhibitory effect of dexamethasone may depend on the suppression of IFN-β production and not on IFN-β-induced RIG-I and MDA5 expressions [10–12], effective treatment strategies for the intervening in these signaling pathways are needed, although this remains to be elucidated in future studies. We believe that intervention within these signaling pathways may lead to the development of future therapeutic strategies for glomerular diseases.

We found the involvement of novel RIG-I-mediated and MDA5-mediated signaling pathways in mesangial inflammation in human MCs that differed from TLR3 triggering, which demonstrated the clinical significance of this issue.

Conflict of Interests

The authors declare that they have no conflict of interests.

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