THE NETWORK OF INFLAMMATORY MECHANISMS IN KIDNEY DISEASE: MECHANISM AND NEW THERAPEUTIC AGENTS

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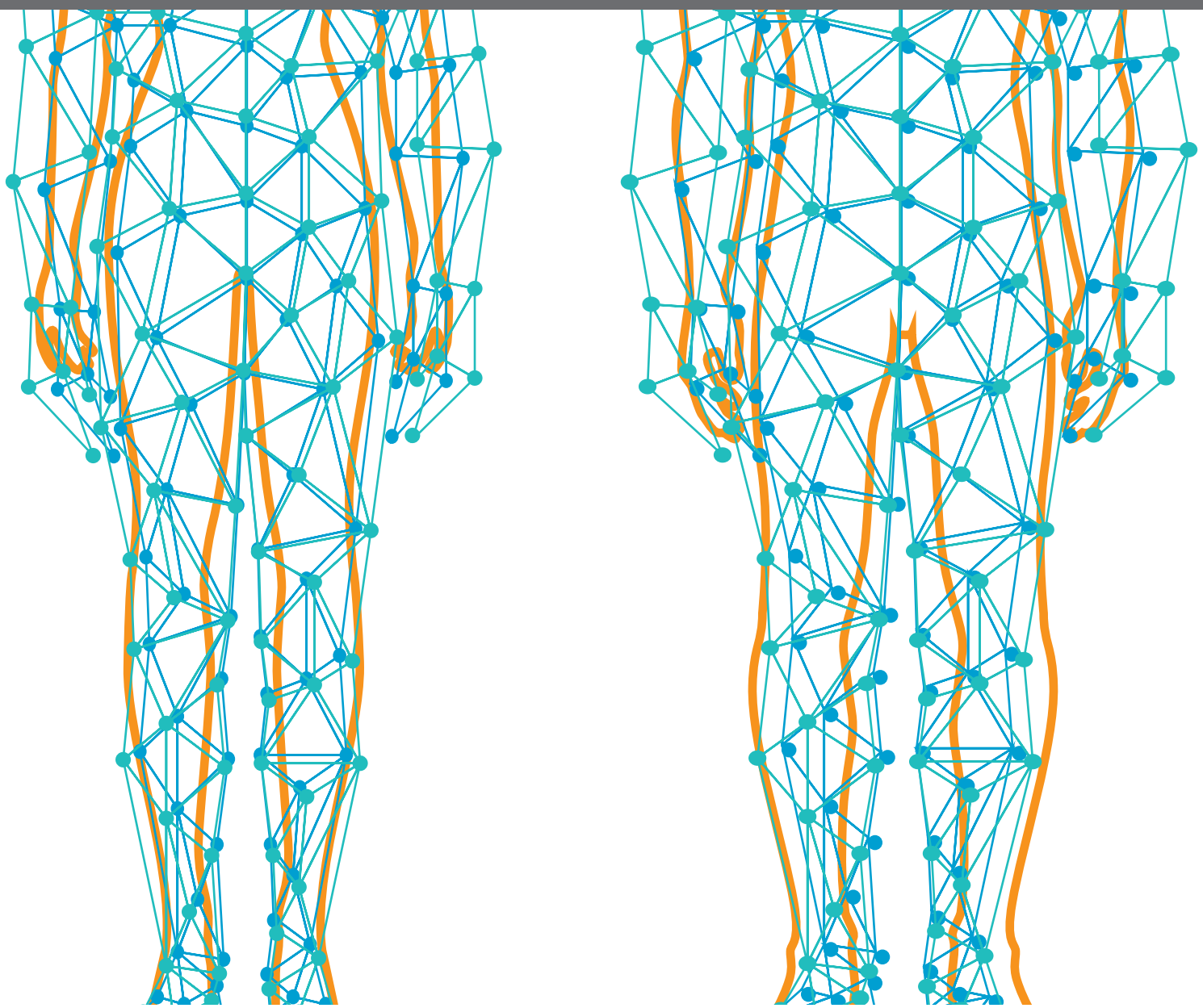

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THE NETWORK OF INFLAMMATORY MECHANISMS IN KIDNEY DISEASE: MECHANISM AND NEW THERAPEUTIC AGENTS

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EDITORIAL
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Editorial: The Network of Inflammatory Mechanisms in Kidney Disease: Mechanism and New Therapeutic Agents

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Keywords: therapeutic agents, kidney disease, autoimmune disease, cytokines, signaling pathways

Editorial on the Research Topic

The Network of Inflammatory Mechanisms in Kidney Disease: Mechanism and New Therapeutic Agents

It has been shown that the progression of kidney disease in humans and animal models usually involves the sustained activation of a number of signaling pathways. In this research theme, immunological mechanisms and genetic analysis that are important in the pathogenesis of various renal diseases were studied, and new perspectives on renal diseases were discussed. This review presents an overview of the papers submitted to this Research Topic.

In autoimmune glomerulonephritis, Nagai reviewed the signaling mechanisms and functional roles of typical T-cell co-inhibitory receptors in the control of autoimmune glomerulonephritis. In models of autoimmune vasculitis affecting the kidney, pathogenesis is characterized by early Th17 dominance followed by Th1 dominance and reduced Treg cell function of autoreactive T cells. Thus, further elucidation of the suppressive effects and pathways in immune kidney disease is needed to develop effective therapies for T cell-mediated autoimmune glomerulonephritis.

Two interesting papers on the pathogenesis of renal fibrosis have been submitted to this Research Topic. Hirooka et al. demonstrated in a mouse model of renal fibrosis that interleukin (IL)-18, a cytokine important for the induction of Th1 responses, plays an important role in renal interstitial fibrosis. Furthermore, Foxp-3 positive cells and regulatory T cells were found to be effective in preventing fibrosis in the renal interstitium through the IL-18 receptor signaling pathway.

Xu et al. showed that cilomilast, a second-generation selective phosphodiesterase-4 inhibitor, inhibited the activation of fibroblasts stimulated by TGF-β1 and reduced the expression of fibronectin, α-SMA, collagen I, and collagen III expression. In addition, cilomilast inhibited the activation of TGF-β1-Smad2/3 signaling in fibroblasts stimulated by TGF-β1.

Yang et al. reported the nephroprotective effect of erythropoietin (EPO) on contrast-induced nephropathy in a mouse model. *In vitro*, EPO was shown to increase protein levels of Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway components and inhibit apoptosis.

In a mouse model of septic acute kidney injury, Wang et al. described the protective effect of heparin against septic acute kidney injury via neutralization of extracellular histones. Based on a variety of perspective, it is suggested that heparin may play a protective role by neutralizing histones, thereby mitigating apoptosis and inflammation.

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Gao et al. analyzed the efficacy and safety of rituximab, one of the first-line drugs for intermediate and high-risk primary membranous nephropathy, in 95 patients during a 6-month observation period. Results of multivariate logistic regression analysis showed that severe proteinuria and persistent anti-PLA2R antibody positivity were independent risk factors for non-remission. The remission rate with rituximab as first-line therapy was higher than that with alternative agents, and only one case of discontinuation due to adverse events was reported, indicating the efficacy and safety of rituximab as first line therapy.

Liu et al. investigated the risk factors for treatment failure in 241 patients with peritonitis, a serious complication of peritoneal dialysis (PD). After logistic regression analysis, they reported that fibrinogen, PD duration, fungal infection, history of hemodialysis, concomitant intestinal obstruction, and diabetes were independent risk factors for poor peritonitis outcome, while high-density lipoprotein was a protective factor.

Selvaraja et al. reported that HLA-DRB1*0405, HLA-DRB1*1502, and HLA-DRB1*1602 were associated with increased risk of developing systemic lupus erythematosus (SLE). HLA-DRB1*0405, HLA-DRB1*1502, and HLA-DRB1*1602 were associated with an increased risk of developing SLE, while HLA-DRB1*1201 and HLADRB1*1202 were associated with a decreased risk of developing SLE. In addition, HLA-DRB1*04 was significantly associated with lupus nephritis and arthritis, and HLA-DRB1*15 with stomatitis. Analysis of the association between HLA-DRB1*04 and clinical and biological factors showed that HLA-DRB1*04 was significantly associated with SLE disease activity index score, antinuclear antibodies, C-reactive protein in blood, and urinary protein. SLE carriers with the HLA-DRB1*04 allele were significantly associated with elevated levels of pro-inflammatory cytokines compared to SLE carriers without the HLA-DRB1*04 allele. Based on these results, the authors indicate that the disease severity of SLE may be determined by the HLA-DRB1 allele.

Overall, this Research Topic introduces the immunological mechanism of action from basic research and the efficacy of drugs and genetic analysis for renal diseases from clinical practice to better understand the new inflammatory mechanisms in renal diseases. Although renal diseases are commonly experienced in clinical practice, autoimmune mechanisms play a variety of roles, the details of which have yet to be elucidated. We hope that basic and clinical researchers will present and evaluate each other's research results through the Research Topics to further elucidate the pathogenesis in this field.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Co-inhibitory Receptor Signaling in T-Cell-Mediated Autoimmune Glomerulonephritis

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Autoimmune glomerulonephritis occurs as a consequence of autoantibodies and T-cell effector functions that target autoantigens. Co-signaling through cell surface receptors profoundly influences the optimal activation of T cells. The scope of this review is signaling mechanisms and the functional roles of representative T-cell co-inhibitory receptors in the regulation of autoimmune glomerulonephritis, along with current therapeutic challenges mainly on preclinical trials. Co-inhibitory receptors utilize both shared and unique signaling pathway, suggesting specialized functions that provide the rationale behind therapies for autoimmune glomerulonephritis by targeting these inhibitory receptors. These receptors largely suppress Th1 immunity, modify Th17 and Th2 immune response, and enhance Treg function. Anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) immunoglobulin (Ig), which is able to block both activating CD28 and inhibitory CTLA4 signaling, has been shown in preclinical and clinical investigations to have effects on glomerular disease. Other inhibitory receptors for treating glomerulonephritis have not been clinically tested, and efficacy of manipulating these pathways requires further preclinical investigation. While immune checkpoint inhibition using anti-CTLA4 antibodies and anti-programmed cell death 1 (PD-1)/PD-L1 antibodies has been approved for the treatment of several cancers, blockade of CTLA4 and PD-1/PD-L1 is associated with adverse effects that resemble autoimmune disorders, including systemic vasculitis. A renal autoimmune vasculitis model features an initial Th17 dominancy followed later by a Th1-dominant outcome and Treg cells that attenuate autoreactive T-cell function. Toward the development of effective therapies for T-cell-mediated autoimmune glomerulonephritis, it would be preferable to pay attention to the impact of the inhibitory pathways in immunological renal disease settings.

Keywords: glomerulonephritis, therapeutics, ITIM, PD-1, CTLA4, renal vasculitis

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INTRODUCTION

T cells are key effectors of the adaptive immune response, playing important roles in the elimination of pathogens and in the development of autoimmune disease. Autoimmune glomerulonephritis occurs as a consequence of autoantibodies and T-cell effector functions that target either antigens intrinsic to the glomeruli [for example, as occurs in anti-glomerular basement membrane (GBM) nephropathy] or non-specific antibodies that become trapped and accumulate in the glomeruli [for example, as occurs in immunoglobulin (Ig) A nephropathy and anti-neutrophil cytoplasmic autoantibody (ANCA)-associated glomerulonephritis] (1, 2). In this regard, peripheral regulation

of T-cell responses is crucial to preventing inappropriate responses to self-antigens leading to autoimmune glomerulonephritis (1).

The optimal activation of T cells is profoundly influenced by co-signaling through cell surface receptors (3). The common feature that identifies receptors as members of the inhibitory class is their ability to attenuate activation signals initiated by other receptors that are often members of the immunoreceptor tyrosine-based activation motif (ITAM) class (4). Loss of inhibitory signaling is often associated with autoreactivity and unchecked inflammatory responses, illustrating the essential role that this system plays in immune regulation (4, 5). Though the human genome is estimated to encode over 300 immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing molecules, of which only a minority has been characterized (6), most reviews discussing the co-receptor signaling pathway as a potential target in autoimmunity have focused on blockade of co-stimulatory receptor signaling (7, 8). The scope of this review is signaling mechanisms and the functional roles of some representative T-cell co-inhibitory receptors in the regulation of autoimmune glomerulonephritis, along with current therapeutic challenges.

CLASSICAL AND NON-CLASSICAL INHIBITORY SIGNALING IN T-CELL RECEPTOR PATHWAY

TCR Signaling Pathway and Co-signaling

The primary signal for conventional T cells is mediated through T-cell receptor (TCR) engagement. TCRs recognize small antigenic peptides presented in the groove of the self-major histocompatibility complex (MHC) (9). As a result of this recognition, TCR complexes aggregate on T-cell surfaces to form stable contacts, resulting in the formation of immunological synapses on antigen-presenting cells (APCs) (9). This aggregation evokes intracellular signaling that involves the activation of Src protein tyrosine kinase, leading to the phosphorylation of CD3- and ζ chain-localized ITAM. Subsequently, the **ζ**-associated protein of 70 kD (ZAP-70) is recruited, resulting in a series of downstream phosphorylation events (10) (**Figure 1**). Another kinase pathway in T cells involves the activation of phosphatidylinositol-3 kinase (PI3K), which phosphorylates a specific membrane-associated inositol lipid. This enzyme is recruited to the TCR complex and generates phosphatidylinositol triphosphate (PIP_3) and diacylglycerol (DAG) from membrane phosphatidylinositol biphosphate (PIP_2). PIP_3 activates signaling enzymes such as PLCγ (phospholipase Cγ) and PKCθ (protein kinase Cθ). However, the primary signal itself does not decide the fate of the immune response (11). Instead, co-stimulatory and co-inhibitory receptors on T cells direct the function and fate. These co-signaling receptors often co-localize with TCR molecules, such that the co-signaling receptors synergize with TCR signaling to promote or inhibit T-cell activation and function (11, 12).

ITIM, ITSM, ITT, and Other Mechanisms

Intracellular protein–protein interaction during cell signaling and activities of cellular enzymes are often regulated by phosphorylation of tyrosine residues. For countering action of phosphorylation by tyrosine kinase, protein tyrosine phosphatases are enzymes that remove phosphate moieties from tyrosine residues to limit and terminate cellular responses that are no longer required (4, 13). One family of immune inhibitory receptors is defined by the presence of a consensus amino acid sequence, the ITIM motif, in the cytoplasmic domain of the proteins (13). The six-amino acid ITIM motif consists of the sequence (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val), where X denotes any amino acid (4). Ligand binding induces clustering of the inhibitory receptors and results in tyrosine phosphorylation that provides a docking site for the recruitment of cytoplasmic phosphatases that have Src-homology-2 (SH2) domains, including SHP-1 (SH2 domain-containing protein tyrosine phosphatase 1) and SHP-2. These phosphatases remove phosphate from tyrosine residues in the activated receptor and adaptors, such as SH2-binding leukocyte phosphoprotein of 76 kD (SLP-76), linker for activation of T cells (LAT), and CD3**ζ** (14) (**Figure 1**).

CTLA4 carries an ITIM-like YVKM motif, which associates with SHP-2 and reduces proximal TCR signaling through dephosphorylation of targets such as the TCR–CD3ζ complex, LAT, and ZAP-70 (15, 16), thereby inhibiting cell cycle progression and cytokine production. PD-1 has an ITIM motif as well as an immunoreceptor tyrosine-based switch motif (ITSM) (17). Both motifs appear to be phosphorylated following interaction with ligands, resulting in the recruitment of SHP-2 and possibly SHP-1; the co-localization of PD-1 with TCR microclusters induces dephosphorylation of CD3ζ, ZAP70, and PKC (17, 18). The TIGIT (T-cell immunoglobulin and ITIM domain) protein contains an ITIM motif and an immunoglobulin tail tyrosine (ITT)-like motif; phosphorylation of the tyrosine residue in either of these motifs is sufficient for signal transduction and inhibitory activity (19, 20). T-cell immunoglobulin-3 (TIM-3) does not have a classical signaling motif in its cytoplasmic tail (21). To simplify the descriptions in the present review, I am avoiding mention of several other remarkable signaling pathways that employ other phosphatases and intracellular motifs of the CTLA4, PD-1, TIM-3, and TIGIT co-inhibitory receptors; those topics have been covered in a number of excellent detailed reviews (3, 4, 11, 22).

Collectively, co-inhibitory receptors regulate T-cell immunity by using both shared and unique signaling pathways (3), suggesting the specialized functions and providing the rationale for therapies that treat autoimmune glomerulonephritis by targeting these inhibitory receptors. The following subsection addresses the expression on T cells, function *in vivo*, and potential role for regulating autoimmune glomerular diseases of CTLA4, PD-1, TIM-3, and TIGIT, summarized in **Figure 2** and **Table 1**.

CTLA-4

Expression, Ligands, and General Function

CTLA4 is a potent negative regulator of T-cell response and was identified as a member of the CD28 family (40).

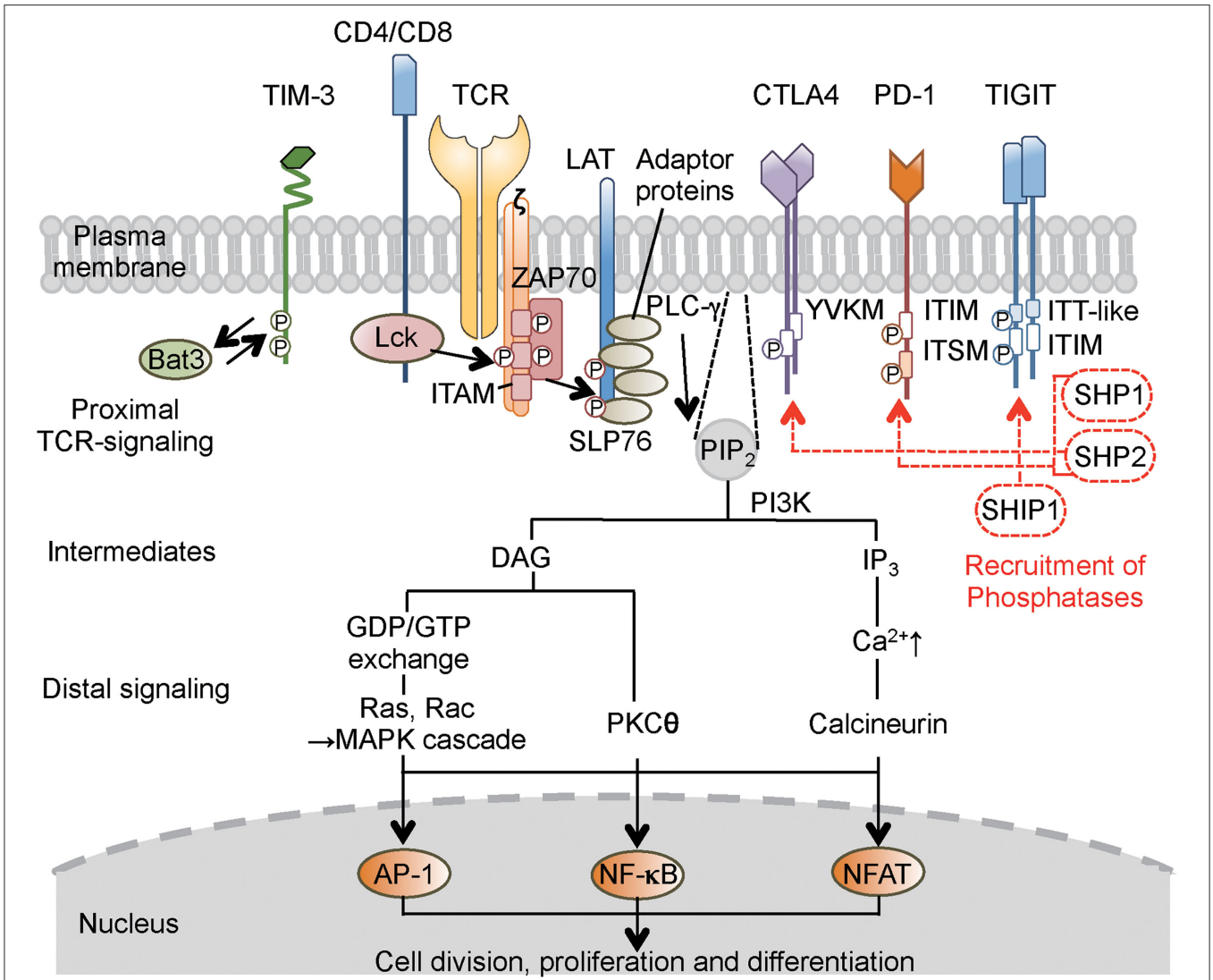

FIGURE 1 | Concise outline of the intracellular signaling events during T-cell activation and roles of co-inhibitory receptors. Binding of the TCR and co-receptors to peptide–MHC (major histocompatibility complex) complexes on the antigen-presenting cells (APCs) initiates proximal signaling events, which result in phosphorylation of the ζ chain, binding and activation of ZAP-70, phosphorylation of LAT and adaptor proteins, production of biochemical intermediates, and activation of distal signaling cascades. MAPK, PKC, and calcineurin are enzymes that activate transcription factors, thereby stimulating the expression of various genes involved in T-cell response. Most inhibitory receptors have an inhibitory motif, represented by ITIM, in their cytoplasmic tails. Ligand binding to these receptors results in the recruitment of phosphatases (SHP-1, SHP-2, or SHIP1), which alter proximal and distal TCR signals. These consequently transmit activating signal (black solid line arrow) and inhibitory signal (red broken line and arrow) in T cells. In the case of TIM-3, which does not have a classical signaling motif, Bat-3 (HLA-B associated transcript 3) binds to the TIM-3 tail and blocks binding of another adaptor molecule under steady state. Ligand binding triggers the dissociation of Bat-3 from the cytoplasmic tail of TIM-3, thus allowing another adaptor molecule to bind and promote the inhibitory function of TIM-3. CTLA4, cytotoxic T-lymphocyte-associated protein 4; PD-1, programmed cell death 1; TIM-3, T-cell immunoglobulin 3; TIGIT, T-cell immunoglobulin and ITIM domain; LAT, linker for activation of T cells; TCR, T-cell receptor; ZAP-70, ζ-associated protein of 70 kD; SLP-76, SH2-binding leukocyte phosphoprotein of 76 kD; ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; ITT, immunoglobulin tail tyrosine; SHP-1,-2, Src-homology-2 domain-containing protein tyrosine phosphatase 1, 2; SHIP1, SH2 domain-containing inositol-5-phosphatase 1; PIP_2, phosphatidylinositol biphosphate; PI3K, phosphatidylinositol-3 kinase; DAG, diacylglycerol; IP_3, phosphatidylinositol triphosphate; GDP/GTP, guanosine diphosphate/triphosphate; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; PKC, protein kinase C; AP-1, activation protein 1; NF-κB, nuclear factor-κB; NFAT, nuclear factor of activated T cells.

Although CD28 is constitutively expressed on all naive $CD4^+$ and $CD8^+$ T cells and Treg cells, CTLA4 is transiently expressed on the surface of activated T cells (41, 42). The B7 family of proteins, B7-1 and B7-2, provides the major co-stimulatory signal for augmenting and sustaining T-cell responses through interaction with CD28 (42). B7-1 and B7-2 are shared ligands of CTLA4; the interaction of CTLA4 with these ligands leads to co-inhibitory signaling (43). In other words, the inhibitory mechanisms of CTLA4 include CD28 out-competition and blockade of intracellular signaling pathways,

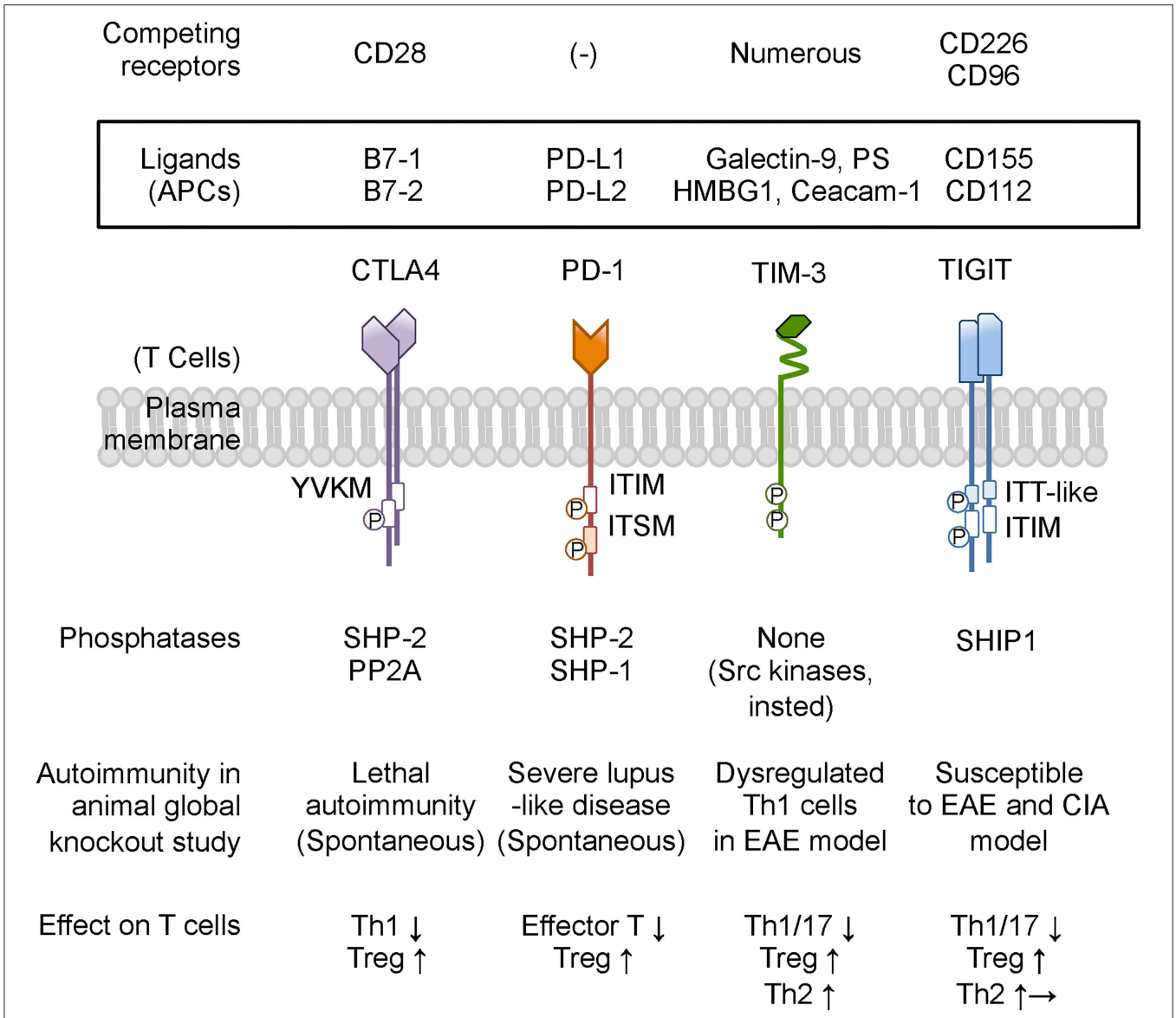

FIGURE 2 | Co-inhibitory receptors on T cells and ligands, phosphatase, and modulation of T-cell function. Co-inhibitory receptors have a variety of physiological and pathological ligands, and the binding is occasionally competed by other immune receptors on T cells. TIM-3 does not have a classical signaling motif in its cytoplasmic tail. However, the cytoplasmic domain of TIM-3 contains tyrosine residues to be targets for phosphorylation and promoting TIM-3-mediated T-cell inhibition by allowing binding of SH2 domain-containing Src kinases. Based on animal studies, CTLA4 and PD-1 are predicted to be associated with more susceptibility to autoimmune disease, while neither TIM-3-deficient nor TIGIT-deficient mice present any spontaneous autoimmune disease phenotype. Co-inhibitory receptors on T cells commonly suppress effector T-cell functions by down-modulating Th1 cells and Th17 cells and by enhancing Treg cells. In addition, TIM-3 and TIGIT shift the cytokine balance to Th2 immunity. PS, phosphatidyl serine; HMGB1, high-mobility group protein B1; Ceacam-1, carcinoembryonic antigen-related cell adhesion molecule 1; PP2A, protein phosphatase 2A; EAE, experimental autoimmune encephalomyelitis; CIA, collagen-induced arthritis. Other abbreviations are shown in **Figure 1** caption.

as CTLA4 has a 10-fold higher affinity than CD28 for B7-1 binding (43).

CTLA4-Ig ("abatacept") is a biological that binds to B7-1 and B7-2, blocking both activating CD28-mediated signaling and inhibitory CTLA4-mediated signaling (44, 45) and effectively inhibiting naive antigen-specific $CD4^+$ T-cell responses (46, 47). While the total $CD4^+$ memory T-cell response was effectively attenuated by administration of CTLA4-Ig (48, 49), examinations of subsets of $CD4^+$ helper T cells revealed that interleukin (IL)-17-secreting $CCR6^+$ memory Th17 cells were resistant to CD28 and CTLA4 blockade (50).

Involvement in Autoimmunity and Glomerular Diseases and Therapeutic Model

CTLA4-deficient mice exhibit severe lymphoproliferative disease, with infiltration of activated T cells into various organs

TABLE 1 | Experimental treatment models targeting T-cell co-inhibitory signaling in autoimmune diseases.

Target receptor	Treatment	Animal model	Effect	References
CTLA-4	CTLA4-Ig fusion	Spontaneous lupus	Suppress autoantibody production and prolong survival	(23)
		Spontaneous lupus GN (NZB/W F1)	When used in combination with cyclophosphamide, reduce renal inflammation and injury	(24)
		Spontaneous immune complex GN ($Lyn^{-/-}$)	Not effective	(25)
		Anti-GBM (mouse/rat)	Controversial; depend on experimental conditions	(26–29)
PD-1	PD-L1-Ig fusion	Autoimmune GN	Reduce number of glomerular T cells and severity of glomerular damage	(30)
		T-cell-induced colitis	Suppress Th1 and Th17 response and ameliorate colitis	(31)
		CIA	Suppress T-cell response and ameliorate arthritis	(32, 33)
TIM-3	Galectin-9 (TIM-3 ligand)	Anti-GBM GN	Suppress T-cell response and ameliorate GN	(34)
		CIA	Suppress Th17 response and ameliorate arthritis	(35)
		EAE	Suppress Th1 response and ameliorate encephalomyelitis	(36)
TIGIT	TIGIT-Ig	Lupus GN	Reduced proteinuria and autoantibody, improve survival	(37)
	TIGIT-Ig and TIGIT tetramer	CIA	Suppress Th1 and Th17 response and ameliorate arthritis	(38)
	Agonistic antibody	EAE	Suppress Th1 and Th17 response and ameliorate encephalomyelitis	(39)

CTLA4, cytotoxic T-lymphocyte-associated protein 4; Ig, immunoglobulin; NZB/W, New Zealand black/white; GN, glomerulonephritis; GBM, glomerular basement membrane; PD-1, programmed cell death 1; PD-L1, programmed cell death ligand 1; CIA, collagen-induced arthritis; TIM-3, T-cell immunoglobulin 3; EAE, experimental autoimmune encephalomyelitis; TIGIT, T-cell immunoglobulin and ITIM domain.

and death within a few weeks of birth (51–53). Given the promising results of CD28 and CTLA4 blockade in small animal models (23), strategies to target this pathway were developed in several clinical trials for the treatment of autoimmunity. CTLA4-Ig has been used clinically for the effective treatment of rheumatoid arthritis (RA) and juvenile idiopathic arthritis, as reviewed in (54), and has been tested against allergen-induced airway inflammation (55), ulcerative colitis (56), systemic lupus erythematosus (SLE) (57, 58), and other autoimmune diseases, as reviewed in (59). The results of those trials indicated that abatacept did not alter the inflammatory response to allergen challenge or show any efficacy in ameliorating colitis symptoms (59). Collectively, abatacept might be efficacious in the treatment of Th1-mediated autoimmune disease, such as RA, but remain less effective in the treatment of Th2- or Th17-mediated autoimmune disease such as asthma and inflammatory bowel disease (7). However, several studies have indicated that CTLA4-Ig protein attenuates glomerular injury in experimental models of crescentic glomerulonephritis (26–29). As a result, abatacept has been tested clinically in patients with granulomatosis with polyangiitis; the drug was well-tolerated in this population, providing a high frequency of disease remission and steroid discontinuation (60). A further clinical trial of abatacept in ANCA-associated vasculitis (AAV) has been progressed (https://clinicaltrials.gov/).

PD-1

Expression, Ligands, and General Function

PD-1 originally was identified as an inducible surface receptor during programed cell death (61) and was shown to be expressed on stimulated T, B, and myeloid cells (62). PD-L1 and PD-L2 are two independent ligands for PD-1. PD-L2 expression is largely confined to dendritic cells (DCs) and monocytes/macrophages, but PD-L1 is more widely distributed on leukocytes and non-hematopoietic cells (63, 64). Expression of PD-L in peripheral tissue may regulate the behavior of infiltrating leukocytes (65). The PD-1/PD-L1 pathway exerts important inhibitory function in primary T-cell proliferation, cytokine production, cytotoxic activity, and cell survival (66, 67). This pathway also promotes development and function of Treg cells (68) and negatively regulates effector T-cell reactivation and function (69).

Involvement in Autoimmunity and Glomerular Diseases and Therapeutic Model

PD-1-deficient mice develop autoantibody-induced disease in a strain-dependent fashion; this autoimmune disease includes lupus-like glomerulonephritis leading to late death (70–72), although the phenotype in these mice is much milder than that of CTLA4-deficient mice. Consistent with human evidence of polymorphisms associated with SLE, T1D (Type 1 diabetes mellitus), and RA, experimental animal models of T1D-prone mice (73) and collagen-induced arthritis (CIA) (32) indicate that PD-1 activation attenuates autoimmune disease.

In the kidney, renal DCs have been shown to express PD-L1 and can be involved in suppressing $CD4^+$ T-cell proliferation (74). Studies in experimental autoimmune glomerulonephritis have shown that blockade of PD-1/PD-1L interaction aggravates glomerular injury and cellular infiltration (64) and that activation of PD-1 using a PD-L1 fusion protein leads to a reduction in disease severity (30). Moreover, recent work with *PD-L1*$^{-/-}$ mice showed that dosing with PD-L1 provided protection in a crescentic glomerulonephritis model via Treg-mediated

suppression of the Th1 immune response (75). However, studies on immune-complex-mediated glomerulonephritis (induced by immunization with a foreign antigen) showed that blockade of the PD-1/PD-L axis (by antibody administration) did not reveal any significant pathological changes (76). This result suggests the need for careful interpretation of the roles of PD-1/PD-L in experimental autoimmune glomerulonephritis. Indeed, clinical trials testing the treatment of glomerulonephritis with PD-1 have not been reported. Thus, the clinical efficacy of modifying this pathway still requires further preclinical investigation.

TIM-3

Expression, Ligands, and General Function

TIM-3 was identified as molecule expressed specifically in interferon (IFN)-γ-producing Th1 and $CD8^+$ cytotoxic T cells, but not in naive T cells (77). Galectin-9 is a soluble S-type lectin that is widely expressed on immune and non-immune cells and has been shown to bind to the IgV domain of TIM-3, resulting in negative regulation of Th1 immunity (36). In addition to galectin-9, phosphatidyl serine, high-mobility group protein B1 (HMGB1), and carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam-1) have been identified as TIM-3 ligands (78–80). It remains to be determined whether the triggering of TIM-3 by individual ligands or by combinations thereof has distinct impacts on TIM-3 function.

Involvement in Autoimmunity and Glomerular Diseases and Therapeutic Model

TIM-3 can be protective in autoimmunity but often is sparsely expressed; in contrast, the protein is highly expressed in cancer and chronic viral infection, resulting in the dampening of protective immunity (22). Even so, multiple reports have shown that TIM-3 blockade results in abrogation of peripheral tolerance of Th1-cell-mediated responses. Anti-TIM-3 blocking murine model develops hyper-acute experimental autoimmune encephalomyelitis (EAE) (77); treatment with soluble TIM-3-Ig results in T-cell hyper-activation and IFN-γ production (81). In addition to its role in regulating effector T-cell responses, TIM-3 also may have a role in regulating the function of $Foxp3^+$ Treg cells (82). Several studies have shown that TIM-3^+ Treg cells have superior suppressive function when compared to TIM-3^- Treg cells (82, 83).

Galectin-9 is a rare example of agonistic treatment based on a natural ligand. Administration of galectin-9 as a soluble protein in mouse ameliorates EAE and CIA (35, 36). The indiscriminate nature of galectin avidity, such that the molecule binds to sugars on multiple different glycoproteins, makes it difficult to definitively attribute these effects to TIM-3 signaling rather than to the manipulation of another galectin-9 binding partner (84). Nevertheless, administration of galectin-9 ameliorates experimental anti-GBM glomerulonephritis; this protective role is associated with inhibition of Th1 and Th17-cell-mediated immune responses and enhanced Th2 immunity in the kidney (34).

Human studies suggest that renal TIM-3 and galectin-9 expression levels are higher in immune-complex-mediated glomerulonephritis, such as IgA nephropathy (85) and lupus nephritis (86) compared to the control group. Some investigations have examined the expression of TIM-3 on peripheral blood cells and in the serum of patients with glomerular diseases (87, 88), but there is little evidence of a role for TIM-3 in other types of autoimmune glomerulonephritis. Clinical trials evaluating the treatment of autoimmune glomerulonephritis using an agent targeting TIM-3 have not been reported. Although the targeting of TIM-3 signaling holds potential for the treatment of T-cell-mediated glomerulonephritis, further preclinical investigation will be required to elucidate effects both on different immune cells and on ligand binding partners other than galectin-9.

TIGIT

Expression, Ligands, and General Function

TIGIT was discovered as a novel member of the CD28 protein family (89, 90). TIGIT is expressed on activated T cells, memory T cells, a subset of Treg cells, and follicular helper T (Tfh) cells, and binds to two ligands, CD155 and CD112, that are expressed on APCs (19, 89, 90). CD226 and CD96 bind to the same ligands, and the CD226–CD155 interaction mediates a co-stimulatory response in cytotoxic T cells (89). TIGIT competes with CD226 by binding with greater affinity to CD155–CD112 to disrupt that co-stimulatory effect, thereby resulting in a dominant inhibitory effect (22). In this regard, the pathway formed by CD226, TIGIT, and their ligands resembles the B7-CD28/CTLA4 pathway: in both cases, a pair of receptors—one positive, one negative–share ligands expressed on APCs (22).

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In human, genomic analyses showed that a polymorphism in CD226 (Gly307Ser) is linked to multiple autoimmune diseases, including T1D, multiple sclerosis, and RA (91, 92). Although TIGIT-deficient mice do not develop spontaneous autoimmunity, these animals display augmented T-cell responses upon immunization (93). The function of TIGIT was examined in EAE and CIA models, with results suggesting that TIGIT is protective for the pro-inflammatory Th1 and Th17 cellular response and contributes to peripheral tolerance (38, 93). In addition to its direct inhibitory role in effector T cells, TIGIT also inhibits immune responses by promoting Treg function and IL-10 production (22). Curiously, several lines of evidence indicates that TIGIT signaling can shift the cytokine balance away from a Th1- and Th17-cell-dominated response and toward a Th2-cell-like response, concurrently with TIGIT-binding-mediated CD155 signaling on APCs (89, 94).

Given the similarity between the B7-CD28/CTLA4 and CD155/CD112-TIGIT/CD226 signaling pathways with regard to their co-signaling frameworks, TIGIT-Ig theoretically should block both activating CD226 signaling and inhibitory TIGIT signaling in a manner similar to that of CTLA-4-Ig. In addition, TIGIT-Ig induces CD155 signaling in cultured DCs *in vitro* and decreases IL-10 production by Th1 cells *in vivo* (89). The specific difference between these two pathways is that B7 is expressed primarily in professional APCs, while CD155 is expressed by a variety of non-professional APCs such as the

vascular endothelium, fibroblasts, and tumor cells (95). When autoimmune disease occurs, the tissue that is infiltrated by T cells contains mainly non-professional APCs, and the CD155/CD112-TIGIT/CD226 pathway might be involved in tissue damage. Still, in both human and animal models, few studies have examined the role of TIGIT signaling in renal-specific disease. Although the treatment of a murine lupus model (NZB/NZW F1 mice) using TIGIT-Ig significantly improved survival, inflammatory responses, and glomerular damage (37), preclinical studies on other glomerular diseases will be needed to permit clinical use of TIGIT-Ig.

THE DEVELOPMENT OF AUTOIMMUNE GLOMERULONEPHRITIS CAUSED BY IMMUNE CHECKPOINT INHIBITORS

In the past decade, cancer therapy has been revolutionized by the development of drugs that promote immune-mediated tumor destruction (96). CTLA-4 and PD-1/PD-L1 are the two best-studied co-inhibitory pathways (97); the use of antibodies as immune checkpoint inhibitors, anti-CTLA4 antibodies, and anti-PD-1/PD-L1 antibodies has been approved for the treatment of several cancers (98–100). While these immunotherapies have shown striking success, blockade of CTLA-4 and PD-1/PD-L1 are associated with adverse effects that resemble autoimmune disorders, including SLE, RA, thyroiditis, and T1D (59, 101). Additionally, renal vasculitis, immune-complex-mediated glomerulonephritis, and pauci-immune glomerulonephritis recently have been reported (102–108). Most systemic vasculitis cases resolved with either holding the immune checkpoint inhibitors and/or administering glucocorticoids (109). These evidences imply relationship between interventional blocking co-inhibitory receptor signaling and development of renal vasculitis, suggesting that this pathway may be a therapeutic target.

RATIONALE FOR TARGETING TH1/17 EFFECTOR AND REGULATORY T CELLS IN AUTOIMMUNE VASCULITIS

As mentioned before, blockade of inhibitory receptors occasionally has resulted in renal vasculitis as well as lupus-like autoimmunity. While autoantibodies play a role in a number of forms of glomerulonephritis, renal vasculitis in humans features the infiltration of T cells and macrophages (110, 111), suggesting a delayed hypersensitivity reaction in kidney. Given that autoreactive $CD4^+$ and $CD8^+$ cells are present in vasculitis patients (112–115), experimental passive transfer studies have defined a role for $CD4^+$ and $CD8^+$ cells in AAV (116, 117). $CD4^+$ effector T cells, particularly upon differentiation to Th17 cells, mediate production of neutrophil chemoattractants by tissue cells via release of IL-17A and renal injury (118, 119). Studies using mice deficient in Th1- and Th17-defining cytokines have shown an initial Th17-dominant lesion followed later by a Th1-dominant outcome (120). Moreover, as human studies implicate abnormal $CD4^+$ $Foxp3^+$ Treg number and function in AAV patients (121–124), depletion of Treg cells led to more anti-neutrophil cytoplasmic protein-specific T cells and more severe glomerulonephritis (125). Approaches for targeting inhibitory receptors might (in theory) include inhibitory receptor-Ig fusion proteins, ligand-Ig fusion proteins, artificial ligands, and agonistic antibodies, as well as the use of bi-specific antibodies to co-ligate inhibitory and activating receptors (59). Among these approaches, as shown in **Table 1**, TIGIT-Ig protein, agonistic anti-TIGIT antibodies, and TIM-3 ligands (e.g., galectin-9), along with PD-L1-Ig and CTLA4-Ig proteins, should be considered candidates for development as bench-to-bedside therapeutics for treatment of T-cell-mediated autoimmune glomerulonephritis through regulation of the function of Th1/Th17 and Treg cells.

CONCLUSION

The studies in knockout mice and clinical experiences of vasculitis caused by immune checkpoint inhibitors treatment give numerous indications that the loss of a functional co-inhibitory receptor leads to sensitivity for autoimmune disease. Clinical utilization of co-inhibitory axes has not progressed in autoimmune disease as it has in cancer. Except for CTLA4-Ig, no clinical trial on co-inhibitory targeted therapy for autoimmune vasculitis and lupus nephritis has been progressed until the middle of 2020. Nevertheless, some recent studies have shown preclinical evidence for the utility of targeting co-inhibitory receptors in lupus and glomerulonephritis. As effector T cells and Treg function have a pivotal role in the development of autoimmune vasculitis, currently available CTLA4-Ig has been tested to evaluate the efficacy of achieving glucocorticoid-free remission in patients with relapsing vasculitis. Clinical efficacy of therapy targeting at PD-1, TIM-3, and TIGIT is expected to be not always consistent because their ligands are different from each other and each co-inhibitory receptor utilizes unique cell signaling as well as shared pathway. Therefore, clinical trials on modulating co-inhibitory signaling by Ig-fusion protein, agonistic antibody, and natural ligands should be carefully designed after sufficient preclinical investigations with clinically relevant animal models show that effector T cells are precisely involved in antigen-specific disease development. Toward the development of effective therapies for T-cell-mediated autoimmune glomerulonephritis, it would be preferable to pay attention to the impact and features of these inhibitory pathways in immunological renal disease settings.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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REVIEW
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The Network of Inflammatory Mechanisms in Lupus Nephritis

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Several signaling pathways are involved in the progression of kidney disease in humans and in animal models, and kidney disease is usually due to the sustained activation of these pathways. Some of the best understood pathways are specific proinflammatory cytokine and protein kinase pathways (e.g., protein kinase C and mitogen-activated kinase pathways, which cause cell proliferation and fibrosis and are associated with angiotensin II) and transforming growth factor-beta (TGF-β) signaling pathways (e.g., the TGF-β signaling pathway, which leads to increased fibrosis and kidney scarring. It is thus necessary to continue to advance our knowledge of the pathogenesis and molecular biology of kidney disease and to develop new treatments. This review provides an update of important findings about kidney diseases (including diabetic nephropathy, lupus nephritis, and vasculitis, i.e., vasculitis with antineutrophilic cytoplasmic antibodies). New disease targets, potential pathological pathways, and promising therapeutic approaches from basic science to clinical practice are presented, and the blocking of JAK/STAT and TIM-1/TIM-4 signaling pathways as potential novel therapeutic agents in lupus nephritis is discussed.

Keywords: TIM-1, KIM-1, nephritis, cytokines, immune response, kidney disease

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INTRODUCTION

As a leading autoimmune disease, systemic lupus erythematosus (SLE) is a chronic inflammatory disease that affects multiple organs. SLE involves activations of dendritic cells (DCs), macrophages, and lymphocytes, which together lead to the production of high-affinity autoantibodies and immune complex formation. The pathogenesis of SLE remains unclear despite extensive clinical and animal studies. Various genes (1) and environmental factors including viral infections, hormones (2, 3), and ultraviolet light are thought to exacerbate SLE. An abnormal production and imbalance of T helper (Th) lymphocyte cytokines was demonstrated to be involved in the development of autoimmune diseases (4), and Th1 cytokines such as interleukin (IL)-2 and -12 and interferon-gamma (IFN-γ) and Th2 cytokines (e.g., IL-4, IL-5, IL-10, and IL-13) are also implicated in the pathogenesis of SLE. The inhibition of these cytokines is a key factor in the development of NZB/WF1 mice, which develop severe lupus-like phenotypes that resemble human SLE (5).

Th17 lymphocytes are a subset of Th cells with an important role in autoimmunity. These lymphocytes are derived from naïve $CD4^+$ T cells and are characterized by the expression of the transcription factor RORγT (retinoic-acid-receptor-related orphan nuclear receptor gamma) (6). Once stimulated by various cytokines, including IL-23 (7), Th17 lymphocytes secrete cytokines such as IL-17 family members, IL-21, IL-22, tumor necrosis factor (TNF)-α, and IL-6 (6). Compared to healthy controls, individuals with SLE exhibited increased numbers of Th17 cells and IL-23 in

their serum (8). Chen et al. observed that the frequency of circulating Th17 cells and the serum levels of IL-17 and IL-23 were higher in patients with loop nephritis compared to controls (9).

Th17 lymphocytes' potent pro-inflammatory effect has been shown to be due to the induction of vascular inflammation and the recruitment of leukocytes, and this is suspected to contribute to several pathological pathways in SLE, including the B-cell activation and autoantibody production observed in SLE (10). The imbalance of cytokines in SLE may be part of a core process of pathogenicity, or it may be a secondary marker of the dysregulation of immune pathways such as those involving Th1-Th2 and Th17-Treg cells (11, 12). IL-6 signaling *via* receptors (IL-6Rs) on activated B cells induces dimerization with the transmembrane protein gp130 and the activation of the receptor-associated Janus kinase (JAK) tyrosine kinases JAK1 and JAK2. This is the most important role of IL-6, as it is involved in multiple autoimmune diseases and contributes directly to the survival of plasma cells in the bone marrow niche (13).

Effector T cells also recognize autoantigens that are present in the kidneys as implanted or endogenous antigens (14–18), and fewer $CD4^+$ and $CD8^+$ cells are recruited to the glomerulus and stroma. The members of the T-cell immunoglobulin mucin-domain (TIM) family encode a protein that has an IgV-like domain and a mucin domain (19), and the three human TIM genes most similar to those in mice are TIM-1, TIM-3, and TIM-4. The roles of TIM proteins in T-cell differentiation, effector function, autoimmunity, and allergy are becoming clear (20), and it was demonstrated that TIM-1 is expressed on activated T cells (21). Another study suggested that TIM-1 on T cells acts as a costimulatory molecule to enhance cell proliferation and cytokine production and to mediate the loss of tolerance (22).

Chemokines and adhesion molecules are reduced by TIM-1 antibodies (18). In intracellular adhesion molecule-1 (ICAM-1) knockout mice treated with TIM-1 antibody, the renal and spleen mRNA expressions of the Th1 chemokines CXCL9 and CXCL10 were reduced and ICAM-1 mediated the recruitment of leukocytes in glomerulonephritis (23). A promising next research task would be to target inflammatory cytokines *via* a blockade of the JAK-signaling transducer and transcriptional activator (STAT) and TIM-1 signaling pathways, in order to better target the development and survival of autoreactive pathogenic plasma cells during the early stages of SLE.

In this review, new therapeutic targets for lupus nephritis, potential pathologies and promising therapeutic approaches to the JAK-STAT and TIM-1-TIM-4 signaling pathways from basic science to clinical practice are presented.

Mechanisms Downstream of the JAK-STAT Pathway

Several signaling pathways are known to be involved in the progression of renal disease in both humans and animal models, and the progression is usually due to a sustained cytokine and JAK-STAT activation of these pathways (24). The JAK-STAT pathway is downstream of the type I and II cytokine receptors. As part of a major signaling cascade, JAK is an effective therapeutic target for a variety of cytokine-driven autoimmune and inflammatory diseases (25, 26). A cytosolic tyrosine kinase, JAK has been demonstrated to be an effective therapeutic target for a wide range of cell-surface receptors, and members of the cytokine receptor common gamma (cg) chain family in particular are involved in signaling (27).

There are four mammalian JAKs: JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2). The activation of JAKs occurs *via* ligand-receptor interactions and results in the phosphorylation of the cytokine receptor; the signaling occurs *via* the generation of docking sites for signaling proteins known as STATs (19). JAKs catalyze the phosphorylation of STATs and promote STAT dimerization and nuclear transport, thereby regulating gene expression and transcription (28, 29). The JAK proteins are structurally related but different in their activation and their downstream effects; their high specificity is thus expected (**Figure 1**).

JAK1 is a receptor (IFN-α/β, IFN-γ, and IL-10) and γc, which is activated by ligands that bind to receptors (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21). JAK2 is activated primarily by the thrombopoietin receptor, IL-3, granulocyte-macrophage-colony stimulating factor (GM-CSF), and IFN-γ. Findings obtained with mouse models of SLE have repeatedly demonstrated the importance of IL-6 in promoting disease expression in SLE (30–32). Tyk2 mediates the signaling induced primarily by IL-12.

Although most of the JAKs are ubiquitously expressed, the expression of JAK3 appears to be restricted mainly to the hematopoietic system and vascular myocytes. JAK3 has an important role in lymphocyte development and function. JAK3 differs from the ubiquitous expression of the other JAK subtypes: it has a restricted tissue distribution, resides primarily on hematopoietic cells, and is associated with cg chains (33). The importance of the JAK3 signaling pathway was highlighted by the findings that mice and humans with genetic deletions or mutations in either the cg subunit or JAK3 develop defects in lymphocyte development, which result in a severe combined-immunodeficiency syndrome phenotype (34).

The Blocking of the JAK-STAT Pathway as a Therapeutic Target

Since the JAK-STAT pathway has a major activating role in a variety of disease processes, concerted efforts have been made to develop specific inhibitors of this pathway. Inhibitors of protein kinases are relatively easy to identify, and the development of JAK inhibitors has received the most attention. The following three JAK inhibitors have been approved by the U.S. Food and Drug Administration (FDA) for clinical use.

Ruxolitinib (Jakafi®, from Incyte) is a potent inhibitor of both JAK1 and JAK2 and was FDA-approved in late 2011 for the treatment of polycythemia vera and myelofibrosis (35). In late 2012, the FDA approved tofacitinib (Xeljanz®, from Pfizer), which was initially designed as a specific inhibitor of JAK1 and JAK3 kinases; tofacitinib has also been administered as an immunosuppressant for the treatment of transplant patients and individuals with autoimmune diseases (36). The JAK2 inhibitor baricitinib (Olumiant, from Eli Lilly) was FDA-approved in

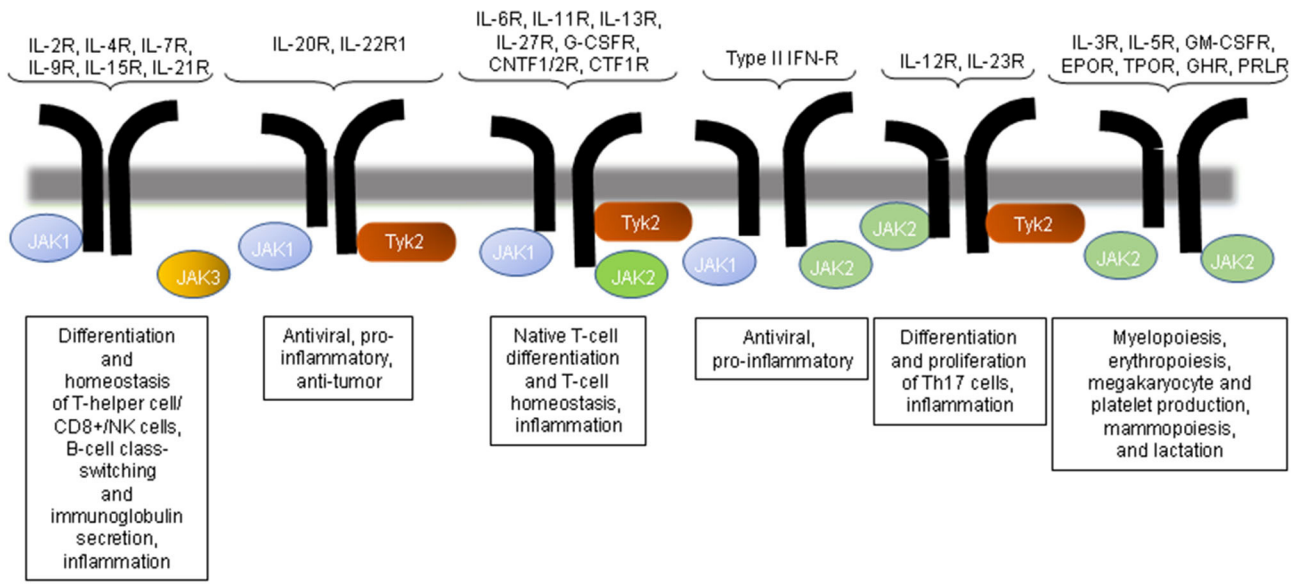

FIGURE 1 | JAK inhibition and immune regulation by the JAK pathway. The inhibition of JAK3 would affect the signaling mediated by only the common gamma chain-associated cytokine receptors (IL-2R, IL-4R, IL-9R, and IL-21R) and regulate T-cell, NK cell, and B-cell function. JAK2 or Tyk2 inhibition would influence multiple cytokine receptor signaling pathways. CNTFR, ciliary neurotrophic factor receptor; EPO, erythropoietin; GH, growth hormone; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; IL, interleukin; NK, natural killer; PRL, prolactin receptor; TPO, thrombopoietin.

June 2018 for the treatment of moderately to severely active rheumatoid arthritis (RA).

Several other JAK inhibitors have been developed as immunosuppressive agents for RA and other autoimmune diseases; e.g., upadacitinib (a JAK1 inhibitor) and filgotinib (a JAK1 inhibitor) were demonstrated to be effective as a treatment of RA (37, 38). Given the effects of JAK-STAT activation on cytokines and chemokines and the specific roles of inflammation in the promotion of progressive renal injury, it is not surprising that JAK-STAT activation is involved in the pathogenesis of both renal disease and acute kidney injury. The JAK-STAT pathway has been studied extensively, and due to its potent immunomodulatory function, the JAK-STAT pathway and its components are promising candidates for immunological interventions for disease control.

Indeed, JAK inhibitor clinical trials have been conducted for a variety of diseases including chronic kidney disease, RA, inflammatory bowel disease, atopic dermatitis, and psoriasis (39–41). There is significant interest in JAK-STAT as a therapeutic target for autoimmune nephritis in particular; the activation of JAK triggers the phosphorylation of IL-6R and gp130, followed by various secondary messengers including STAT3, mitogen-activated protein kinases (MAPKs), and Akt, all of which provide growth and proliferation signals and the activation of transcription factors (42).

Cytokines are glycated proteins with immunomodulatory functions that have important functions in infection and inflammation. Representative cytokines are members of the IL-6 family (which consists of IL-6, IL-11, IL-27, oncostatin M, cardiotrophin-1, and neuropoetin) (43). These cytokines are homo- or heteroduplexes of the signaling β-receptor gp130, which is expressed on ubiquitin. They are characterized by their quantified biological effects. A further transduction of signaling is carried out by the JAK/STAT, MAPK, and phosphatidylinositol-3-kinase (PI3K) pathways (44). Genetic excision or polymorphisms of key suppressors of JAK-STAT signaling, such as suppressors of cytokine signaling, have been implicated in elevated serum IL-6 levels and in the risk of SLE development in humans (45, 46).

JAKs also play an important role in transmitting signals from IL-6Rs, and IL-6 is involved in both SLE and the maintenance of a pool of potentially autoreactive plasma cells. The blockade of JAK signaling with selective and potent JAK2 inhibitors may therefore weaken the supportive effect of IL-6 on the maintenance of autoreactive plasma cells in SLE. Targeting the cytokine/growth factor pathway—which is important for plasma cell generation and the development of SLE—has been supported by several studies targeting the IL-6 pathway and receptors for the treatment of SLE (47); however, targeting IL-6 and IFN-γ failed to produce significant renal effects in either case.

The first *in vivo* study of the therapeutic use of the JAK/STAT pathway in lupus was performed in 2010 by Wang et al. (48). In that study, mice treated with the tyrosine kinase inhibitor AG-490 showed more inflammation (i.e., glomerulonephritis, interstitial nephritis, vasculitis, and even extra-renal features of the salivary glands as an extra-renal feature) than mice treated with the vehicle (inflammation). The inhibition of chemokines, IFN-γ, and major histocompatibility complex class II molecules on the surface of renal cells was observed. The AG-490 treatment also reduced the levels of blood urea nitrogen (BUN), serum

creatinine, and proteinuria, and it reduced the depositions of IgG and C3 in glomerular cells. The study's immunohistochemical examination revealed a reduced expression of STAT1 in glomerular cells, tubular cells, and interstitial cells of the mice.

The effects of a selective JAK2 inhibitor (CEP-33779) on mice with lupus nephritis (LN) were assessed in a pivotal study conducted by Lu et al. (49). CEP-33779 protected MRL/lpr mice from the development of renal damage and ameliorated established disease in the mice, as well as in NZB/WF1 mice. In mice with pre-existing conditions, CEP-33779 resulted in increased survival, decreased proteinuria, the resolution of histological features of renal disease, and a decreased level of pSTAT3. Interestingly, CEP-33779 also reduced the levels of long-lived plasma cells in the spleen (at all doses) and in the bone marrow (at the highest dose). This effect may have therapeutic implications in human LN, given that long-lived plasma cells are involved in the production of antibodies. Conversely, treatment with CEP-33779 did not affect the levels of spleen short-lived plasma cells, which may be associated with a reduction in immunosuppression-related side effects (i.e., infections) and potentially associated with a better response to the vaccine (38, 50).

A specific blockade of JAK2 may also contribute to the treatment of SLE pathology, including arthritis and dermatitis. Multiple cytokines (IL-6, IL-12, and α/β-type IFNs) are suspected to have important roles in the initiation, progression, and development of SLE (51–54). These three cytokines are signaled through receptors regulated by JAK kinases. IL-6 signaling *via* IL-6R on activated B cells induces dimerization with gp130 and the activation of the receptor-associated JAK tyrosine kinases JAK1 and JAK2. This is the most important function of IL-6, since IL-6 is involved in multiple autoimmune diseases and contributes directly to the survival of plasma cells in the bone marrow niche (13).

In addition, multiple studies using mouse models of SLE have repeatedly demonstrated the importance of IL-6 in promoting disease expression in SLE (30, 31, 33, 52, 53). As noted earlier, the activation of JAK causes the phosphorylations of IL-6R and gp130, followed by growth and proliferation signals. JAK activates secondary messengers and transcription factors (e.g., STAT3, MAPK, and Akt) (43). Targeting the IL-6 pathway and receptors is currently being tested for the treatment of SLE (43, 55, 56).

Based on experimental and preclinical data, the oral selective JAK1 and JAK2 inhibitor baricitinib (which has been approved for the treatment of RA) was recently studied in 314 patients with epidural SLE, an epidural disease primarily involving the skin and joints, in a randomized, 24-week, placebo-controlled phase II trial (57). The patients were randomized 1:1:1 to two doses of baricitinib (4 mg or 2 mg/day) or placebo. The percentage of patients who achieved the resolution of arthritis or skin lesions was significantly higher in the 4-mg baricitinib group compared to the placebo group. Among the patients who received baricitinib, the SLE Disease Activity Index 2000 score at week 24 had decreased by >4 points and the British Isles Lupus Assessment Group A or B disease activity score did not worsen, and the Physician's Global Assessment.

In the trial (57), the percentage of patients whose disease activity and SLE Responder index-4 (as defined) did not worsen (64%) was also significantly higher than that in the placebo group (48%). The improvement in the number of tender joints was significantly higher in the 4-mg baricitinib group vs. the placebo group (−6.9 vs. −5.6 joints). However, the extent and severity of skin lesions (as assessed by the area and severity index of cutaneous lupus erythematosus) did not improve with baricitinib treatment compared to the placebo group. There were also no significant differences in the changes in anti-dsDNA antibodies and complement levels between the baricitinib and placebo groups.

Although the occurrence of adverse events was similar among the three groups in the trial (57), serious infections were more common in the 4 mg baricitinib group (6%) than in the 2-mg baricitinib group (2%) and placebo group (1%). One patient with SLE who was positive for antiphospholipid antibodies and treated with 4 mg baricitinib developed a deep vein thrombosis (accounting for 1% of patients treated with 4 mg baricitinib). Although the effect of baricitinib in reducing joint tenderness is very small, the results of this trial provided a positive signal for further phase III trials of JAK inhibitors for various symptoms of SLE.

Two multicenter, randomized, placebo-controlled phase III clinical trials of baricitinib in non-renal SLE are underway (NCT03616912 and NCT03616964). Solcitinib (GSK2586184), a selective JAK1 inhibitor, was going to be tested in a Phase II trial (NCT01777256) in patients with active non-renal SLE; the trial was stopped early after the recruitment of 50 patients, due to inadequate efficacy. No significant effect on the mean expression of IFN transcriptional biomarkers was observed (58). In addition, drug reactions exhibiting eosinophilia and systemic symptoms associated with solcitinib were observed in two patients (4%) and reversible hepatic dysfunction was documented in four patients (8%) (58, 59). More clinical data are needed to confirm the selective effects of selective JAK inhibitors and their efficacy and toxicity.

Based on the limited information available in the literature (57, 60), JAK inhibitors are expected to provide an alternative treatment option for patients with non-life threatening lupus who are refractory to standard therapeutic management, such as those with joint or skin disease. Many new JAK inhibitors are currently in development and will be tested in patients with SLE, and it is hoped that more-effective and less-toxic drugs will soon be available to continue to improve the prognosis of SLE patients.

KIM-1 as a Urinary Biomarker in Lupus Nephritis

Kidney injury molecule-1 (KIM-1) and TIM-1, which are the same molecule, are relatively recently discovered transmembrane proteins with Ig-like and mucin domains in their ectodomain. TIM-1 modulates $CD4^+$ T-cell responses and is also expressed by damaged proximal tubules in the kidney (where it is known as KIM-1). KIM-1 is upregulated more than any other protein in the proximal tubules of the kidneys and with various forms of injury (61, 62) (**Figure 2**). KIM-1 is a phosphatidylserine receptor

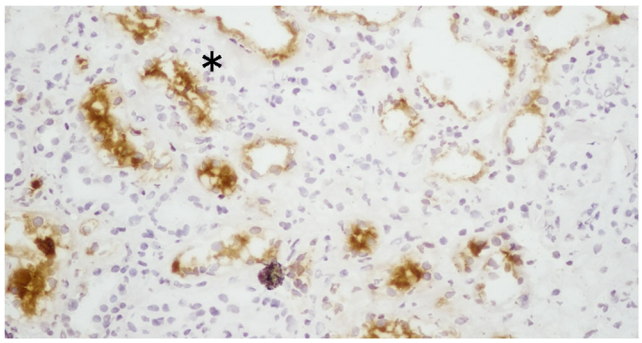

FIGURE 2 | Expression of KIM-1 (kidney injury molecule-1) in the proximal tubules of patients with lupus nephritis. The expression of KIM-1 in this renal biopsy of a patient with LN was stained by immunohistochemistry. KIM-1 was not present in the glomeruli, but it was present in the damaged and dilated tubular cells. *The expression of KIM-1 is observed on the apical surface of proximal tubular cells in the kidney.

that mediates the phagocytosis of apoptotic bodies and oxidized lipids (63). A chronic expression of KIM-1 leads to progressive renal fibrosis and chronic renal failure (64), which is speculated to be due to oxidized lipids; KIM-1 is associated with phagocytic functions that take up toxic substances such as oxidized lipids.

In addition to its role in phagocytosis, KIM-1 can activate signaling through the PI3K pathway (65). The role of KIM-1 signaling in proximal tubular cells and the link between KIM-1 phagocytosis and phosphorylation remain to be determined. Yang et al. observed that KIM-1-mediated phagocytosis functions downregulate the inflammation and innate immune responses in acute ischemic and toxic injury (66). It is thought that KIM-1 has a role in tubular interstitial damage (67). The expression of tubular KIM-1 is specific to ongoing tubular cell damage and de-differentiation (68, 69), and urinary concentrations of KIM-1 are thought to reflect this expression. KIM-1 is also associated with renal interstitial fibrosis and inflammation in certain types of renal disease (70).

Regarding prognostic factors, Austin et al. reported that tubular atrophy and fibrosis are associated with poor prognosis in LN (69). LN is often associated with comorbid acute and chronic pathological renal changes, and understanding the extent of renal damage without invasive testing is important in determining a patient's renal prognosis. The majority of tubular KIM-1 (∼90%) in various human renal diseases is of proximal origin, as was identified by double-labeling studies with aquaporin-1 (a marker for proximal tubules) (71). KIM-1 is localized in the apical membrane of dilated tubules in acute and chronic tubular injury (72). The localization of KIM-1 expression appears to be related to the susceptibility of specific tubular segments to different types of injury (72). The selective KIM-1 expression by injured proximal tubular cells provides a strong impetus for using KIM-1 as a biomarker of damage.

Elevated urinary KIM-1 levels are strongly related to the tubular KIM-1 expression in experimental settings and in human renal disease (71, 72). We observed a significant correlation between urinary KIM-1 levels and the activity in LN by and enzyme-linked immunosorbent assay (ELISA) in humans and mice (61, 73). In the former study, we assessed the urinary KIM-1 level and tubular KIM-1 expression in kidney biopsies of SLE patients and their association with histological markers of renal damage (61), and we found that the urinary KIM-1 levels were significantly correlated with proteinuria ($R = 0.39$, $p = 0.004$) and with tubular damage ($R = 0.31$, $p = 0.01$). To assess the diagnostic value of urinary Kim-1 as a novel marker for crescent formation and interstitial infiltration, we used a receiver operating characteristic curve analysis to determine a cut-off level for urinary KIM-1 levels. At urinary KIM-1 levels >11.2 ng/day, the assay had 62.5% specificity and 100% sensitivity for the diagnosis in patients with cellular crescent formation. At urinary KIM-1 levels >3.2 ng/day, the assay had 60.8% specificity and 87.5% sensitivity for the diagnosis in patients with interstitial infiltration (61). Elevated urinary KIM-1 levels were strongly associated with tubular KIM-1 expression in both an experimental setting and human renal disease, and it was revealed that urinary KIM-1 is a very promising biomarker for the presence of tubular interstitial pathology and damage (61, 74, 75).

Several studies have shown that in patients with other forms of renal injury (including ischemia, inflammation, and nephrotoxic drug injury), the renal cortical and medullary expression of tubular KIM-1 in damaged tubules is up-regulated after the disease induction (74, 75). In clinical practice, it is essential to evaluate patients' kidney status. A renal biopsy is a standard diagnostic tool for the evaluation of kidney lesions in SLE, but due to its invasive nature, a kidney biopsy has potential risks and as a rule, it is not routinely performed. Moreover, a small amount of tissue may not be representative of the entire kidney (76). It is thus highly desirable to identify early and reliable biomarkers of kidney lesions in SLE (77, 78).

Mechanisms Downstream of the TIM-1/TIM-4 Signaling Pathway

Different anti-TIM-1 antibodies that are specific to the IgV domain of TIM-1 have different effects on immune cell activation and response, due mainly to their different binding activities. A high-affinity anti-TIM-1 antibody, 3B3, forms a stable TIM-1 complex and brings TIM-1 into the TCR-CD3 complex, which enhances T-cell function and helps to form a large molecular activation cluster for complete T-cell activation (79). The low-affinity antibody RMT1-10 has an inhibitory effect and may not support the formation of a stable TIM-1-TCR-CD3 complex (80).

Foxp3-expressing regulatory T cells (Tregs) helped regulate the autoimmune response and provide protection against a murine model of LN (81). Treatment with the low-affinity antibody RMT1-10 increased the number of $foxp3^+$ cells in the thoracic cavity and the percentage of $foxp3^+CD4^+CD25^+$ cells in the spleen of the mice. RMT1-10 modulates the immune-response regulatory B cells (Bregs) and $CD19^+CD5^+$ cells, and IL-10-producing cells may be involved in the effects of TIM-1 by increasing the percentage of $CD19^+CD5^+$ and IL-10-producing cells. TIM-1 expression has been reported in activated T cells

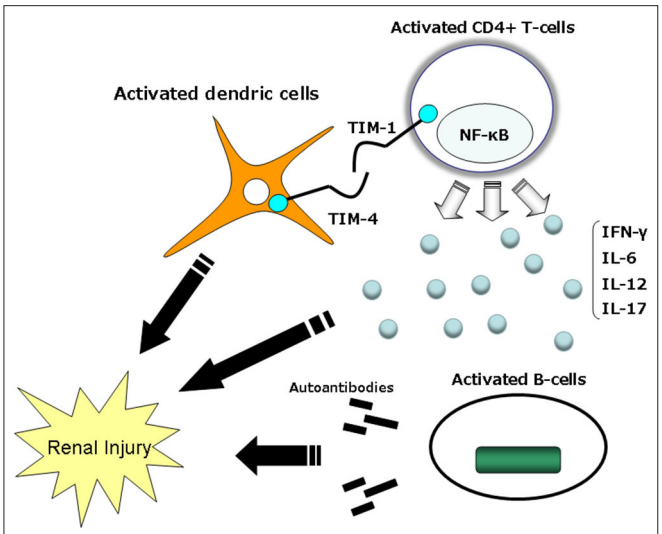

FIGURE 3 | Mechanisms by which TIM-1–TIM-4 might modulate immune function. TIM-1 expression is observed to be higher on Th2 cells than Th1 cells. TIM-4 on the surface of mature dendric cells interacts with TIM-1 on the surface of activated T cells and delivers a stimulatory signal into the T cell, thus enhancing T-cell expansion and effector functions. TIM, T-cell immunoglobulin mucin domain.

(82), DCs (83), and B cells (84). In association with this loss of IL-10 production in Bregs, the mice developed features of systemic autoimmune disease, including activated T cells with autoantibody formation and high IFN-γ production (85).

The Blocking of TIM-1/TIM-4 Pathway Agents

Macrophages and $CD4^+$, $CD8^+$, and CD4-CD8-$B220^+$ T cells are present in the kidneys of individuals with LN. Leukocyte recruitment is influenced by cytokines and chemokines, which correlate with the degree of tissue damage and predict disease progression (86, 87). Autoantibodies are important, and the T-cell population of T-cell-deficient MRL-Faslpr mice, which are prone to lupus, do not develop autoantibodies or immune complex disease (88–90). The tissue injury in LN is thus mediated by both autoantibodies and autoreactive lymphocytes (73, 91). TIM-1 can bind to TIM-4, which is expressed on antigen presenting cells (APCs) (83) (**Figure 3**). TIM-1-TIM-4 interactions on macrophages contribute to T-cell activation and macrophage-induced autoimmune nephritis (19, 81, 92). In the direct pathway, TIM-1 expressed on activated T cells cross-links with TIM-4 and directly activates macrophages.

TIM-4 is not expressed on T cells, but it is expressed on APCs, especially mature lymphoid DCs (92). TIM-4 binds to activated T cells expressing TIM-1, and TIM-1 binds to DCs expressing TIM-4; all of the fusion protein binding is mediated by TIM. It was demonstrated that RMT1-10 could be specifically blocked by a monoclonal antibody specific to TIM-1 (92).

The antibody RMT1-10 was shown to inhibit both Th1 and Th17 responses without a significant inhibitory effect on Th2 responses, Tregs or Bregs, as a low-activity antibody; treatment with RMT1-10, when administered after the development of autoimmunity and the progression of renal damage, suggesting that manipulation of TIM-1 may have potential therapeutic applications for LN. We have examined the low-affinity antibody RMT1-10 in experimental studies (73, 73, 74). In a murine lupus model, treatment with RMT1-10 attenuated the progression of lupus nephritis by prolonging survival and affecting a range of important mediators (73).

The renal manifestations of the systemic autoimmune disease SLE are characterized by the expression of autoantibodies in response to nuclear antigens, and they are associated with immune injury and a local inflammatory tissue response (93). Reduced autoantibody production is associated with a reduced recruitment of glomerular macrophages and reduced depositions of glomerular IgG and complement in brought about by RMT1-10. The serum anti-DNA antibody of IgG2a, whose switching is also known to be dependent on Th1 cytokines, was significantly reduced in RMT1-10-treated mice (94); circulating anti-DNA antibodies of IgG3 were associated with glomerulonephritis in MRL-Faslpr mice (73). These results suggest that an anti-TIM-1 antibody may affect not only the cytokine response, but also the ability to produce antibodies and immunoglobulins in LN.

CONCLUSION

The mechanisms of JAK-STAT and TIM-1/TIM-4 signaling pathways in controlling the inflammatory network in LN have been briefly explained herein. The JAK-STAT pathway sends signals from extracellular ligands such as cytokines, chemokines, growth factors, and hormones directly to the cell nuclei. Because the JAK-STAT pathway plays a major activating role in a variety of disease processes, extensive efforts have been made to develop specific inhibitors of this pathway. The JAK-STAT pathway and its components have been used in immunology for the regulation of various diseases, and this pathway is a good candidate for targeted interventions. The increased evidence of JAK-STAT activation in the pathogenesis of renal injury establishes a new set of targets for potential interventions in this disease. The TIM-1/TIM-4 pathway contributes to pro-inflammatory cytokines and triggers T-cell activation and macrophage activation. In the direct pathway, TIM-1 on activated T cells cross-links with TIM-4 and directly activates macrophages. In the indirect pathway, TIM-1 on activated T cells triggers IFN-γ production, resulting in the activation of macrophages. TIM-1 plays an important role in the development of systemic autoimmunity and its effects on end organs. The low-affinity antibody RMT1-10 inhibits both Th1 and Th17 responses without having a significant inhibitory effect on Th2 responses, Tregs or Bregs. As a result, TIM-1 appears to have potential as a therapeutic agent for LN.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Foxp3-Positive Regulatory T Cells Contribute to Antifibrotic Effects in Renal Fibrosis via an Interleukin-18 Receptor Signaling Pathway

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Renal interstitial fibrosis is a common lesion in the process of various progressive renal diseases. Interleukin (IL)-18 is a proinflammatory cytokine that plays an important role in the induction of Th1 responses and is associated with renal interstitial fibrosis, but the mechanism of fibrosis remains unclear. Here we used IL-18 receptor alpha knockout (IL-18Rα KO) mice to investigate the role of an IL-18Rα signaling pathway in renal fibrosis in a murine model of unilateral ureteral obstruction. IL-18 Rα KO mice showed decreased renal interstitial fibrosis and increased infiltration of CD4+ T cells and Foxp3+ regulatory T cells (Tregs) compared to wildtype (WT) mice. The expression of renal transforming growth factor beta 1 (TGF-β1, which is considered an important cytokine in renal interstitial fibrosis) was not significantly different between WT and IL-18Rα KO mice. The adoptive transfer of CD4+ T cells from the splenocytes of IL-18Rα KO mice to WT mice reduced renal interstitial fibrosis and increased the number of Foxp3+ Tregs in WT mice. These results demonstrated that Foxp3+ Tregs have a protective effect in renal interstitial fibrosis via an IL-18R signaling pathway.

Keywords: IL-18, IL-18 receptor, renal fibrosis, unilateral ureteral obstruction, regulatory T cells

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INTRODUCTION

Renal interstitial fibrosis is a common and important lesion in the process of various progressive renal diseases that progress to renal atrophy. The pathological features of renal interstitial fibrosis are renal tubular atrophy, a reduction or disappearance of peritubular capillaries, and an increase in the extracellular matrix (ECM) (1–3). Interleukin (IL)-18 is a proinflammatory cytokine and member of the IL-1 family, and it is produced by macrophages, dendritic cells, epithelial cells, keratinocytes, and other cell types (4–6). IL-18 is stored intracellularly as a biologically inactive precursor (pro-IL-18), similar to IL-1β, and is secreted extracellularly as the bioactive mature form of IL-18 after being cleaved by IL-1β-converting enzyme (caspase 1). IL-18 recognizes a heterodimeric receptor, which consists of unique α [IL-1 receptor(R)-related protein] signaling chain and non-binding β (IL-1R-accessory protein-like) signaling chain (7). IL-18 promotes the production of interferon gamma (IFN-γ) and strongly induces a Th1 response (8).

In the kidney, IL-18 is expressed in the renal tubular epithelium. Patients with chronic kidney disease or nephrotic syndrome exhibit elevated levels of IL-18 (9–12). IL-18 is associated with renal interstitial fibrosis, and IL-18 neutralization has been shown to prevent renal injury and fibrosis in

unilateral ureteral obstruction (UUO) mice (13). However, the mechanism of IL-18 during renal obstruction remains unclear. We conducted the present study to: (1) determine whether renal interstitial fibrosis is reduced in IL-18Rα knockout (KO) mice undergoing UUO, and (2) elucidate the mechanisms underlying fibrosis.

MATERIALS AND METHODS

Ethics Statement

The animal protocols were approved by the Kindai University Animal Care Committee and were performed in accordance with the Kindai University Animal Care Guidelines (KAME-22-014, 1/4/2010).

Animals

IL-18Rα-deficient mice on a C57BL/6 background were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan). The C57BL/6 mice used as the wildtype control (WT) were purchased from the Shizuoka Laboratory Animal Center (Shizuoka, Japan). All mice were bred in our specific pathogen-free animal facility. Eight-week-old female mice were used in this study.

UUO Protocol

For the UUO model, the mouse was anesthetized with inhaled isoflurane and underwent left ureteral obstruction (the controls underwent a sham operation). The left ureter was isolated and completely ligated with a 3–0 silk suture. Sham-operated animals underwent the same surgery without ureteral ligation. Operated mice were re-anesthetized and culled at day 3 (WT and IL-18RαKO; $n = 6$ and 5), day 7 ($n = 5$), or day 14 ($n = 6$) after surgery. The left kidneys were harvested for analysis. Blood was collected in heparinized tubes for the measurement of blood urea nitrogen (BUN) and IL-18.

Histological Analysis

Kidney tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with periodic acid-Schiff-stained (PAS) reagent. Tubular injury was evaluated based on a semiquantitative scale by determining the percentage of cortical tubules in which epithelial necrosis, loss of brush border, cast formation, and tubular dilation were evaluated: 0 = normal kidney; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; 4 = 76–100% tubules injured.

CD4+ T cells, kidney injury molecule-1 (Kim-1), and type IV collagen were demonstrated by the immunoperoxidase staining of frozen 6-μm-thick periodate-lysine-paraformaldehyde-fixed kidney sections, as described (14). F4/80+ cells as macrophages, α-smooth muscle actin (α-SMA) and Forkhead box protein 3 (Foxp3) as regulatory T cells (Tregs), and cleaved caspase-3 were identified in 4-μm-thick formalin-fixed sections as described (14, 15). The numbers of CD4+ T cells, F4/80+ cells, Foxp3+ cells, and cleaved caspase-3+ cells were assessed in 10 fields per slide at × 400 magnification, and the results are expressed as cells per high-power field (c/hpf). Tubular Kim-1 immunostaining was quantified by counting the number of positively stained tubules in 10 fields per slide at a magnification of × 400. A positive tubule cross-section was defined as having two or more stained cells. The positive area of type IV collagen and α-SMA were assessed in 10 fields per slide at × 400 magnification with a fluorescence microscope and analyzer (model BZ-X700, Keyence, Osaka, Japan).

The primary monoclonal antibodies used were rat monoclonal antibody GK1.5 for CD4+ T cells (Pharmingen, San Diego, CA), F4/80 hybridoma culture supernatant (HB198; American Type Culture Collection, Manassas, MD), rat monoclonal antibody for TIM-1 (R&D Systems, Minneapolis, MN), mouse monoclonal antibody for α-SMA (Sigma-Aldrich, St. Louis, MO), rabbit polyclonal antibody for type IV collagen (ab6586; Abcam, Cambridge, United Kingdom), mouse/rat monoclonal antibody for Foxp3 (FJK-16s; eBioscience, Hatfield, United Kingdom), and rabbit antibody recognizing the cleaved form of caspase-3 (Cell Signaling Technology, Beverly, MA).

Real-Time PCR Analysis

We performed a real-time polymerase chain reaction (PCR) as described (14) for the measurement of the intrarenal mRNA expressions of IFN-γ, monocyte chemoattractant protein-1 (MCP-1/CCL2), matrix metalloproteinase-2 (MMP-2), Foxp3, and 18SrRNA by using FastStart DNA master Sybr Green I (Applied Biosystems, Foster City, CA) and the expressions of IL-6, IL-10, IL-12, IL-18R, tumor necrosis factor-alpha (TNF-α), transforming growth factor-β1 (TGF-β1), Kim-1, and 18S RNA by using Taqman gene (Applied Biosystems) on whole

TABLE 1 | Primer sequences for analysis of mRNA expression.

	Forward primer	Reverse primer
18SrRNA	GTAACCCGTTGAACCCCATTC	GCCTCACTAAACCATCCAATCG
IFN-γ	TGCTGATGGGAGGAGATGTCT	TTTCTTTCAGGGACAGCCTGTT
MCP-1	AAAAACCTGGATCGGAACCAA	CGGGTCAACTTCACATTCAAAG
MMP-2	ACCCAGATGTGGCCAACTAC	GAGCAAAGGCATCATCCACT
Foxp3	GGCCCTTCTCCAGGACAGAC	TCCACAGTGGAGAGCTGATGC

IL, interleukin; IFN, interferon; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; Foxp, Forkhead box protein.

TABLE 2 | Gene database number for analysis of mRNA expression.

	Forward primer
18SrRNA	NM_026744.3
IL-6	Mm00446190
IL-10	Mm99999062_m1
IL-12p40	Mm00434174_m1
IL-18R1 (IL-18Rα)	Mm00515180_m1
TNF-α	Mm99999068_m1
TGF-β1	Mm03024053_m1
Kim-1	Mm00506686_m1

IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; Kim, kidney injury molecule.

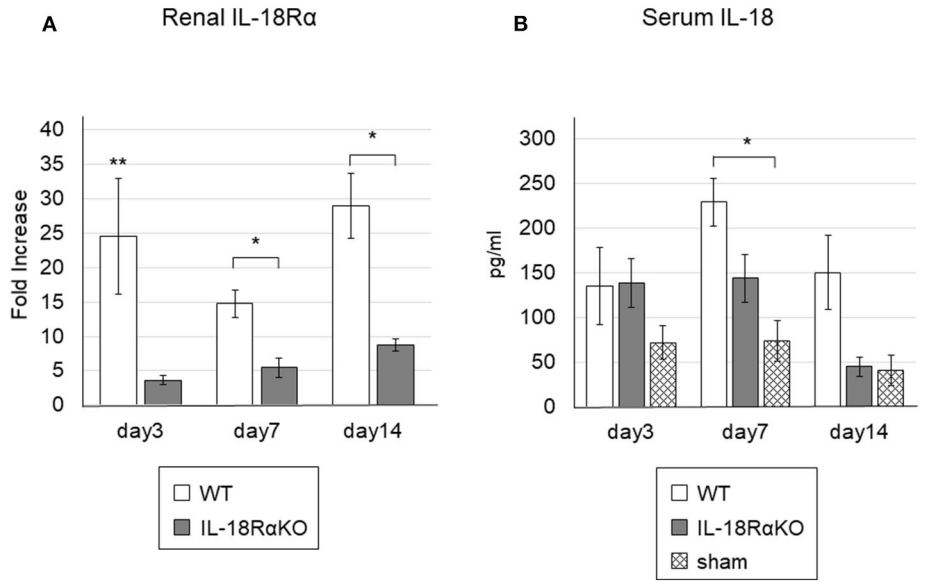

FIGURE 1 | Gene expressions of IL-18Rα in the mouse kidney and the levels of serum IL-18. **(A)** Gene expressions of IL-18Rα measured by real-time PCR. **(B)** The levels of IL-18 in the blood. The data are mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$.

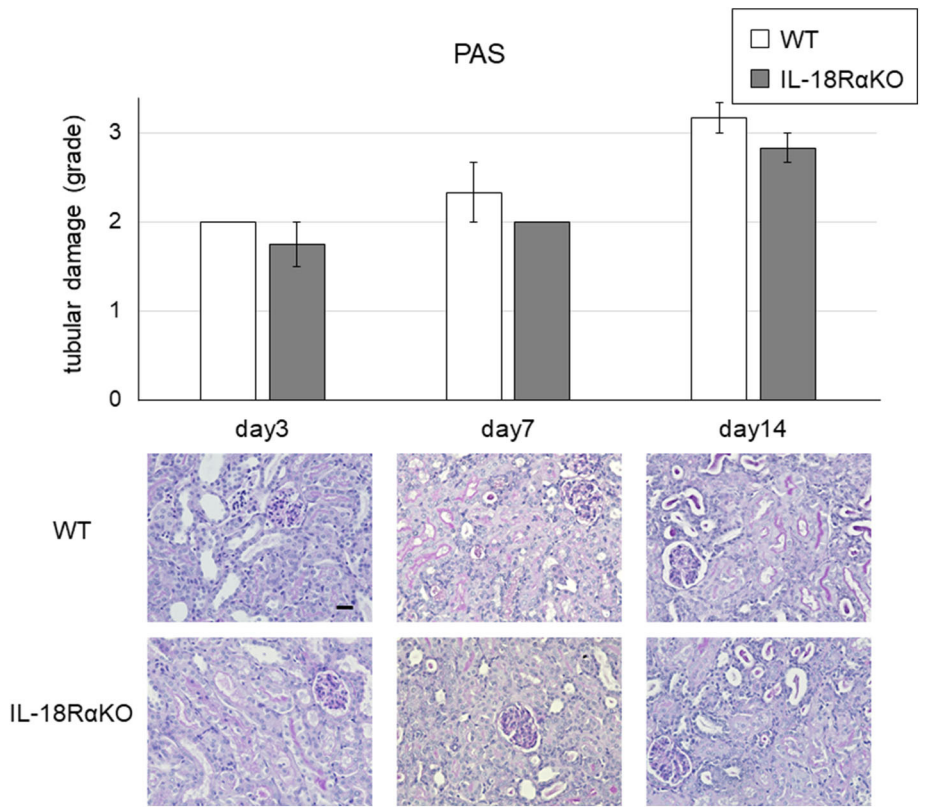

FIGURE 2 | Representative light microscopy of periodic acid Schiff (PAS)-stained renal sections after unilateral ureteral obstruction (UUO). Tubular injury in the cortex of the kidney was scored (see the Materials and Methods section for the scoring method). The data are mean ± SEM. Original magnification × 400. Scale bar, 50 μm.

kidney tissue. The sequences of the primers and the gene database number are listed in **Tables 1**, **2**. The relative amount of mRNA was calculated using the comparative Ct (ΔΔCt) method. All specific amplicons were normalized against 18SrRNA, which was amplified in the same reaction as an internal control using commercial reagents (Applied Biosystems) and is expressed as the fold increase relative to the data of the sham-operated mice.

Serum IL-18 Quantitation by ELISA

The serum IL-18 levels in the mice were determined by an enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Diego, CA) as described (14).

Cell Sorting and Adoptive Transfer Experiments

CD4+ T cells were isolated using a BD FACSAria special-order research product (Becton Dickinson, Lincoln Park, NJ) for 90–95% purity from splenocytes in IL-18Rα KO mice for the adoptive transfer. Approximately 2×10^6 CD4+ T cells were injected intravenously into WT mice 3 days before they were subjected to the UUO operation. WT mice that received a transfer of CD4+ T cells were sacrificed at day 7 after surgery (n = 10). WT mice transferred with CD4+ T cells were compared to non-transferred WT mice. The flow cytometry antibody was FITC-anti-CD4 (BD Bioscience). An isotype-matched irrelevant monoclonal antibody was used. Cells that fluoresced at levels above the negative control were considered positive.

Statistical Analyses

The results are expressed as the mean ± SEM. Groups were compared by the Mann-Whitney U-test. Multiple comparisons were analyzed using Dunn's multiple comparisons test. We analyzed the data using GraphPad Prism software (GraphPad, La Jolla, CA). Differences were accepted as significant when the $p < 0.05$.

RESULTS

Renal IL-18R Expression and Serum IL-18 Levels

The renal IL-18Rα expression as measured by real-time PCR is shown in **Figure 1A**. The renal IL-18Rα expression in IL-18RαKO mice was significantly decreased on days 3, 7, and 14 compared to the expression in WT mice. **Figure 1B** illustrates the serum IL-18 levels measured by ELISA. The serum IL-18 levels in WT mice were significantly increased on day 7 compared to those in sham-operated mice. On day 14, IL-18RαKO mice tended to have lower serum IL-18 levels compared to WT mice, although the difference was not statistically significant ($P = 0.07$), and their serum IL-18 levels were similar to those of sham-operated mice.

Functional and Structural Aggravation From UUO

The semiquantitative tubular injury scores obtained by PAS staining are shown in **Figure 2**. The intensity of tubular injury gradually increased from day 3 to day 14 after ureteral ligation. There was no significant difference in the tubular injury score between WT mice and IL-18RαKO mice. **Figure 3** shows the BUN levels as an indications of kidney function. No significant difference in BUN levels was observed between WT and IL-18RαKO mice.

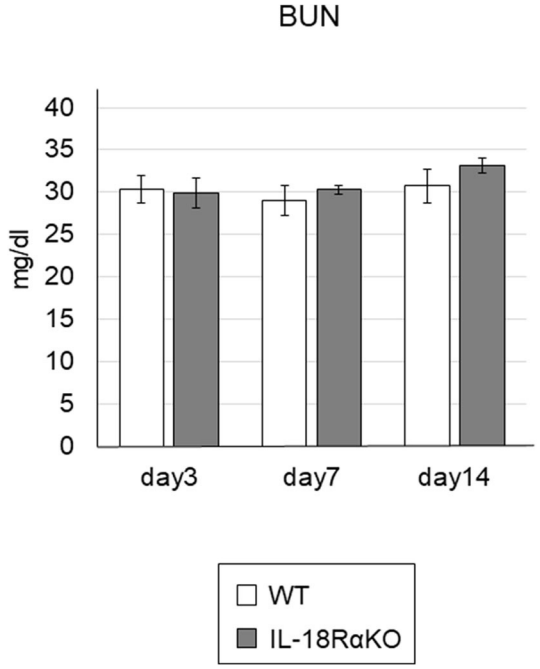

FIGURE 3 | The levels of BUN. BUN was measured from blood samples as an indicator of kidney function. Data shown are the mean ± SEM.

Expression of Type IV Collagen

Figure 4 shows representative results of the immunohistochemical staining for type IV collagen. The interstitial type IV collagen expression in IL-18RαKO mice was significantly decreased on day 7 compared to that in WT mice. On day 14, IL-18Rα KO mice tended to have lower expression of type IV collagen compared to WT mice, although the difference was not statistically significant ($P = 0.09$).

Expression of α-SMA

Examples of the immunohistochemical staining for α-SMA are shown in **Figure 5**. The interstitial α-SMA expression gradually increased from day 3 to day 14 after ureteral ligation. On days 7 and 14, the interstitial α-SMA expression in IL-18RαKO mice was significantly reduced compared to that in WT mice.

Expression of Kim-1

Figure 6 provides examples of the immunohistochemical staining for tubular Kim-1. Kim-1+ tubules were not observed in the sham-operated mice, whereas the Kim-1 expression was increased in WT and IL-18RαKO mice. Kim-1+ tubules in IL-18RαKO mice were decreased significantly on days 3 and 7 compared to WT mice. We also investigated the Kim-1 mRNA expression as measured by real-time PCR and observed that the

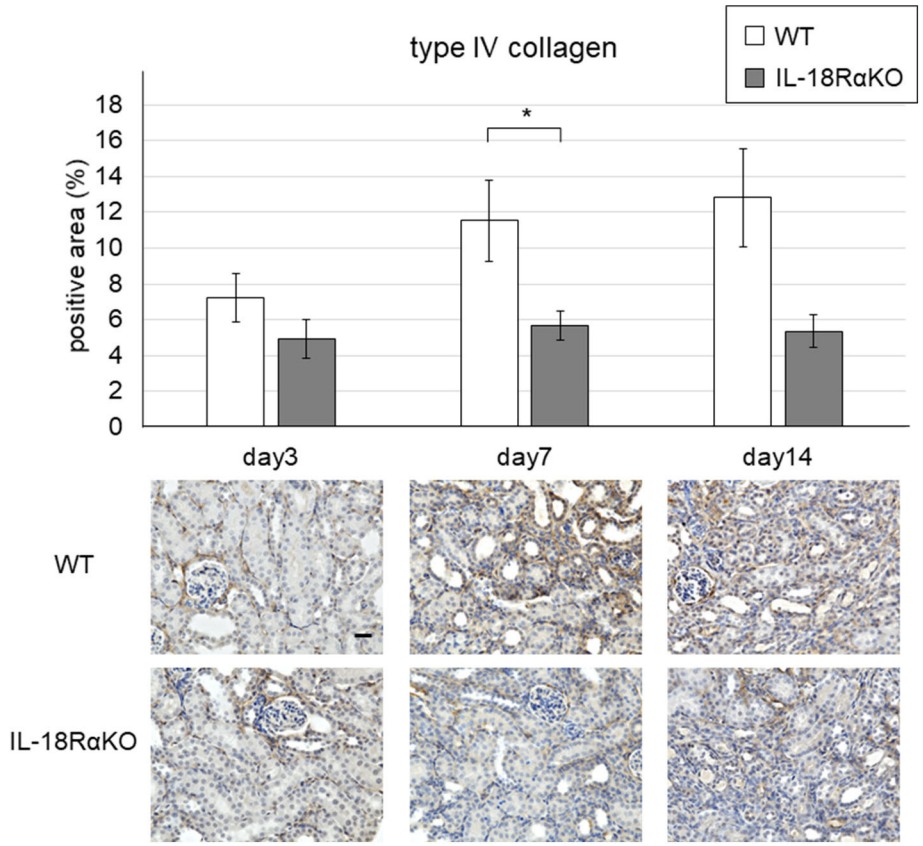

FIGURE 4 | Expression of type IV collagen in the mouse kidney. The interstitial type IV collagen expression in IL-18RαKO mice was significantly reduced compared to that in WT mice on day 7 after unilateral ureteral obstruction. Data shown are the mean ± SEM. Original magnification × 400. Scale bar, 50 μm. *$p < 0.05$.

expression in IL-18RαKO mice was decreased significantly on days 7 and 14 compared to WT mice (**Table 3**).

The Infiltration of CD4+ T Cells and Macrophages

We investigated the infiltration of CD4+ T cells and macrophages in the renal interstitium (**Figure 7**). The number of interstitial CD4+ T cells in IL-18RαKO mice on days 7 and 14 was significantly increased compared to WT mice. The number of F4/80+ macrophages was increased after ureteral ligation. There was no significant difference in macrophage infiltration between WT mice and IL-18RαKO mice.

Expression of Foxp3

Figure 8A shows immunohistochemical staining for Foxp3. The number of Foxp3+ cells in IL-18RαKO mice was significantly increased on day 14 compared to WT mice. We also determined the Foxp3 mRNA expression measured by real-time PCR and observed that the expression in IL-18RαKO mice was increased significantly on days 3, 7, and 14 compared to WT mice (**Table 3**).

Expression of Cleaved Caspase-3

Examples of the immunohistochemical staining for cleaved caspase-3, a marker of apoptosis, are given in **Figure 8B**. The number of cleaved caspase-3+ tubular cells was increased after ureteral ligation. On days 7 and 14, a significant reduction in the number of cleaved caspase-3+ tubular cells were present in IL-18RαKO mice compared to WT mice.

Expression of Renal mRNA

The values of the renal mRNA expressions as measured by real-time PCR are shown in **Table 3**. There was no significant difference in the mRNA expressions of IFN-γ, TNF-α, TGF-β1, IL-6, IL-10, IL-12, MCP-1, or MMP-2 between WT mice and IL-18RαKO mice.

Splenocyte Adoptive Transfer Reduced Interstitial Fibrosis

The results of the above experiments suggested that IL-18Rα knockout reduced the renal interstitial fibrosis in the UUO model mice. In the IL-18RαKO kidneys, the number of CD4+ T cells was increased compared to the WT kidneys. Based on these results, in order to determine whether CD4+ T cells affect interstitial fibrosis, we transferred CD4+ T cells extracted from IL-18RαKO mouse splenocytes into WT mice.

We then observed that WT mice transferred with CD4+ T cells had significantly lower levels of interstitial type IV collagen and α-SMA expression compared to the WT mice without

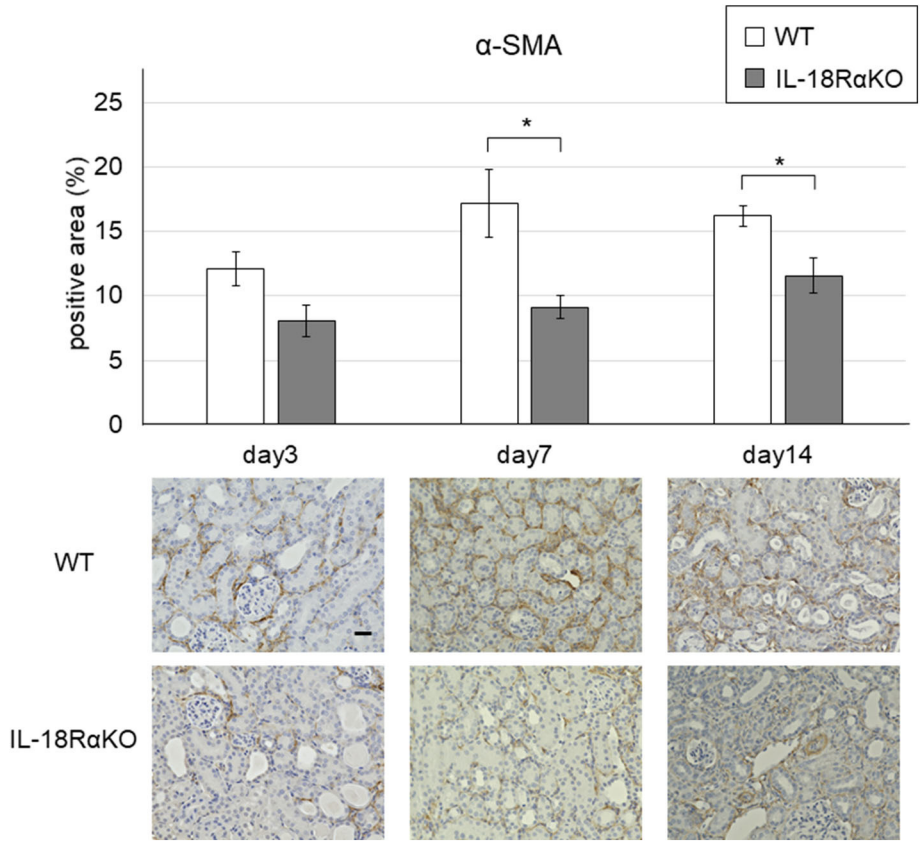

FIGURE 5 | Expression of α-SMA in the mouse kidney. The interstitial α-SMA expression in IL-18RαKO mice was significantly reduced compared to WT mice on days 7 and 14 after unilateral ureteral obstruction. The data are mean ± SEM. Original magnification × 400. Scale bar, 50 μm. *$p < 0.05$.

transferred CD4+ T cells (**Figures 9A,B**). The numbers of CD4+ T cells and Foxp3+ cells were significantly increased and the number of cleaved caspase-3+ cells was significantly decreased in transferred WT mice compared to non-transferred WT mice (**Figures 9C–E**).

DISCUSSION

We have reported that MRL/lpr mice (a well-known model of lupus) cross-bred with mice that are deficient in IL-18Rα exhibited a reduction in autoantibodies, nephritis, and death (16). We also observed that when IL-18RαKO mice experienced a lipopolysaccharide (LPS)-induced acute kidney injury (AKI), they had markedly ameliorated renal function (17). However, a paradoxical finding was obtained in our past studies and other investigations; i.e., that the blockade of IL-18R is associated with an exacerbation of several diseases (14, 18–20). We previously reported that splenic and renal suppressors of cytokine signaling (SOCS)1 and SOCS3 were downregulated in IL-18RαKO mice compared to WT mice with cisplatin-induced acute kidney injury (14). In addition to the inflammatory response, IL-18Rα may induce an anti-inflammatory response by affecting the expression of the cytokine signaling inhibitors SOCS1 and SOCS3. Thus, IL-18/IL-18R interaction may regulate a dynamic balance between destructive and repair signals in renal injury (18). Whether the IL-18/IL-18Rα cytokine signaling pathway acts nephroprotectively may depend on the disease model. In the present study, we investigated whether IL-18R knockout and blockade aggravated or ameliorated renal interstitial fibrosis, and our findings revealed that IL-18RαKO mice had significantly reduced tubular cell apoptosis and significantly suppressed interstitial fibrosis compared to WT mice during obstructive injury. There was no significant difference between WT and IL-18RαKO mice in the results of PAS staining. However, the tubular injury was assessed on a 4-point semi-quantitative scale, which may have obscured the differences in PAS staining between WT and IL-18RαKO mice. We measured BUN levels and found no significant differences between WT and IL-18RαKO mice. This may have been due to compensation by the healthy side of the kidney, which is less likely to reflect renal injury on the obstructed side.

In our previous report using sepsis model mice, we found an increased expression of IL-18 in CD4+ T cells of the spleen (17). Although the origin of IL-18 production was not investigated in this study, a previous report suggests that tubular epithelial cells are the predominant source of IL-18

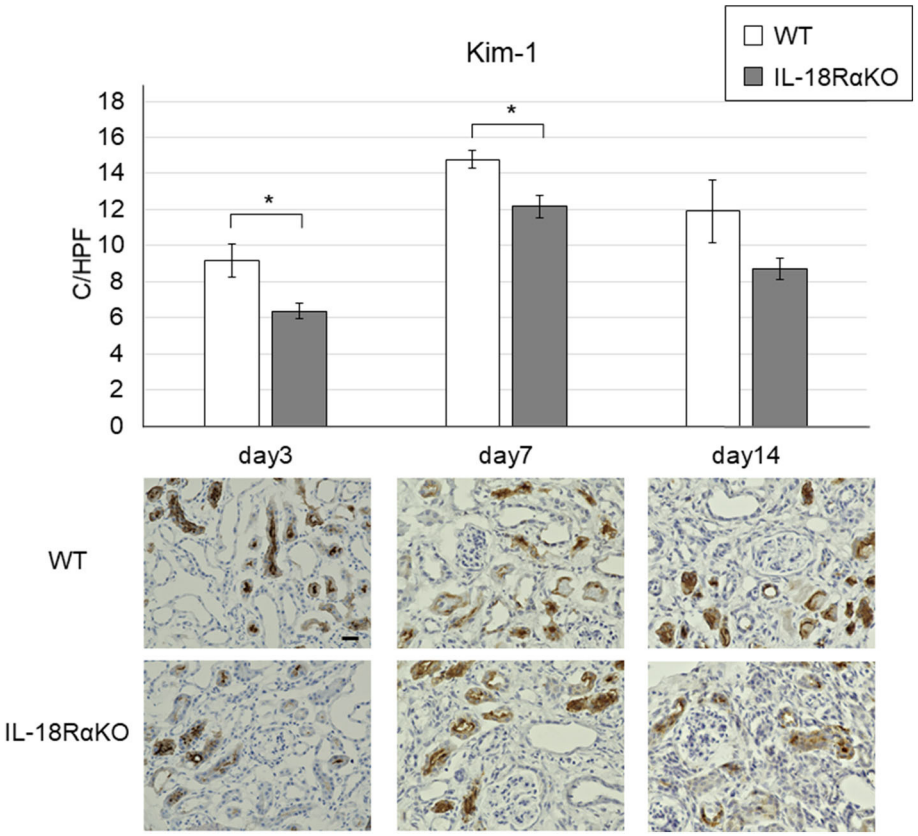

FIGURE 6 | Expression of Kim-1 in the mouse kidney. Kim-1+ tubules were significantly decreased in IL-18RαKO mice on days 3,7, and 14 compared to WT mice. The data are mean ± SEM. Original magnification × 400. Scale bar, 50 μm. *$p < 0.05$.

TABLE 3 | Expression of renal mRNA.

	day3	day7	day14
	WT vs. IL-18RαKO	**WT vs. IL-18RαKO**	**WT vs. IL-18RαKO**
IL-6	123.3 ± 31.36 vs. 93.4 ± 19.0	24.1 ± 4.3 vs. 26.8 ± 5.4	53.8 ± 5.7 vs. 47.0 ± 6.2
IL-10	82.9 ± 26.2 vs. 39.4 ± 11.5	34.7 ± 4.3 vs. 31.8 ± 5.4	31.4 ± 8.0 vs. 41.7 ± 10.0
IL-12	3.5 ± 0.7 vs. 4.0 ± 0.7	5.0 ± 0.5 vs. 6.0 ± 0.8	16.7 ± 1.8 vs. 25.3 ± 4.9
IFN-γ	4.2 ± 0.2 vs. 3.5 ± 0.7	9.1 ± 0.4 vs. 7.2 ± 0.6	12.2 ± 2.0 vs. 12.3 ± 3.0
TNF-α	18.1 ± 4.1 vs. 20.5 ± 5.7	12.4 ± 2.2 vs. 13.9 ± 2.2	22.1 ±2.6 vs. 23.0 ± 1.8
TGF-β1	9.1 ± 2.4 vs. 7.4 ± 1.4	5.1 ± 0.7 vs. 4.4 ± 0.6	7.8 ± 0.6 vs. 6.2 ± 0.8
MCP-1	29.3 ± 4.7 vs. 24.1 ± 5.5	30.5 ± 1.3 vs. 50.4 ± 8.6	35.2 ± 5.6 vs. 31.4 ± 3.2
MMP-2	8.1 ± 1.1 vs. 6.2 ± 1.0	41.1 ± 4.2 vs. 30.4 ± 5.3	30.1 ± 3.7 vs. 33.6 ± 6.4
Kim-1	2778.1 ± 595.3 vs. 1687.8 ± 334.7	385.7 ± 49.8 vs. 206.9 ± 25.5**	272.9 ± 47.4 vs. 137.5 ± 18.8*
Foxp3	1.36 ± 0.1 vs. 2.0 ± 0.1**	4.4 ± 0.4 vs. 8.7 ± 1.5*	12.4 ± 1.3 vs. 20.8 ± 3.0*

*Cytokines, chemokines, and fibrosis-related molecules were measured by real-time PCR in WT and IL-18RαKO mice on days 3, 7, and 14 after unilateral ureteral obstruction. In each experiment, the expression levels were normalized to the expression of 18SrRNA and are expressed relative to the values of sham-operated mice. The data are mean fold-increase ± SEM. *p <0.05, **p < 0.01.*

production during renal occlusion (21). We compared serum IL-18 levels and renal IL-18Rα expression in IL-18RαKO and WT mice. IL-18RαKO mice tended to have lower serum IL-18 levels than WT mice, but the difference was not statistically significant. This may have been due to the small sample size. Brian et al. showed an increase in renal IL-18 levels and IL-18R expression in WT mice compared to IL-18RKO mice after UUO and reported that IL-18 stimulates its own production during renal obstruction via a positive feedback loop involving IL-18R. Based on their findings we consider that inhibition of the

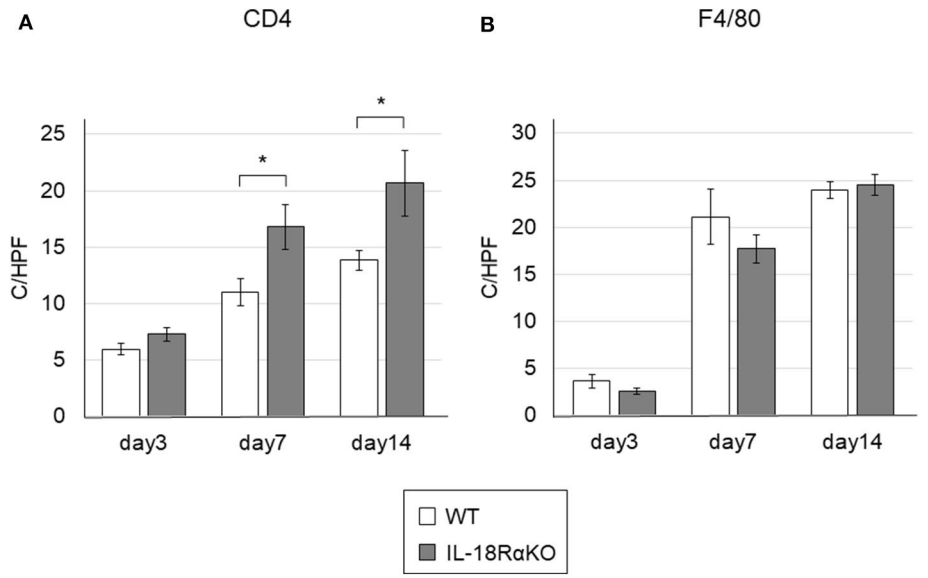

FIGURE 7 | The infiltration of CD4+ T cells and macrophages in the mouse kidney. **(A)** The numbers of interstitial CD4+ cells in IL-18RαKO mice on days 7 and 14 were significantly increased compared to WT mice. **(B)** The numbers of F4/80+ cells as macrophages were not significantly different between WT mice and IL-18RαKO mice. The data are mean ± SEM. $*p < 0.05$.

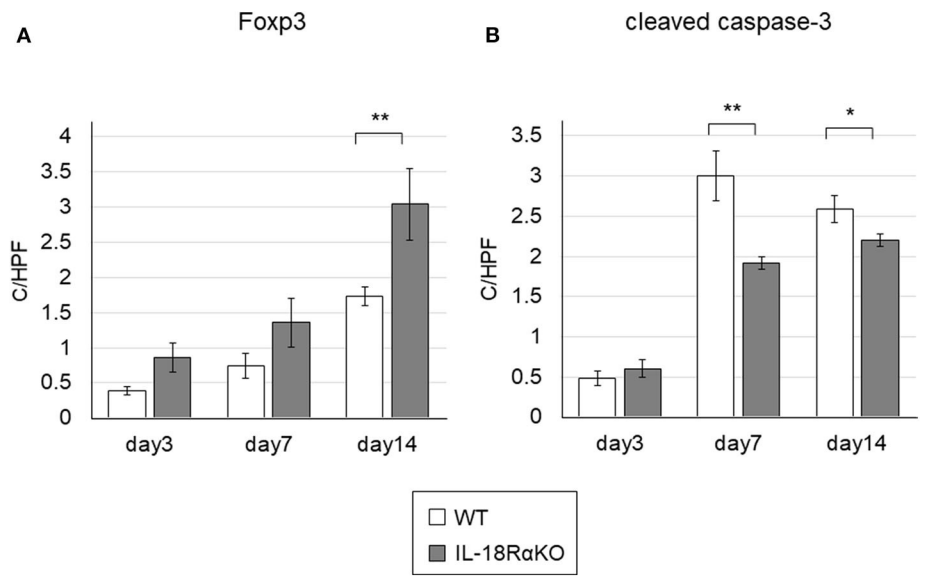

FIGURE 8 | Foxp3+ cells and cleaved caspase-3+ cells in the mouse kidney. **(A)** The number of interstitial Foxp3+ cells in IL-18RαKO mice was increased significantly on day 14 compared to WT mice. **(B)** The numbers of cleaved caspase-3+ tubular cells in IL-18RαKO mice were significantly decreased on days 7 and 14 compared to WT mice. The data are mean ± SEM. $*p < 0.05$, $**p < 0.01$.

IL-18/IL-18Rα signaling pathway by IL-18Rα deficiency blocks intracellular signaling and reduces the expression of cytokines, including IL-18, in the cell nucleus. In our results, renal IL-18Rα expression in IL-18RαKO mice was significantly reduced compared to that in WT mice. Even in the KO model, IL-18Rα KO mice appeared to express low levels of renal IL-18Rα. We analyzed the renal expression of IL-18Rα using the ΔΔCt method. The ΔΔCt method is a comparison of mRNA expression levels between experimental and sham-operated mice, and using this method, we consider that faint levels of expression might be observed as non-specific mRNA expression. The results of this analysis showed that, although IL-18 expression was observed at low levels, it was significantly reduced in IL-18RαKO mice compared to WT mice. Therefore, we do not expect that IL-18 expression affected the conclusions drawn from this study.

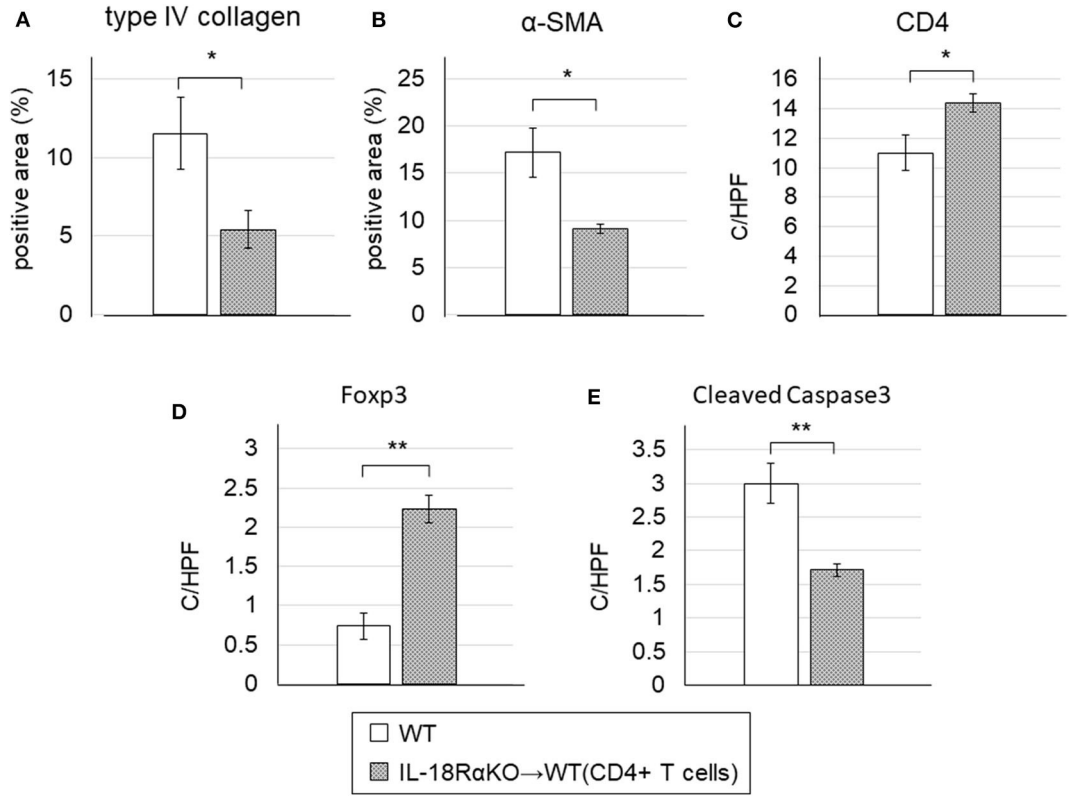

FIGURE 9 | Effects of the adoptive transfer of CD4+ T cells from IL18RαKO mouse splenocytes. CD4+ T cells (2×10^6/mouse) from splenocytes of IL-18RαKO mice were injected intravenously into WT mice 3 days before they mice were subjected to the UUO protocol. WT mice that received a transfer of CD4+ T cells were sacrificed on day 7 after UUO and compared with non-transferred WT mice. WT mice transferred with CD4+ T cells had significantly lower interstitial **(A)** type IV collagen and **(B)** α-SMA expression compared to the non-transferred WT mice. The numbers of **(C)** CD4+ cells and **(D)** Foxp3+ cells were significantly increased and **(E)** the number of cleaved caspase-3+ cells was significantly decreased in the transferred WT mice compared to the non-transferred WT mice. The data are mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$.

Renal fibrosis is believed to be the result of an immune response involving myofibroblast accumulation and matrix deposition (22). TGF-β is a mediator that plays a central role in renal fibrosis, and its inhibition reduces renal fibrosis in animal models (23). Intracellular signal transduction of TGF-β is mediated mainly by smad2/3 phosphorylation, and it acts on ECM accumulation (23). The epithelial mesenchymal transition (EMT) is the mechanism underlying TGF-β-induced renal fibrosis. The tubular EMT is a biological process by which renal tubular cells lose their epithelial phenotype and acquire new mesenchymal features (24). TGF-β plays an important role not only in the induction of the EMT but also in the induction of the endothelial-to-mesenchymal transition (EndMT), and the induction of the EndMT requires an interaction with inflammatory signal transduction pathways such as those involving TNF-α and IL-1β (25). It is also speculated that MCP-1 and MMP-2 contribute to progressive fibrosis (26, 27), whereas IFN-γ is considered to be an antifibrotic cytokine that attenuates renal fibrosis (28, 29).

Herein we investigated various fibrosis-related markers including TGF-β1 by performing a PCR analysis, but we did not obtain any results that could explain the inhibitory effect of IL-18RαKO on renal fibrosis. We also measured TGF-β1 protein levels by western blotting and found no significant difference between WT and IL-18RαKO mice (data not shown). These results suggested that IL-18R is involved in renal interstitial fibrosis by a mechanism that is independent of TNF-α and TGF-β1. Similarly, Bani-Hani et al. reported that transgenic mice with neutralized IL-18 activity exhibited a suppressed EMT and renal fibrosis without demonstrating alterations in TGF-β1 or TNF-α activity (13). Our present findings showed suppression of tubular cell apoptosis in IL-18RαKO mice. The proapoptotic activity of IL-18 has been shown to be mediated through a Fas/FasL-dependent pathway (30).

To elucidate the mechanism of IL-18R-induced renal interstitial fibrosis, we focused on the increase of CD4+ T cells in the kidney of IL-18Rα KO mice. We speculated that Foxp3+ Tregs may play a protective role in tubular cell apoptosis and interstitial fibrosis. We tested these hypotheses by conducting the adoptive transfer of CD4+ T cells from the splenocytes of IL-18Rα KO mice into WT mice, and the results demonstrated the suppression of fibrosis with an increase of Foxp3+ cells in the kidneys of the transferred WT mice. In addition, CD4+ T cells from IL18RαKO mice may have been involved in the

induction of Foxp3+ Tregs through a mechanism that remains to be identified.

Tregs are a subset of CD4+ T cells and are characterized by the expression of the Foxp3. Tregs are 5–10% of peripheral CD4+T cells in normal mice and healthy humans, and they play important roles in immune homeostasis and in the suppression of unwanted inflammatory responses to self-antigens (31–33). In animal models, Tregs have been shown to have a renal-protective effect through the suppression of renal inflammation (34–39). In an ischemia-reperfusion injury mouse model, the depletion of Tregs led to worse neutrophil infiltration, tubular damage, and renal function (36, 37). A protective effect of Tregs has also been reported in nephrotoxic renal injury and septic AKI, and Tregs are expected to be the focus of a novel therapeutic approach for these renal diseases (38, 39). Little has been reported on the role of Tregs in obstruction-induced renal interstitial fibrosis via IL-18R.

Our study has some limitations. The cause of the increase in CD4+ T cells in the kidneys of IL-18RαKO mice was not fully examined. In addition, we did not investigate the transcription factors potentially responsible for the differentiation of naive CD4+T cells, such as T-bet, GATA3, and RoRγt, or markers associated with the switch from the Th1 to the Th2 phenotype, such as IL-4, IL5, and Il-13. In addition to CD4+ T cells, IL-18Rα is also expressed on CD8+ T cells and NK cells and is activated by IL-18 (40), but we did not examine the effects of IL-18RαKO on CD8+ T cells and NK cells in the UUO model. Our results did not fully clarify the mechanism by which Tregs are induced in IL-18RαKO mice. In a report showing that IL-12p40 and IL-18 were necessary for viral control and recovery from ectromelia virus infection, Wang et al. reported that splenic Tregs were increased in IL-18-deficient mice (41). IL-6, together with TGF-β, induces the generation of Th17 cells from naive T cells and inhibits Tregs differentiation (42). Thiolat et al. reported that treatment with anti-IL-6 antibodies increased Tregs in the spleens of mice with collagen-induced arthritis (CIA) and the patient sera of rheumatoid arthritis patients (43). In the CIA model, we previously reported that IL-18RαKO mice had reduced IL-6 levels in serum and splenocytes and reduced IFN-γ production in splenic CD4+ T cells compared to the WT mice (44). In the present study, we did not examine the cytokine profile of CD4+ T cells, including IL-6, which may affect Tregs differentiation, in IL-18RαKO mice. Although the reason for the increase in Tregs is not clear, IL-6 may be involved in IL-18Rα-mediated effects on Tregs. Further experiments are required to compare the detailed cytokine profiles of CD4+ T cells in WT and IL-18RαKO mice. In addition, experiments to transplant CD4+ T cells from the bone marrow and kidneys of IL-18RαKO mice into WT mice would be necessary to investigate whether renal or extra-renal IL-18 is more important.

In our present study, although the mechanisms underlying the induction of Tregs and the suppression of fibrosis by Tregs remain unclear, our findings are important in the establishment of the pathology of renal interstitial fibrosis mediated by IL-18 and in the design of therapeutic strategies.

In conclusion, we determined the effect of IL-18RKO in UUO model mice. This study is one of the few that has investigated the relationship between IL-18R and renal interstitial fibrosis. Our results suggest that Foxp3+ Tregs have a protective effect in the pathology of renal interstitial fibrosis via IL-18R. Our findings may provide a new therapeutic target for the control of renal interstitial fibrosis by IL-18.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Kindai University Animal Care Committee Kindai University School of Medicine.

AUTHOR CONTRIBUTIONS

YH and YN designed, performed, and interpreted the experiments. YH drafted the manuscript. YH and KN performed the experiments. YH, YN, and AI analyzed the data. YN, MS, KK, MF, and IM edited the manuscript. All authors contributed to the manuscript's revision, read, and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Heparin Attenuates Histone-Mediated Cytotoxicity in Septic Acute Kidney Injury

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Histones are considered potential risk factors that contribute to the development of septic acute kidney injury (SAKI) by inducing apoptosis and inflammation. This study aimed to explore the protective effects of heparin on septic acute kidney injury through the neutralization of extracellular histones (EH) and to uncover the underlying mechanism. C57BL mice (16 each) were randomly divided into the sham group, the sepsis group (established by cecal ligation and puncture operation, CLP), and the heparin intervention group. Mice in the heparin intervention group received a subcutaneous injection of unfractionated heparin (0.03 IU/g) 4 h after CLP. At 6 h after the operation, nine mice from each group were sacrificed by the removal of the eyeballs to harvest blood samples; the upper half of the right kidney was used as the study sample. Mice renal tubular epithelial cells cultivated in six-well plates were equally divided into five groups. We cultured cells treated with either histone (40 U), histone (40 U) + heparin (25 IU/ml), histone(40U) + lipopolysaccharides (LPS; 10 μg/ml), or histone (40 U) + LPS (10 μg/ml) + heparin (25 IU/ml) for 6 h. For the histone + heparin group and the histone + LPS + heparin group, histone (and LPS) were treated with heparin simultaneously. Mice in the heparin intervention group showed decreased levels of EH4, neutrophil gelatinase-associated lipocalin (NAGL), kidney injury molecule-1 (KIM-1), tumor necrosis factor-α (TNF-α), and interleukin (IL)-6 in the blood serum, longer average 72-h survival rate, significantly decreased kidney tissue edema, and a clearer glomerular structure coupled with decreased protein and mRNA expression levels of kidney apoptosis-related proteins (cleaved Caspase-3/Caspase-3 and Bax/Bcl-2) compared with those in the sepsis group at 6 h after CLP ($P < 0.05$). Meanwhile, cells in the heparin intervention group exhibited lower expression levels of serum EH4 and inflammatory cytokines, a lower apoptosis rate, and decreased expression of apoptosis-related proteins, both at protein and mRNA levels, than those in the histone-stimulated group at 6 h after stimulation ($P < 0.05$). Heparin may alleviate apoptosis and inflammation through the neutralization of histones, thus playing a protective role against septic acute kidney injury.

Keywords: heparin, histone, HK-2 cells, apoptosis, septic acute kidney injury

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INTRODUCTION

Extracellular histones (EH) have been shown to exhibit cytotoxicity that can induce apoptosis in HK-2 cells *in vitro* (1). Exposure to antibody directed at histone H4 in cells pretreated with H4 has been shown to effectively alleviate the damaging effects of histones on cell integrity and inflammation (2). Zarjou et al. have reported that EH can lead to a series of acute kidney injury (AKI) manifestations *in vivo*, such as endothelial cell activation, increased vascular permeability, and leukocyte recruitment (3). Using a murine model of septic AKI (SAKI), animals receiving anti-histone antibody exhibited much less injury than those without treatment (4), indicating that histones may be potential targets for SAKI, although the underlying mechanisms involved in SAKI are not entirely clear. Apoptosis, especially apoptosis of HK-2 cells, has drawn much research attention. It has been assumed that the increased production of reactive oxygen species (ROS) may activate cell apoptosis (5).

Heparin is known to carry the highest negative charge among all biomolecules known. Accordingly, histones have a stronger binding affinity for heparin than for DNA molecules (6). Thus, heparin may attenuate EH-mediated cytotoxicity through the neutralization of opposite charges, both in HK-2 cells and in a SAKI mice model. However, limited research attention has been given to the protective effect of heparin in neutralizing histones that induce cytotoxicity. In this study, the roles histones and heparin play in apoptosis and inflammation were analyzed using *in vivo* and *in vitro* approaches. In addition, the relationship between histones and ROS in cell apoptosis was evaluated using HK-2 cells.

METHODS

Reagents

EH was purchased from Roche (China). Heparin was purchased from Tianjin Biochemical Pharmaceutical Company. Anti-Bcl-2 antibody, anti-Bax antibody, anti-Caspase-3 antibodies, horseradish peroxidase (HRP)-labeled goat anti-mouse IgG antibody, and HRP-labeled goat anti-rabbit IgG antibody were purchased from Abcam (USA). Cleaved Caspase-3 antibody was purchased from CST/Cyxnet (USA). Mouse tumor necrosis factor (TNF)-α ELISA kit and TransStart Top Green qPCR Supermix were purchased from Beijing TransGen Biotech. The mouse histone H4 ELISA kit, mouse NAGL ELISA kit, and mouse KIM-1 ELISA kits were purchased from Shanghai Guangrui Biotech. Purified anti-β-actin and the fluorescein isothiocyanate (FITC)–annexin V apoptosis detection kit with 7-amino-actinomycin D (7-AAD) were purchased from Biolegend (USA). Fetal bovine serum was purchased from GIBCO (USA). DCFH-DA probe was purchased from Shanghai Beyotime Biotechnology.

Murine Model

Adult male C57BL/6J mice, aged 6–8 weeks and weighing 19–21 g, were purchased from Peking University Experimental Animals Center (Beijing, China), housed in a pathogen-free environment in the animal center of Tianjin Medical University, and allowed to acclimate to their surroundings for 1 week. Standard mice chow and water were available to the animals during the course of the experiment *ad libitum*. The mice were equally divided into three groups ($n = 12$ mice/group): sham, sepsis, and heparin intervention groups. The surgical procedure was performed as follows: male mice were fasted for at least 12 h and then anesthetized by intraperitoneal injection of tribromoethanol (10 mg/kg). For the sepsis and the heparin intervention groups, the cecum was exposed after mid-line laparotomy and ligated immediately below the ileo-cecal valve without causing intestinal obstruction. After being punctured twice with an 18G needle, the cecum was placed back in the peritoneal cavity, and the abdominal wall was closed in two layers. Then, the heparin intervention group was also administered with heparin (3 mg/kg) by intraperitoneal injection at 4 h after the CLP operation. For the sham group, the cecum was exposed, and then the abdominal wall was closed in two layers. All the three groups were treated with normal saline just after operation to mimic clinical therapy. At 6 h after the operation, nine mice from each group were sacrificed by the removal of eyeballs to harvest the blood, after which the kidneys were removed immediately, and the upper pole of the right kidney was used as the study sample. The levels of kidney injury factors (NAGL and KIM-1) in the mice serum and kidneys were evaluated separately by ELISA and PCR. We also administered calf thymus histone (10 mg/kg) by intraperitoneal injection into the mice ($n = 3$), and the survival of these four groups at 72 h was recorded. Our experiments were carried out in strict accordance with the international ethical guidelines and National Institutes of Health Guide concerning the care and use of laboratory animals, with protocol being approved by the Institutional Animal Care and Utilization Committee of Tianjin Medical University.

Cell Culture

HK-2 cells were obtained from Tianjin Institute of Urology and were cultured in an incubator (SANYO, Japan) at Tianjin Institute of Cardiology under standard conditions (37°C, 5% CO_2); the experiments were carried out after two passages. The cells were divided into three groups: control, histone, and heparin intervention groups. Each group contained an approximately equal number of cells. Cells in the control group received no intervention, whereas cells in the histone group were treated with EH (H4). Meanwhile, cells in the heparin intervention group were treated with heparin in the presence of histone. In order to explore the effects of lipopolysaccharides (LPS) on the apoptosis of HK-2 cells, three additional treatment groups were used: LPS, LPS + histone (with LPS and histone added simultaneously), and LPS + histone + heparin (with LPS, histone, and heparin added simultaneously) groups.

ELISA Measurement

The levels of H4, TNF-α, IL-6, NAGL, and KIM-1 from supernatants or blood serum were measured by ELISA kits, according to the manufacturer's protocols. Plates were read in a microplate reader at OD 450. All the samples were tested in triplicate.

TABLE 1 | Primer sequences used in RT-PCR.

	Forward primer	Reverse primer
GAPDH	TGCACCACCAACTGCTTAG	GATGCAGGGATGATGTTC
Bax	AGCTGCAGAGGATGATTGCT	ATGGTTCTGATCAGCTCGGG
Bcl-2	CCACCTGTGGTCCATCTGA	GACTGGACATCTCTGCGAA
Caspase-3	TGCTCACGAAAGAACTGTACT	GACAGCTTTCTCATTTGGCATA

Flow Cytometry Analysis

Cell apoptosis was tested by flow cytometry using an annexin V–FITC apoptosis detection kit. HK-2 cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 100 μl of 1× binding buffer mixed with 5 μl of annexin-V–FITC and 2.5 μl of 7-AAD staining solution for 15 min in the dark at room temperature. After 15 min of incubation, an additional 400 μl of binding buffer was added, and then the cells were analyzed using a flow cytometer (BD, USA). The production of ROS was tested by flow cytometry using an DCFH-DA probe. HK-2 cells were washed twice with PBS and resuspended in 100 μl PBS mixed with 10 μM DCFH-DA for 30 min in the dark at room temperature. Then, the cells were washed with PBS three times, and 300 μl PBS was added before analysis by flow cytometry. 7-AAD and annexin-V assay Q2 + Q3 were used to perform the apoptosis rate (7).

PCR Evaluation of Gene Expression

Trizol reagent (Life Technologies, USA) was used to extract total RNA from cells. Subsequently, cDNA was synthesized by FastQuant RT Kit (TianGen Biotech, Beijing), using the GeneAmp PCR System 2400 (Perkin Elmer, USA). SYBR Green Master Mix was used to amplify gene targets by real-time fluorescence quantitative PCR (qPCR) in accordance with the manufacturer's protocol and the 7500 Fast Real-Time PCR System (ABI, USA) to perform qPCR analysis. The specific primer sequences used for gene amplification are shown in **Table 1**. The cycling conditions were as follows: 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The expression levels of targeting mRNAs were normalized to that of housekeeping gene GAPDH and expressed as a fold of control.

Western Blotting

Apoptosis-mediated proteins were analyzed by western blotting. The total protein was extracted by radio-immunoprecipitation assay and phenylmethane sulfonylfluoride following the standard protocols for extracting protein. The protein concentrations were quantified using the BCA Protein Assay kit. Samples were electrophoresed in 10 or 8% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane. Then, the membrane was blocked in 5% dried milk at 4°C overnight. The membrane was washed with Tris-buffered saline Tween-20 (TBST) three times, followed by incubation with a secondary antibody at room temperature for 40 min. After washing with TBST again, to observe protein signals, substrate luminol reagent and HRP substrate solution were added onto the membrane, 1 ml/membrane, and membrane signals were revealed by an enhanced chemiluminescence immunoblot detection system. The staining intensity of the bands was quantitated by densitometry through ImageJ software. The antibodies used in our study are described above in the "Reagents." Protein expression levels were defined as gray value, standardized to the housekeeping gene β-actin, and expressed as a fold of control. All experiments were performed in triplicate and three times independently.

Hematoxylin and Eosin Staining Measurement

To observe the effect of heparin on organ histomorphology in CLP-induced septic mice, kidneys were inflated and fixed with 4% methanol-free formaldehyde for 48 h and embedded in paraffin. Tissue sections of 5-mm thickness were cut and stained with hematoxylin and eosin (H&E) following the standard staining protocols for histological analysis. Tubular injury scores were assigned to H&E-stained kidney sections by an experienced kidney pathologist who was blinded to the identity of the samples. We adopted published criteria as described for each parameter (8). Tubular injury degeneration was defined as including vacuolization, luminal cell casts, and acellular/atrophic changes, and scoring was as follows: 0 = none detected, 1 = 1–10% tubules involved, 2 = 10–25% tubules involved, 3 = 25–50% tubules involved, and 4 = >50% tubules involved. Tubulointerstitial inflammation was defined as the presence of lymphocytes in perivascular and interstitial cortical areas, and scoring was as follows: 0 = no significant inflammation, 1 = 1–10% foci in perivascular areas, 2 = 10–25% of cortex involved, 3 = 25–50% of cortex involved, and 4 = >50% of cortex involved.

Statistical Analysis

SPSS (version 22.0) was used to conduct all statistical analysis. Statistical analysis was accomplished based on the data from three independent experiments. Data are presented as mean ± standard deviation (SD). Statistical differences between the means of multiple groups were analyzed by one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Heparin Administration Can Improve the Survival Rate of SAKI Mice Induced by CLP

Mice were divided into four groups: sham group, sepsis group, heparin intervention group, and histone group. The heparin intervention group was also administered with heparin (3 mg/kg) by intraperitoneal injection at 4 h after the CLP operation. The histone group was administered with calf thymus histone (10 mg/kg) by intraperitoneal injection. The 72-h survival rate of the four groups was monitored in our study. As illustrated in **Figures 1A,C**, while all sham-operated mice survived to the end of the observation period (except one that died on day 2), all mice in the sepsis group died within 3 days. As illustrated in **Figure 1B**, the survival rate of mice in the sepsis group and the histone injection group were lower than that in the sham group. The survival rate of mice in the heparin intervention

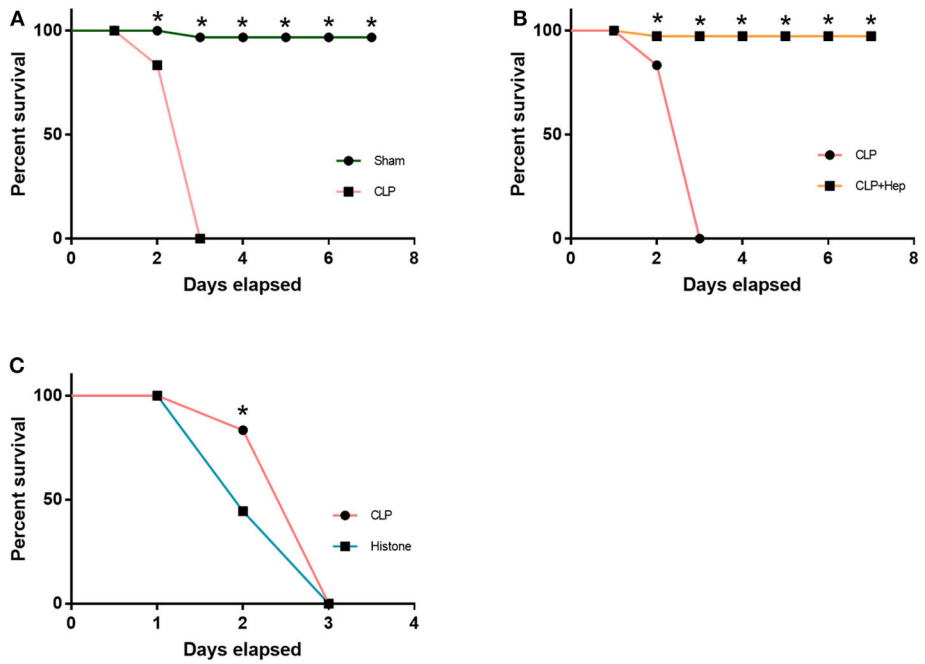

FIGURE 1 | Heparin improves the survival rate. The 72-h survival rates of the sham group, cecal ligation and puncture operation (CLP) group, CLP + heparin group, and histone group (instead of CLP, received 10 μg/ml histone intraperitoneally) were observed. **(A,C)** The CLP group and the histone injection group had a lower survival rate than the Sham group. **(B)** The heparin intervention group dramatically improve the 72-h survival rate. Data are presented as mean ± SD, $n = 7$ per group of the representative data from three independent experiments; $^*P < 0.05$.

group was essentially improved compared with that in the sepsis group.

Heparin Can Ameliorate the Abnormal Morphology Observed in the Kidneys of SAKI Mice

The kidney structure changes were shown by H&E staining. As illustrated in **Figure 2**, mice in the sepsis group presented anomalies in kidney structure. However, mice in the heparin intervention group had significantly improved anomalies. Renal tubular injury, quantified based on the tubular injury scores of H&E-stained kidney sections, was also significantly lower in the heparin intervention group, indicating that heparin is sufficient to protect the kidneys from septic damage.

Heparin Can Decrease Levels of NAGL and KIM-1 in SAKI Mice Induced by CLP

The *in vivo* expression levels of NAGL and KIM-1 in the kidneys of mice were determined using ELISA and PCR. Our data showed that, at 6 h after CLP, the levels of NAGL and KIM-1 were significantly higher in the sepsis group than in the sham group and that heparin could dramatically decrease the protein expression levels of both NAGL and KIM-1 (**Figure 3**).

Heparin Can Decrease H4 Levels and Pro-inflammatory Cytokine Release in HK-2 Cells Treated With Histone

ELISA was performed to evaluate the EH levels in the blood serum of mice in all three groups. Our results showed that mice in the sepsis group exhibited higher expression levels of H4 in serum (**Figure 4**), indicating that the levels of EH increased during the development of SAKI. Administration of heparin effectively decreased the expression of H4.

Cytokines are known to play important roles in the regulation of the immune response and inflammation. IL-6 and TNF-α are the main pro-inflammatory factors that regulate early responses. TNF-α is the most well-studied pro-inflammatory cytokine and has been shown to be involved in sepsis. Here we evaluated the *in vivo* release of inflammatory cytokines by ELISA. The levels of TNF-α and IL-6 in the serum from mice in the sepsis group (and in the cultured cells of the histone group for *in vitro* analysis) were higher (**Figure 4**) compared with those in the sham group, and heparin effectively decreased the levels of TNF- α and IL-6 in the samples of the heparin intervention group.

Heparin Can Regulate Apoptotic Proteins in HK-2 Cells Treated With Histone

Apoptotic proteins, including Caspase-3, cleaved Caspase-3, Bax, and Bcl-2, have been reported to be involved in apoptotic pathways. We evaluated the levels of apoptotic proteins *in vivo* by western blot analysis. As shown in **Figure 5**, the ratio of cleaved

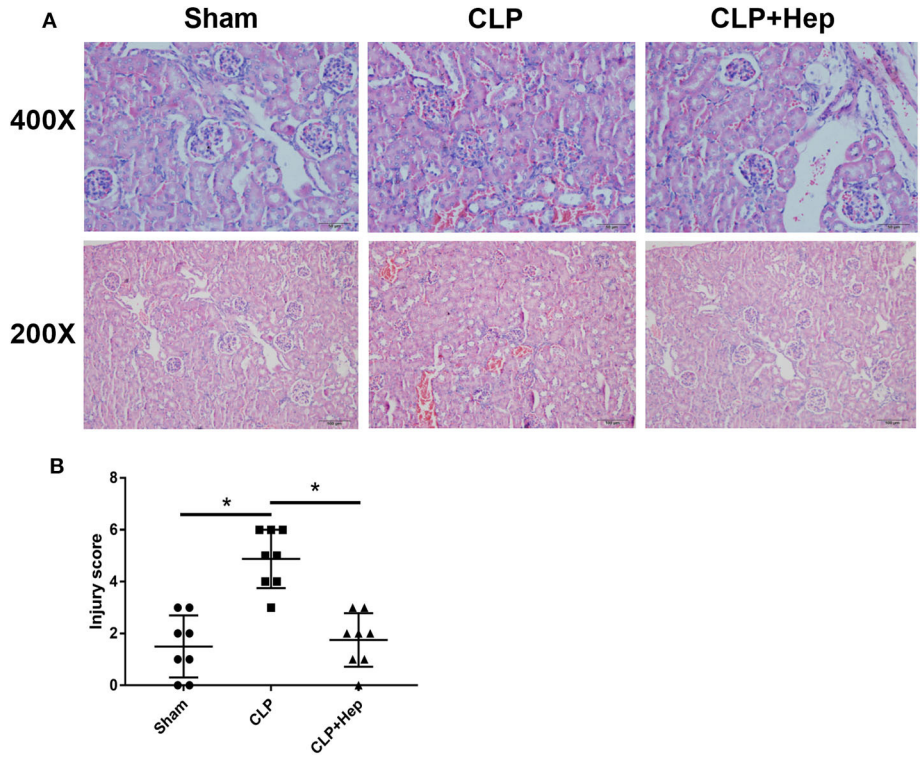

FIGURE 2 | Heparin ameliorates the histology of kidney in a septic mice model induced by cecal ligation and puncture operation. **(A)** Kidney histology changes of the sham group, sepsis group, and heparin intervention group were examined by hematoxylin and eosin (H&E) staining. Representative images of H&E-stained kidneys of the three groups at low (lower panels) and high (upper panels) magnification; scale bars are shown in figures. **(B)** Tubular injury scores were assigned to H&E-stained kidney sections to show kidney histology changes (*$P < 0.05$). The histopathological findings shown are representative of a similar histology observed in more than three mice examined for each group.

Caspase-3/Caspase-3 and Bax/Bcl-2 expression was increased in mice of the sepsis group, and heparin dramatically reduced the levels of apoptotic proteins. Consistent with the data obtained from western blotting analysis, our PCR results also showed that, although the ratio of Bax/Bcl-2 gene expression was dramatically increased in mice of the sepsis group, the ratio decreased in samples from the heparin intervention group.

Pre-study Was Performed to Determine the Time Point and the Concentration in HK-2 Cells Treated With Histone

In our pre-study, as shown in **Figure 6**, HK-2 cells were treated with histones at different concentrations for 6 h. Our data showed that the proportion of apoptotic cells (Q2 + Q3) in the control group was only 29.05 ± 1.22%; the proportion of apoptotic cells (Q2 + Q3), following treatment with 10 U, 20 U, and 40 U of histone, was determined to be 20.40 ± 1.67, 42.13 ± 1.47, and 58.8 ± 5.69%, respectively. The increase in apoptotic cells following treatment at a higher concentration of histone (40 U) was more pronounced than in cells exposed to a moderate amount of histone (20 U), so we chose 40 U as an efficient concentration for histone. As shown in **Figure 7**, HK-2 cells were treated with 40 U histone and cultured for 2, 4, and 6 h before analysis by flow cytometry. The proportion of apoptotic cells (Q2 + Q3) increased in cells treated with histone from each time point compared to the same time points in the control group without histone treatment, and heparin could significantly alleviate apoptosis in cells treated with histone from each time point. For the convenience of the study, we chose 6 h as an efficient time point. As shown in **Figure 8**, HK-2 cells treated with 40 U histone were cultured with heparin at different concentrations for 6 h before the flow cytometry analysis. The proportion of apoptotic cells (Q2 + Q3) was significantly lower following exposure to heparin at concentrations of 25 and 37.5 IU/ml, compared to apoptotic cells observed following the 12.5 IU/ml treatment ($P < 0.05$), so we chose 25 IU/ml as an efficient concentration for heparin.

Heparin Can Decrease EH4 Levels and Pro-inflammatory Cytokine Release in HK-2 Cells Treated With Histone

HK-2 cells were divided into five groups. Besides the control (Con) group, we cultured HK-2 cells treated with either histone (40 U), histone (40 U) + heparin (25 IU/ml), histone (40 U) + LPS (10 μg/ml), or histone (40 U) + LPS (10 μg/ml) + heparin (25 IU/ml) for 6 h. For the histone + heparin group and the histone + LPS + heparin group, histone (and LPS) was treated

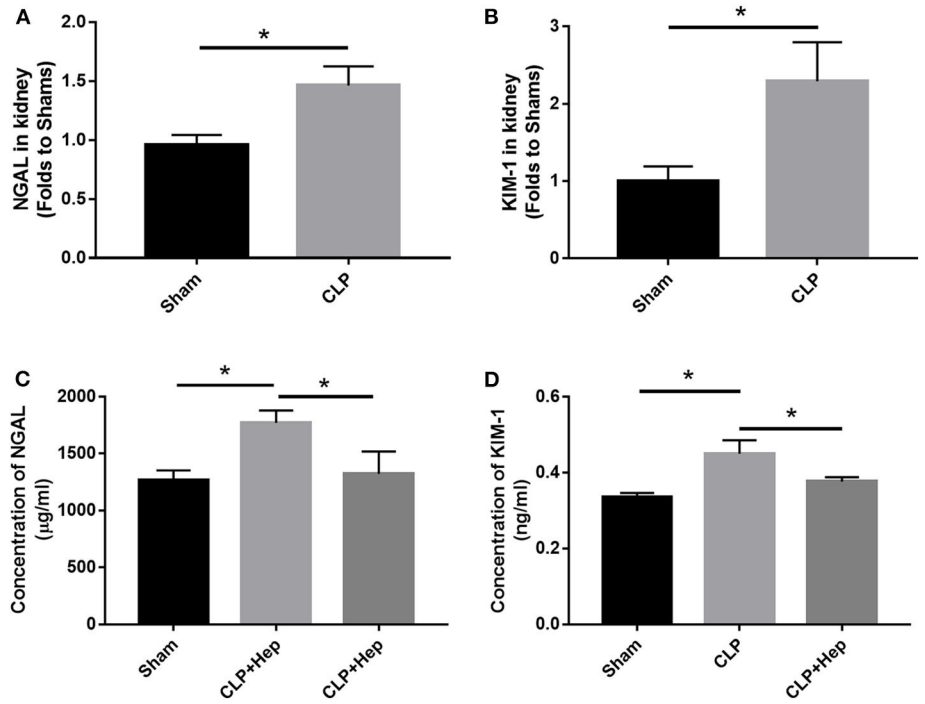

FIGURE 3 | Heparin decreases the expressions of NGALF and KIM-1 *in vivo*. **(A,B)** PCR analysis showing the mRNA expression levels of NGALF and KIM-1 in the kidney in each mouse group. **(C,D)** ELISA revealed the protein expression level of circulating NGALF and KIM-1 in the blood serum in each mouse group. Data are presented as mean ± SD, $n = 3$ per group of the representative data from three independent experiments; $^*P < 0.05$.

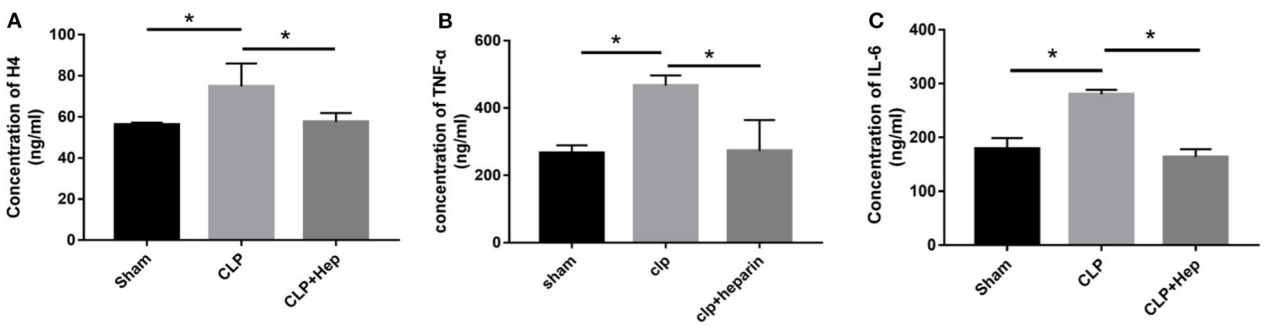

FIGURE 4 | Heparin decreases the expressions of inflammation-related factors and level of EH4 *in vivo*. At 6 h before the onset of surgery, blood samples acquired by removing the mice's eyeballs were used to determine the expression levels of H4 **(A)**, TNF-α **(B)**, and IL-6 **(C)** in in blood serum by ELISA. Data are presented as mean ± SD, $n = 3$ per group of the representative data from three independent experiments; $^*P < 0.05$.

with heparin simultaneously. At 6 h later, the levels of EH4, IL-6, and TNF-α present in the cell culture supernatant of all the five groups were evaluated. The ELISA data showed that the cell culture supernatant of the histone (and LPS) stimulation group had significantly higher levels of EH4, IL-6, and TNF-α than those present in the control group (**Figure 9**). The ELISA data also showed that the cell culture supernatant of the heparin intervention group had significantly lower levels of EH4, IL-6, and TNF-α than those present in the histone (and LPS) stimulation group (**Figure 9**). Our results clearly demonstrated that heparin could decrease the levels of histone and pro-inflammation factors in the culture supernatant of HK-2 cells pretreated with histone (and LPS).

Heparin Can Inhibit Apoptosis Induced by Histone Along With LPS in HK-2 Cells

In our pre-study, as shown in **Figure 10**, HK-2 cells were treated with LPS at an increasing concentration of 0.5, 1, 5, and 10 μg/ml. Only cells treated with 10 μg/ml LPS had an increased proportion of apoptotic cells (Q2 + Q3) compared to

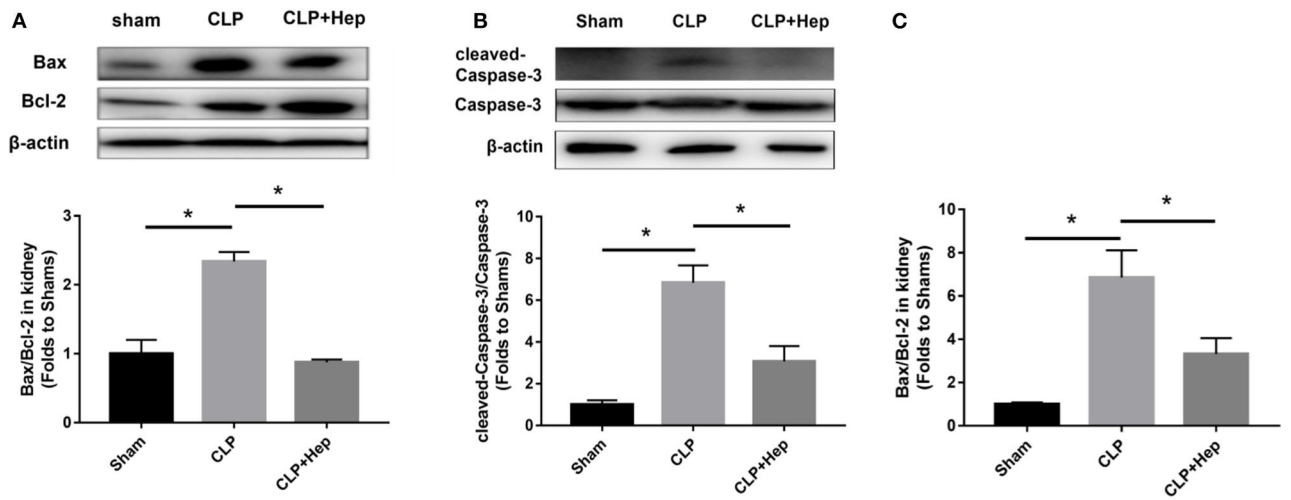

FIGURE 5 | Heparin decreases the expression levels of apoptosis-mediated proteins *in vivo*. **(A,B)** The protein expression of Bax/Bcl-2 and cleaved Caspase-3/Caspase-3 in HK-2 was analyzed by western blotting and quantified by densitometry. The mice were subjected to cecal ligation and puncture operation (CLP) surgery. At 2 h before the onset of CLP, the mice were treated with heparin. The levels of protein in the kidney were determined by western blotting and quantified by densitometry. **(C)** The mRNA expression of Bax/Bcl-2 was analyzed by SYBR Green qPCR. The ratio of Bax/Bcl-2 in the sepsis group was higher than that in the sham group. The ratio of Bax/Bcl-2 in the heparin intervention group was lower than that in the sepsis group. Data are presented as mean ± SD, $n = 3$ per group of the representative data from three independent experiments; $^*P < 0.05$.

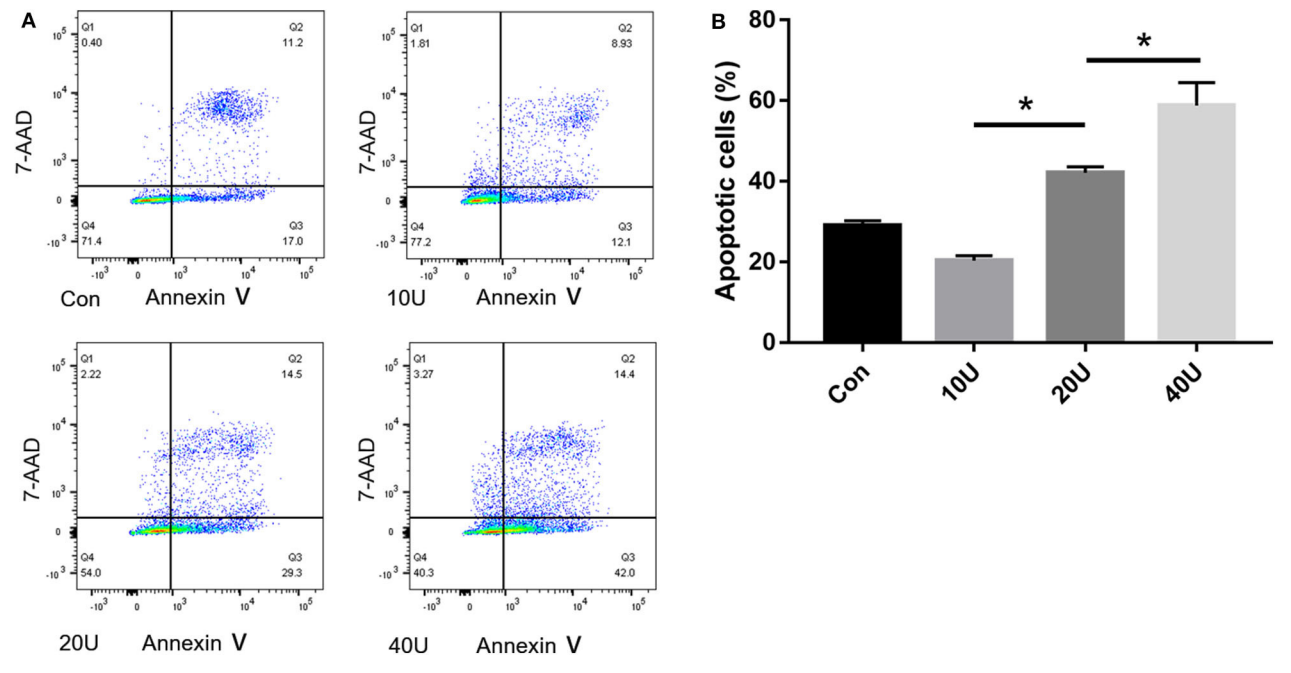

FIGURE 6 | Pre-study to find an efficient concentration of histone. **(A)** HK-2 cells were treated with histones (10 U/20 U/40 U) at 6 h. Flow cytometry was used to evaluate apoptosis rate. **(B)** The apoptosis rate of 20 U and 40 U are higher in comparison to the control. The apoptosis rate of 40 U group is higher than that in 20 U group. There is no significant difference between 10 U group and the control group ($P < 0.05$). Data are presented as mean ± SD ($n = 3$ per group) of the representative data from three independent experiments; $^*P < 0.05$.

the control group ($P < 0.05$). In parallel, western blotting showed that only cells treated with 10 μg/ml LPS had an increased ratio of Bax/Bcl-2 compared to the control group ($P < 0.05$). We further cultured HK-2 cells treated with either histone (40 U), histone (40 U) and heparin (25 IU/ml), histone (40 U) and LPS (10 μg/ml), or histone (40 U) + LPS (10 μg/ml) + heparin (25 IU/ml) for 6 h. As shown in **Figure 11**, the proportion of apoptotic cells (Q2 + Q3) in the control, histone, and

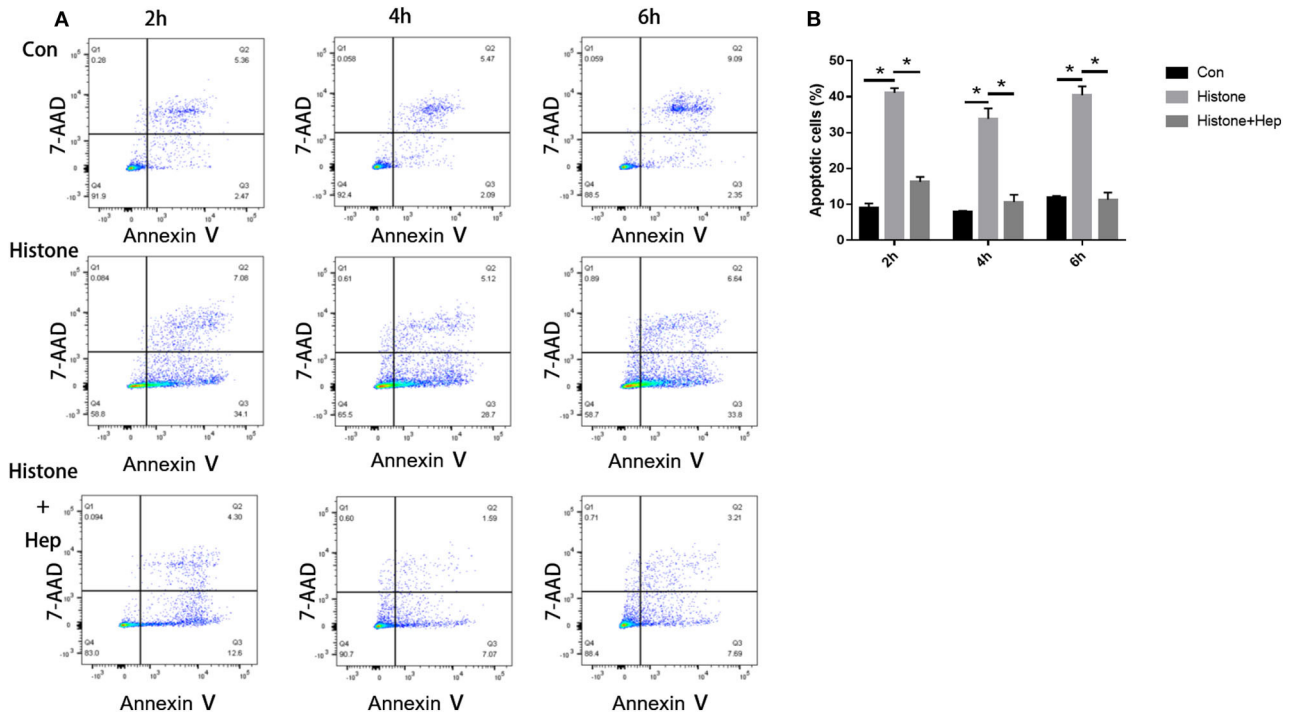

FIGURE 7 | Pre-study to find an efficient time point. **(A)** Cells were treated with 40 U histone cultured for 2, 4, and 6 h. Flow cytometry was used to evaluate apoptosis rate **(B)** Data showed that heparin could significantly reduce the apoptosis rate in each group ($P < 0.05$). Data are presented as mean ± SD, $n = 3$ per group of the representative data from three independent experiments; *$P < 0.05$.

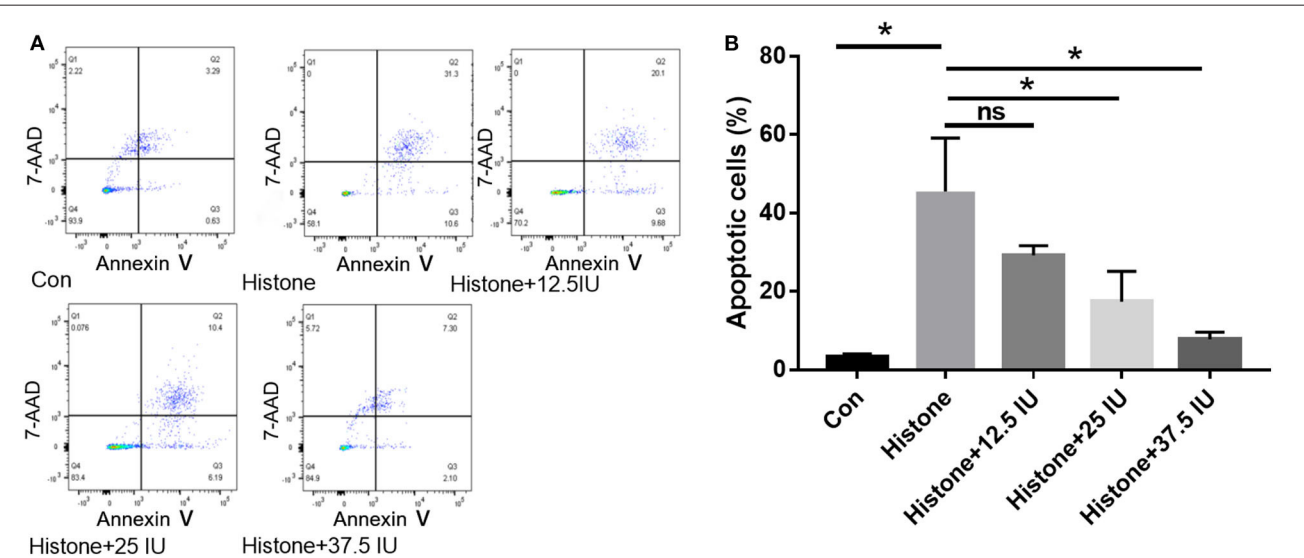

FIGURE 8 | Pre-study to find an efficient concentration of heparin. **(A)** HK-2 cells treated with 40 U histone were administered with different concentrations of heparin and cultured for 6 h. Flow cytometry was used to evaluate apoptosis rate. **(B)** The proportion of apoptotic cells was significantly lower at doses of 25 and 37.5 IU/ml in comparison to 12.5 IU/ml ($P < 0.05$). Data are presented as mean ± SD, $n = 3$ per group of the representative data from three independent experiments; *$P < 0.05$.

histone + LPS groups was 26.88 ± 1.44, 43.67 ± 1.88, and 58.50 ± 3.47%, respectively. Heparin remarkably alleviated cell apoptosis induced by histone, and the proportion of apoptotic cells (Q2 + Q3) was 16.63 ± 4.72 and 19.60 ± 3.47% in the histone + heparin group and the histone + LPS + heparin group, respectively.

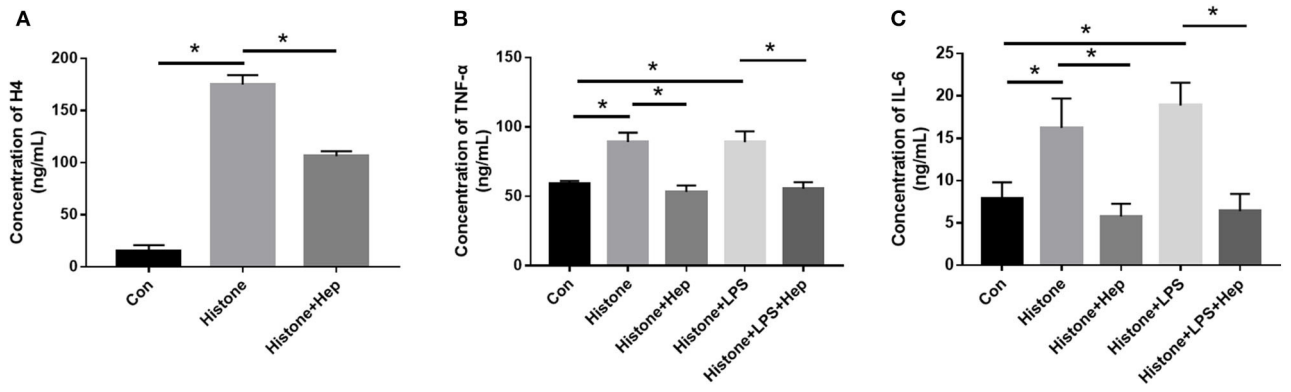

FIGURE 9 | Heparin decreases the expression of inflammation-related factors and level of EH4 in HK-2 cells treated with histone. At 6 h after cultivation, the expression levels of H4 **(A)**, TNF-α **(B)**, and IL-6 **(C)** in cell supernatants were determined by ELISA. Data are presented as mean ± SD ($n = 3$ per group of) the representative data from three independent experiments; *$P < 0.05$.

Heparin Can Regulate Apoptotic Proteins in HK-2 Cells Treated With Histone

As shown in **Figure 12**, the ratio of cleaved Caspase-3/Caspase-3 and Bax/Bcl-2 expression was increased in the cells of the histone group, and heparin remarkably reduced the levels of apoptotic proteins. In parallel with the data obtained from western blotting analysis, our PCR results also showed that, although the ratio of Bax/Bcl-2 gene expression was dramatically increased in the cells of the histone group, the ratio decreased in samples from the heparin intervention group. The *in vitro* ratios of cleaved Caspase-3/Caspase-3 and Bax/Bcl-2 protein expression were increased in the cells of the LPS (10 μg/ml) + histone-treated group, and heparin dramatically lowered the ratios (**Figure 12**).

ROS Production Is Increased During Apoptosis Development of HK-2 Cells Treated With Histone

As shown in **Figure 13**, we assessed the production of ROS in HK-2 cells receiving different treatments after 1, 2, 4, and 6 h. After a 2-h histone (40 U) treatment, ROS production was significantly higher in treated cells than that in the control group, and ROS production was significantly lower in cells from the heparin intervention group compared to that in the cells from the histone-treated group. There was no statistical significance in the production of ROS among the control group, the histone-treated group, and the heparin intervention group at 1, 4, and 6 h.

DISCUSSION

Sepsis-3 is defined as life-threatening organ dysfunction caused by disorders of the immune response (9, 10). Hence, the alleviation of organ dysfunction is crucial to improve septic prognosis. About 40–50% of patients with sepsis have AKI (11). Although the apoptosis observed in tubular epithelial cells is considered to play an important role in contributing to SAKI, the underlying disease pathogenesis has not been fully elucidated.

Histones are the main structural proteins of eukaryotic chromatin. Consisting of five protein variants, namely, H1, H2A, H2B, H3, and H4, histones can be released into the extracellular matrix under pathological conditions (12). Since both H3 and H4 have been shown to be key components within the histone octamer complex and exhibit a high level of toxicity, H4 was selected as the representative histone for measurement of histone levels in our experiments (13). Under normal conditions, histones are involved in the composition of chromosomes and in the regulation of different physiological functions, such as gene expression, mitosis, and DNA repair (14). In pathological situations, histones can be released into the cytoplasm *via* two different pathways: (1) activated neutrophils that release neutrophil extracellular traps to defend from pathogens and (2) passive release from dead cells (15). While a small amount of histones can defend from pathogens, a larger amount of histones can induce inflammatory responses and organ dysfunction (16). It has been shown that high levels of circulating histones are major mediators of multiple organ dysfunction syndrome (17). Our previous clinical experimental studies have also shown that H4 concentration is inversely correlated with the prognosis of patients with sepsis (18, 19).

Highly conserved in eukaryotic cells (20), histone proteins are highly positively charged due to enriched lysine and arginine residues (21). Heparin is a mucopolysaccharide ester composed of D-glucosamine, L-iduronic acid, N-acetylglucosamine, and glucuronic acid, which are responsible for the high negative charge of the molecule (22). Clinical meta-analysis studies have shown that heparin can decrease the 28-day mortality rate and APACHE II score among septic patients and that the severity and the dysfunction of coagulation can be alleviated by heparin without any evident increased risk of bleeding (23, 24). Recently, heparin/low molecular weight heparin is reported to have a beneficial effect on mortality through neutralization of extracellular cytotoxic histones in COVID-19 (25). In animal studies, non-anticoagulant heparin has been shown to decrease the levels of inflammatory cytokines (26) as well as improve the survival of septic mice in a dose-dependent manner. A

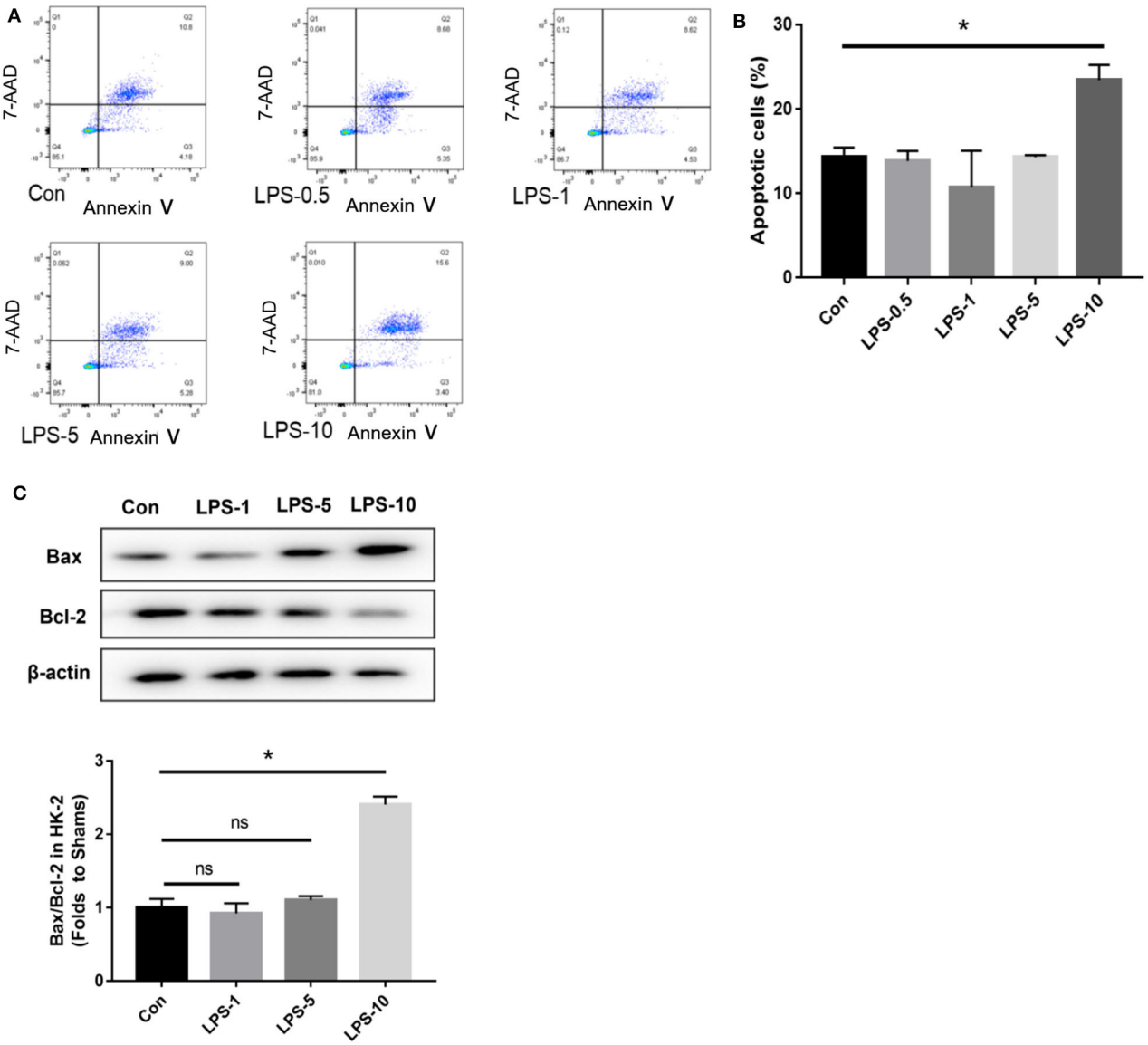

FIGURE 10 | The cytotoxic effect of lipopolysaccharides (LPS) on HK-2 cells. The HK-2 cells were treated with LPS at doses of 0.5, 1, 5", and 10 μg/ml. **(A,B)** A flow cytometry analysis showed that only the only 10 μg/ml group increased the proportion of apoptotic cells (Q2 + Q3) in comparison to the control group. **(C)** HK-2 cells were analyzed by western blotting and quantified by densitometry. The ratio of Bax/Bcl-2 was higher only at the dose of 10 μg/ml compared with the control group. Data are presented as mean ± SD ($n = 3$ per group) of the representative data from three independent experiments; $^*P < 0.05$.

number of studies have found that heparin may neutralize EH, thereby alleviating the toxicity of EH (6, 27, 28). In this study, we established a murine model of SAKI with CLP and used heparin as an intervention. Our results showed that both histone injection and sepsis could decrease the 72 h-survival rate; heparin enhances the survival rate and protect kidney histology. It is indicated that histone plays a major role in the pathogenesis of SAKI, and heparin may be a potential therapy. We also observed that the sepsis group had a higher level of EH4 paralleled with a higher level of kidney injury factors and pro-inflammatory factors. However, the heparin intervention group had a remarkably lower concentration of EH4 paralleled with a lower level of kidney injury factors and pro-inflammatory factors compared with the sepsis group. Consistently, western blotting and PCR data showed that the protein and the gene expression levels of apoptotic proteins also increased in the sepsis group and decreased in the heparin intervention group. The results suggested that a positive correlation was expressed between the level of histone and the degree of kidney injury in mice of SAKI; heparin may alleviate kidney injury through neutralization of histone.

We explored next the mechanism in which histone induces kidney injury and how heparin played in this process. Histone can produce cytotoxic effects as well as directly contribute to

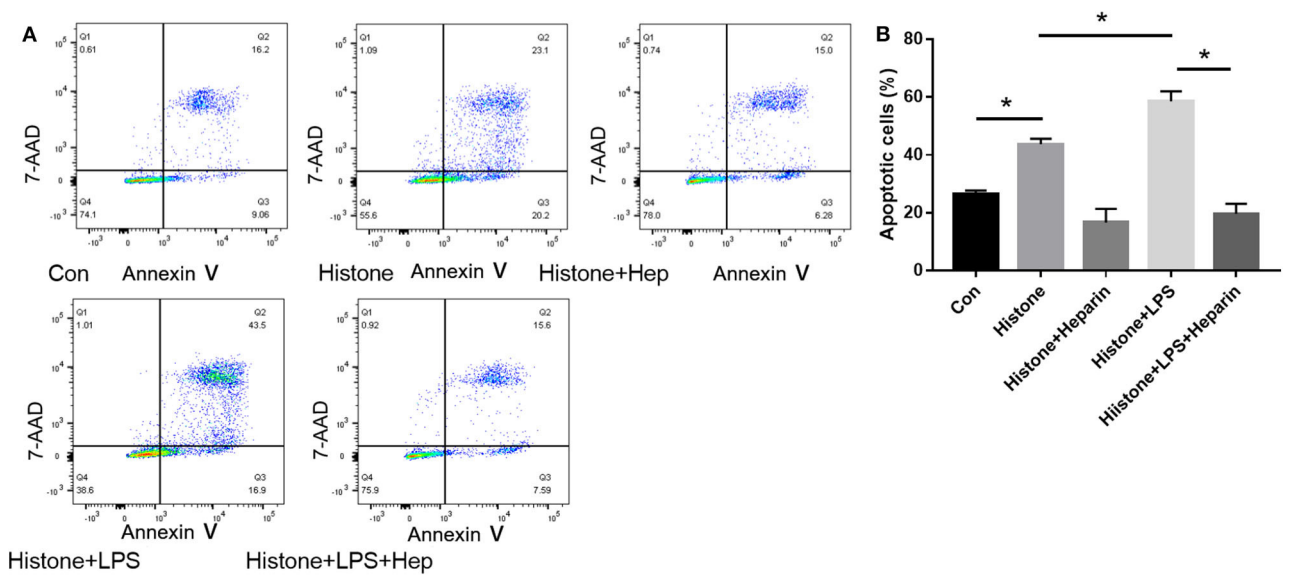

FIGURE 11 | Histone, along with lipopolysaccharides (LPS), increases the apoptosis rate and heparin alleviates it. **(A)** We equally divided cells into the control (con) group, histone (40 U) group, histone (40 U) + LPS (10 μg/ml) group, histone (40 U) + heparin (25 IU/ml) group, and histone (40 U) + LPS (10 μg/ml) + heparin (25 IU/ml) groups and evaluated the proportion of apoptotic cells (Q2 + Q3) in each group. **(B)** For histone and histone + LPS groups, the proportion of apoptotic cells (Q2 + Q3) increased significantly ($P < 0.05$) in comparison to the control group. Heparin remarkably reduced the cell apoptosis rate both in histone and histone + LPS groups ($P < 0.05$). Data are presented as mean ± SD, $n = 3$ per group of the representative data from three independent experiments; *$P < 0.05$.

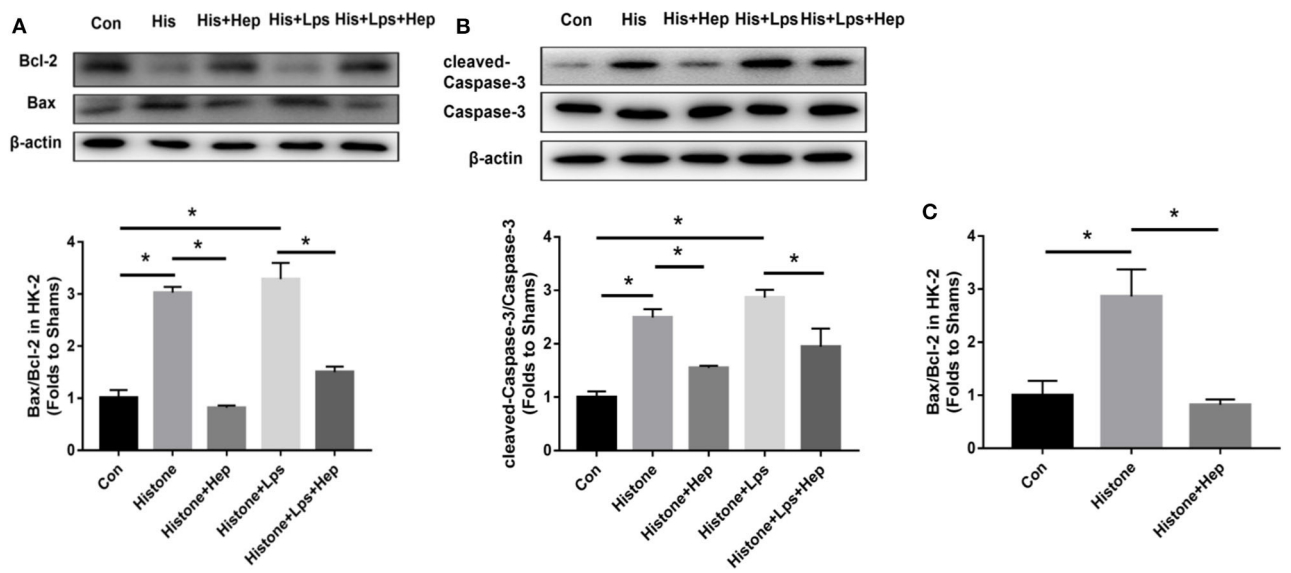

FIGURE 12 | Heparin decreases the expression levels of apoptosis-mediated proteins induced by histone in HK-2 cells. **(A,B)** The protein expression of Bax/Bcl-2 and of cleaved Caspase-3/Caspase-3 in HK-2 were analyzed by western blotting and quantified by densitometry. Administration of histone enhanced the ratio of cleaved Caspase-3/Caspase-3 and the ratio of Bax/Bcl-2 in the kidney. Heparin could decrease the ratio of cleaved Caspase-3/Caspase-3 and the ratio of Bax/Bcl-2 in comparison with the histone group. **(C)** The mRNA expression of Bax/Bcl-2 was analyzed by SYBR Green qPCR. The administration of histone increased the ratio of Bax/Bcl-2 in the kidney. Heparin could decrease the ratio of Bax/Bcl-2 in comparison with the histone group. Data are presented as mean ± SD, $n = 3$ per group of the representative data from three independent experiments; *$P < 0.05$.

apoptosis (29), resulting in endothelial activation and, ultimately, alteration of vascular homeostasis (30). It has been proven that apoptosis, especially the apoptosis of renal tubular epithelial cells, greatly affects the process of SAKI (31, 32). For this reason, we explored the role histone and heparin played on renal tubular epithelial cells. A flow cytometry analysis was performed to

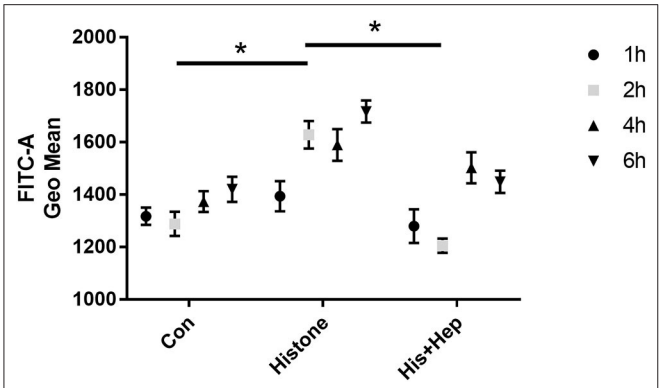

FIGURE 13 | Heparin decreases the production of ROS in HK-2 cells treated with histone. In the histone + heparin group, histone and heparin were administered to the HK-2 cell line simultaneously. The cells were collected from each group at various time points (1, 2, 4, and 6 h). Histone enhanced the level of ROS production in 2 h of treatment, and heparin could decrease its level. Data are presented as mean ± SD, $n = 5$ per group of the representative data from three independent experiments; $^*P < 0.05$.

evaluate the apoptosis of renal tubular epithelial cells of mice (HK-2 cells). In the pre-study, efficient time point and effective concentration of histone, heparin, and LPS were found. At the time point of 6 h, 40 U histone, 25 IU heparin, and 10 μg/ml LPS were selected as study subjects. At 6 h after stimulation, the flow cytometry results clearly demonstrated that histones increased the apoptosis rate, and heparin could alleviate it, coinciding with western blotting and PCR results of apoptosis-related protein. The ELISA results showed that heparin could decrease the levels of histone and pro-inflammation factors in the culture supernatant of HK-2 cells pretreated with histone. We concluded that histone has a cytotoxic effect on renal tubular epithelial cells through cell apoptosis and inflammatory response; heparin could alleviate these damages through neutralization of histone. Then, we talked about the positive role to renal tubular epithelial cells under the background of SAKI. As we all know, LPS is the main component of the cell wall of Gram-negative bacilli, which can trigger the activation of an inflammatory response and the release of cell mediators. It has been used to stimulate cells to induce sepsis in many studies (33, 34). We stimulated HK-2 cells with histone and LPS to simulate the pathologic state of SAKI, and it could be seen that heparin still dramatically alleviates the apoptosis and inflammation response of HK-2 cells. It is partly indicated that heparin may become a potential therapy for SAKI patients.

Additionally, we assessed the production of ROS in HK-2 cells with different treatments of histone and heparin after 1, 2, 4, and 6 h. Our results showed that, while ROS production was significantly higher in cells after a 2-h treatment with histone, ROS production was alleviated in cells in the intervention group compared with the histone-treated group. To some extent, our results suggested that the cytotoxic effect of EH on HK-2 cells may be related to mitochondrial oxidative stress that may have been manifested through the endogenous apoptosis pathway (32). Changes in myocardial mitochondria have been suggested to be the causative factor rather than sepsis-related inflammation (35). Therefore, whether ROS production can induce or influence apoptosis requires further studies.

All in all, our data showed that histone may induce SAKI through cell apoptosis and inflammation response, and heparin could play a protective role by attenuating it.

Limitations

There are limitations in our study. Considering that our experimental data were insufficient in analyzing the mechanism of apoptosis, further validation is required. More quantitative data and evidence of apoptosis, for example, by TUNEL stain or immunofluorescence, are also limited to show in our renal section. We just indicated that histone play a more essential role *in vitro* than LPS in SAKI when the concentration of LPS is 10 μg/ml; more effective concentrations of LPS should also be taken into consideration. The positive role of heparin on HUVEC or primary mouse kidney cells of SAKI *in vitro* should be explored in a future study. While most studies addressed the correlation between EH and sepsis based on experimental animal and cell studies, there have been only very few clinical studies. Whether histone is a therapeutic target for SAKI patients requires more validation from clinical data.

CONCLUSION

Taken together, our study demonstrated that histone may contribute to the development of SAKI through the activation of the apoptotic pathway and by increasing the inflammatory response. Heparin may represent a potential treatment for SAKI due to its ability to decrease apoptosis and the inflammatory response through attenuation of histones.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Utilization Committee of Tianjin Medical University.

AUTHOR CONTRIBUTIONS

GL, SS, and LW contributed to the conception of this study. ZW and LW performed the experiment. CC, HJ, and YZ contributed to analysis and manuscript preparation. ZW, LW, YG, and CC performed the data analysis and drafted manuscript. GL, SS, and XL edited, revised, and approved final version of manuscript.

All authors contributed to the article and approved the submitted version.

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Cilomilast Ameliorates Renal Tubulointerstitial Fibrosis by Inhibiting the TGF-β1-Smad2/3 Signaling Pathway

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Background: Renal tubulointerstitial fibrosis is the key pathological feature in chronic kidney diseases (CKDs) with no satisfactory therapies in clinic. Cilomilast is a second-generation, selective phosphodiesterase-4 inhibitor, but its role in renal tubulointerstitial fibrosis in CKD remains unclear.

Material and Methods: Cilomilast was applied to the mice with unilateral ureteric obstruction (UUO) and renal fibroblast cells (NRK-49F) stimulated by TGF-β1. Renal tubulointerstitial fibrosis and inflammation after UUO or TGF-β1 stimulation were examined by histology, Western blotting, real-time PCR and immunohistochemistry. KIM-1 and NGAL were detected to evaluate tubular injury in UUO mice.

Results: *In vivo*, immunohistochemistry and western blot data demonstrated that cilomilast treatment inhibited extracellular matrix deposition, profibrotic gene expression, and the inflammatory response. Furthermore, cilomilast prevented tubular injury in UUO mice, as manifested by reduced expression of KIM-1 and NGAL in the kidney. *In vitro*, cilomilast attenuated the activation of fibroblast cells stimulated by TGF-β1, as shown by the reduced expression of fibronectin, α-SMA, collagen I, and collagen III. Cilomilast also inhibited the activation of TGF-β1-Smad2/3 signaling in TGF-β1-treated fibroblast cells.

Conclusion: The findings of this study suggest that cilomilast is protective against renal tubulointerstitial fibrosis in CKD, possibly through the inhibition of TGF-β1-Smad2/3 signaling, indicating the translational potential of this drug in treating CKD.

Keywords: chronic kidney disease, cilomilast, TGF-β1, Smad2/3, renal tubulointerstitial fibrosis

INTRODUCTION

Chronic kidney disease (CKD) has become a major public health problem in many countries. Almost all forms of chronic renal diseases can ultimately result in end-stage renal diseases (ESRDs), leading to a significant impact on quality of life as well as a substantial social burden (1). Renal tubulointerstitial fibrosis is characterized by the deposition of extracellular matrix (ECM), excessive accumulation of activated myofibroblasts, and infiltration of inflammatory cells (2, 3). Unfortunately, no satisfactory therapeutic strategies for inhibiting or reversing renal

tubulointerstitia fibrosis are clinically available. Therefore, there is an urgent need to find new effective therapeutic drugs for renal tubulointerstitial fibrosis.

Phosphodiesterase 4 (PDE4) isozymes belong to the PDE superfamily and selectively hydrolyse 3′,5′-cyclic adenosine monophosphate (cAMP) with high affinity (4). In recent years, PDE4 inhibition has been applied to study its probable therapeutic value in the nervous system (5), respiratory system (6) and immune system (7). Furthermore, PDE4 inhibition has been found to attenuate lung fibrosis (8) and dermal fibrosis (9). Previous studies have shown that PDE4 is widely expressed in renal tubules (4). The inhibition of PDE4 has suppressive effect on tubular damage in acute renal failure (10–12). Recently, a report showed that one PDE4 inhibitor, rolipram, played an antifibrotic role in CKD possibly via acting on C/EBP-β and PGC-1α in tubular epithelial cells (13). Cilomilast is another PDE4 inhibitor that is currently being investigated in a phase III clinical trial for the treatment of chronic obstructive pulmonary disease (COPD). It has beneficial effects on COPD (14), tumors (15), acute lung injury (16), and acute kidney injury (12). Furthermore, cilomilast has been reported to attenuate pulmonary fibrosis (17). However, the effect of cilomilast on renal tubulointerstitial fibrosis has not been studied.

Transforming growth factor-β1 (TGF-β1) is an essential fibrogenic factor that plays a crucial role in the renal fibrotic process (18). Emerging evidence suggests that TGF-β1 initiates renal tubular epithelial cell transdifferentiation to myofibroblasts, enhancing collagen and fibronectin (FN) synthesis and extracellular matrix deposition (19–21). TGF-β1 receptor activation stimulates the translocation of decapentaplegic homolog 3 (Smad3) to the nucleus, where it regulates the transcription of target genes (22). Smad7 has an inhibitory effect on TGF-β1, Smad2, and Smad3 (23). The activation of TGFβ1-Smad2/3 signaling or the loss of inhibitory Smad7 triggers fibrotic cascades (24). In this study, we investigated the therapeutic effects of cilomilast on renal tubulointerstitial fibrosis using a mouse model of obstructive uropathy. Additionally, we further explored the antifibrotic action of cilomilast and its effect on regulating the TGFβ1-Smad2/3 pathway in renal fibroblasts. We found that cilomilast attenuated the development of renal tubulointerstitial fibrosis possibly by inhibiting the TGFβ1-Smad2/3 signaling pathway.

METHODS

Animal Models of Chronic Kidney Fibrosis

In the UUO experiment, 8-week-old male C57BL/6 mice weighing 20–25 g were divided into 3 groups (control: $n = 6$; UUO-treated: $n = 6$; and UUO+cilomilast-treated: $n = 6$). Mice were anesthetized with 2% isoflurane, and the left ureter was ligated at the ureteropelvic junction with a 4-0 silk suture through a median ventral incision. Cilomilast was intraperitoneally (i.p.) delivered to mice at 30 $mg \cdot kg^{-1} \cdot day^{-1}$ based on previous report (25, 26). Cilomilast or vehicle was administered to mice from −2 to 7 days before and after UUO surgery (**Figure 1A**). After 7 days of UUO, all mice were sacrificed by cervical dislocation and the kidney tissues were harvested for further analysis. All animal procedures were approved by the Nanjing Medical University Institutional Animal Care and Use Committee (registration number: IACUC-1809017).

Histological Analysis

Kidney tissues were fixed in 4% PFA, embedded in paraffin, and cut into sections (4-μm-thick), which were stained with Masson's trichrome. Masson's trichrome staining was used to assess collagen deposition in the obstructed kidney tissue. Next, 8–10 randomly selected fields were observed under the microscope, and then, each mouse kidney tissue was evaluated in a blinded manner.

Immunohistochemistry (IHC) of Animal Kidney Samples

IHC was performed as previously described (27). Briefly, paraffin-embedded animal kidney sections (4 μm) were blocked with 5% BSA for 1 h and incubated at 4°C overnight with rabbit monoclonal primary antibodies against FN (Abcam, ab2413, Cambridge, MA, USA, 1:250), α-smooth muscle actin (SMA; ab7817, Abcam, Cambridge, MA, USA, 1:400), TNF-α (ab215188, Abcam, Cambridge, MA, USA, 1:100), TGF-β1 (ab215715, Abcam, Cambridge, MA, USA, 1:500), neutrophil gelatinase-associated lipocalin (NGAL; ab63929, Abcam, Cambridge, MA, USA, 1:1000) and F4/80 (ab100790, Abcam, Cambridge, MA, USA, 1:100). After washing with TBST buffer three times, sections were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody for 60 min. The localization of peroxidase conjugates was determined using a DAB kit (ZLI-9018, Zsbio, China). Slides were examined under a microscope, and the signals were analyzed using Image-Pro Plus software analysis tools.

Immunofluorescence Staining

The cells were fixed with PBS containing 4% PFA for 30 min. After blocking with 5% BSA for 1 h, the slides were incubated overnight at 4°C with an anti-FN antibody (#26836, CST, Danvers, MA, USA, 1:250), which was diluted with 5% BSA overnight at 4°C. Subsequently, the cells were incubated with anti-rabbit secondary antibodies (ab150077, Abcam, Cambridge, MA, USA, 1:250) for 1 h at room temperature and were then stained with the nuclear-specific stain DAPI (Beyotime Institute of Biotechnology) for 3 min at room temperature. Then, the cells were washed three times in PBS and imaged. The slides were viewed with a Carl Zeiss LSM 5 PASCAL laser scanning confocal microscope.

Cell Culture and Treatments

NRK-49F cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DMEM and fetal bovine

Abbreviations: CKD, chronic kidney disease; UUO, unilateral ureteric obstruction; ESRD, end-stage renal diseases; cAMP, cyclic adenosine monophosphate; COPD, chronic obstructive pulmonary disease; IHC, immunohistochemistry; ECM, extracellular matrix; PDE4, phosphodiesterase 4; TGF-β1, transforming growth factor-β1; FN, fibronectin; α-SMA, α-smooth muscle actin; Col-I, collagen I; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; SEM, standard errors of the mean.

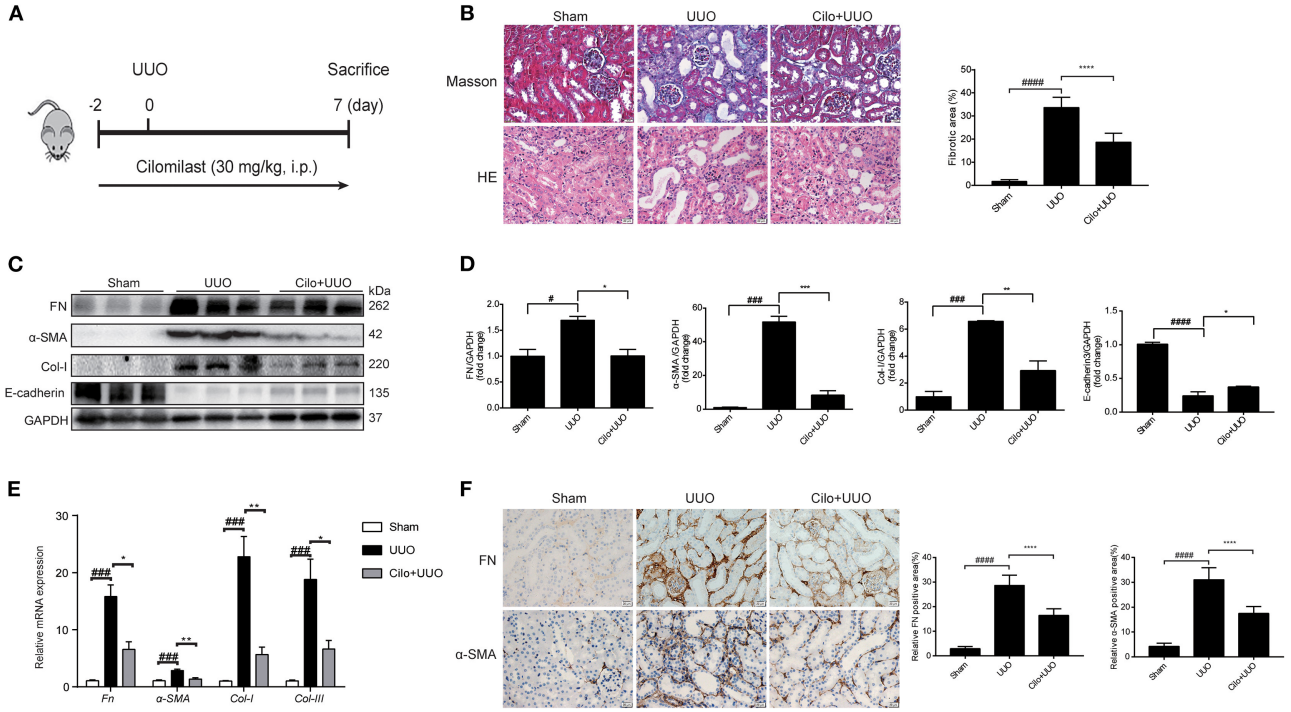

FIGURE 1 | Cilomilast improved UUO-induced renal tubulointerstitial fibrosis. Mice were pre-treated with cilomilast by i.p. injection for 2 days and then treated with cilomilast by i.p. injection for 7 days after UUO surgery. Mice in the UUO group were treated with an equal volume of saline, and mice in the sham group were subjected to the same procedure without the ureteral ligation. **(A)** A schematic diagram showing the procedure of UUO-induced renal tubulointerstitial fibrosis in mice. **(B)** Masson's trichrome staining and HE staining of kidney tissue sections. Scale bar =20 μm (left). Fibrotic area in mice were analyzed (right). Six random fields were taken from each kidney. **(C)** Western blot analyses of renal FN, Col-I, α-SMA and E-cadherin protein expressions in kidney tissues. **(D)** Densitometry of the western blot results in **(C)**. **(E)** qRT-PCR analyses of *Fn*, *Col-I*, *Col-III* and *α-SMA* mRNA expression ($n = 6$). **(F)** Representative images and quantitative assessment of the expression and distribution of FN and α-SMA in kidney tissues using immunohistochemical staining. Scale bar =20 μm (left). Relative FN and α-SMA positive area in mice were analyzed (right). Six random fields were taken from each kidney. The data are presented as the mean ± SEM. Statistically significant differences were determined by one-way ANOVA and two-way ANOVA. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.001$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

serum were purchased from Wisent Corporation (Wisent, Canada). The cells were grown in DMEM supplemented with 10% fetal bovine serum (GIBCO), penicillin (100 U/mL) and streptomycin (100 μg/mL) and maintained at 37°C in 5% CO_2 in a humidified incubator. The cells were grown to 80% confluence. Cells were pre-treated with cilomilast (5 μM) for 1 h, and TGF-β1 (5 ng/mL) was added to 2% fetal bovine serum medium to stimulate NRK-49F cells for 24 h. In a separate exprement, NRK-49F cells were pre-treated with cilomilast (5 μM) and SB-431542 (1 μM) (a inhibitor of Smad2/3) for 1 h, and then TGF-β1 (5 ng/mL) was added to stimulate cells for 24 h.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from kidney cortexes and cells by TRIzol (Invitrogen, Carlsbad, CA) based on the manufacturer's protocol (28). We reverse transcribed total RNA (1 μg) into cDNA using a PrimeScript™ Reverse Transcriptase System. Quantitative real-time PCR was subsequently carried out with SYBR Green Master Mix (Vazyme) on a QuantStudio 3 Real-time PCR System (Applied Biosystems, Foster City, CA). The cycling programme consisted of a preliminary denaturation (95°C for 10 min) followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative mRNA levels were normalized to the levels of GAPDH and calculated with the comparative threshold cycle (ΔΔCt) method. The primer sequences are shown in **Table 1**.

Western Blotting

We lysed cells or homogenized tissues using protein lysis buffer [50 mmol/L Tris, 150 mmol/L NaCl, 10 mmol/L EDTA, 1% Triton X-100, 200 mmol/L sodium fluoride, supplemented with 1× protease inhibitor cocktail (Roche, 04693132001)]. Then, the samples were centrifuged (14,000 rpm) at 4°C for 15 min. The supernatant was collected and the protein concentration was determined using a BCA protein assay kit (Beyotime, China). Total protein was separated by SDS-PAGE gel and transferred onto PVDF membranes. Then the membranes were blocked by TBS-T (0.1% Tween 20 in TBS) containing 5% non-fat milk for 1 h at room temperature and incubated with primary antibodies against FN (#26836, CST, Danvers, MA, USA, 1:1000), α-SMA (ab7817, Abcam, Cambridge, MA, USA, 1:1000), Collagen-I (Col-I; ab34710, Abcam, Cambridge, MA, USA, 1:1000), kidney injury molecule-1 (KIM-1; ab190696, Abcam; Cambridge, MA, USA, 1:5000), NGAL (ab63929, Abcam, Cambridge, MA, USA,

TABLE 1 | Primer sequences for qRT-PCR.

Gene (species)	Primer sequence
FN (Mice)	F: GGACCTCCTCATCTACATTCG
	R: GTTCCCTCCACAGTTCAAAAG
α-SMA (Mice)	F: CCACCGATCCAGACAGAGTAC
	R: TCCACGAAACCACCTATAACA
Col-I (Mice)	F: CTCAAGGTCACGGTCACGAAC
	R: CCTGGCAAAGACGGACTCAAC
Col-III (Mice)	F: GGACCAGGCAATGATGGAAAAC
	R: GGACCAGGGAAACCCATGACA
TGF-β1 (Mice)	F: CTGAGTGGCTGTCTTTTGA
	R: TGGAGTTTGTTATCTTTGCTG
KIM-1 (Mice)	F: TCAGCTCGGGAATGCACAACC
	R: CTCCAGGGAAGCCGCAGAAAA
NGAL (Mice)	F: ACACTCACCACCCATTCA
	R: CACCACGGACTACAACCA
IL-1β (Mice)	F: TCGTGAATGAGCAGACAG
	R: AGAGGCAAGGAGGAAAAC
IL-6 (Mice)	F: GTCACCAGCATCAGTCCCAAG
	R: CCCACCAAGAACGATAGTCAA
TNF-α (Mice)	F: CAGACCCTCACACTCACAAACCAC
	R: CCTTGTCCCTTGAAGAGAACCTG
Mcp-1 (Mice)	F: GTGCTGACCCCAAGAAGGAATG
	R: TGAGGTGGTTGTGGAAAAGGTAGTG
IL-18 (Mice)	F: CATGTCAGAAGACTCTTGCGTCA
	R: TTATATTCCGTATTACTGCGGTTGT
Icam-1 (Mice)	F: GTGATGCTCAGGTATCCATCCA
	R: CACAGTTCTCAAAGCACAGCG
GAPDH (Mice)	F: AAGAAGGTGGTGAAGCAGG
	R: GAAGGTGGAAGAGTGGGAGT
FN (Rat)	F: GGACCTCCTCATCTACATTCG
	R: GTTCCCTCCACAGTTCAAAAG
α-SMA (Rat)	F: GTCTCAAACATAATCTGGGTCA
	R: GATAGAACACGGCATCATCAC
Col-I (Rat)	F: GAAGCAAAGTTTCCTCCAAGA
	R: GCCCAGAAGAATATGTATCACC
Col-III (Rat)	F: GGTTTGGAGAATCTATGAATGGTGG
	R: GCTGGAAAGAAGTCTGAGGAAGG
IL-1β (Rat)	F: AGGAGAGACAAGCAACGACA
	R: CTTTTCCATCTTCTTCTTTGGGTAT
IL-6 (Rat)	F: AGTTGCCTTCTTGGGACTGATGT
	R: GGTCTGTTGTGGGTGGTATCCTC
Mcp-1 (Rat)	F: CTGTGCTGACCCCAATAAGGAA
	R: GAGGTGGTTGTGGAAAAGAGAGTG
GAPDH (Rat)	F: GGCTCTCTGCTCCTCCC
	R: CCGTTCACACCGACCTT

1:1000), TGF-β1 (#3711, CST, Danvers, MA, USA, 1:1000), Smad2 (#5339, CST, Danvers, MA, USA, 1:1000), Smad2/3 (#8685, CST, Danvers, MA, USA, 1:1000), p-Smad2 (#18338, CST, Danvers, MA, USA, 1:1000), Smad3 (#9513, CST, Danvers, MA, USA, 1:1000), p-Smad3 (#9520, CST, Danvers, MA, USA, 1:1000), p-Smad2/3 (#8828, CST, Danvers, MA, USA, 1:1000), Smad7 (25840-1-AP, Proteintech, Chicago, IL, USA, 1:1000), E-cadherin (#3195, CST, Danvers, MA, USA, 1:1000)and GAPDH (#3683, CST, Danvers, MA, USA, 1:1000), followed by the addition of HRP-labeled secondary antibodies (#7074, CST, Danvers, MA, USA, 1:2500). Densitometry was analyzed with ImageJ software (NIH, Bethesda, MD, USA).

ELISA for TGF-β1

Mouse TGF-β1 in kidney tissue homogenates was evaluated by an ELISA kit (E-EL-M0044c, Elascience, China) according to the manufacturer's protocol.

Cell Counting Kit-8 (CCK-8) Assay

Cell viability was determined by CCK-8 assay kit (KGA317, KeyGen Biotech, China) (27). Briefly, NRK-49F cells were treated with cilomilast (5–40 μM) for 24 h, and then 10 μL CCK-8 reagent was added to medium and incubated for 2 h. The absorbance was detected at 450 nm.

Statistical Analysis

All data are presented as the means ± standard errors of the mean (SEMs). Differences between 2 groups were analyzed using two-tailed Student's *t*-test and incorporated into GraphPad Prism 6 software (GraphPad Software). ANOVA was used for comparisons among multiple groups. $P < 0.05$ was considered significant.

RESULTS

Cilomilast Treatment Attenuates UUO-Induced Renal Tubulointerstitial Fibrosis

First, we used a UUO model to explore the effect of cilomilast treatment on renal tubulointerstitial fibrosis (**Figure 1A**). Firstly, Masson's trichrome staining showed that cilomilast treatment led to a remarkable reduction in collagen deposition. In addition, HE staining showed that cilomilast significantly reduced tubule brush border disruption and tubular atrophy, indicating the attenuation of UUO-induced tubular injury (**Figure 1B**). Then, western blot and qRT-PCR analysis confirmed the deceased expression of FN, α-SMA (a marker of myofibroblasts) and Col-I and a significant increase in protein levels of E-cadherin after cilomilast treatment (**Figures 1C–E**). Furthermore, by using immunohistochemistry, we further found cilomilast treatment markedly inhibited the UUO-induced upregulation of FN and α-SMA expression (**Figure 1F**). These results demonstrated that cilomilast improved renal tubulointerstitial fibrosis.

Cilomilast Treatment Reduced TGF-β1 in the Kidneys of UUO Mice

TGF-β1 is involved in renal tubulointerstitial fibrosis and is produced in large quantities during renal fibrogenesis. Therefore, we examined whether the decreased fibrosis in obstructed kidneys in cilomilast-treated mice was associated with change of TGF-β1 production. By qRT-PCR assays, we found that the enhanced expression of TGF-β1 in UUO mice was significantly blunted after cilomilast treatment

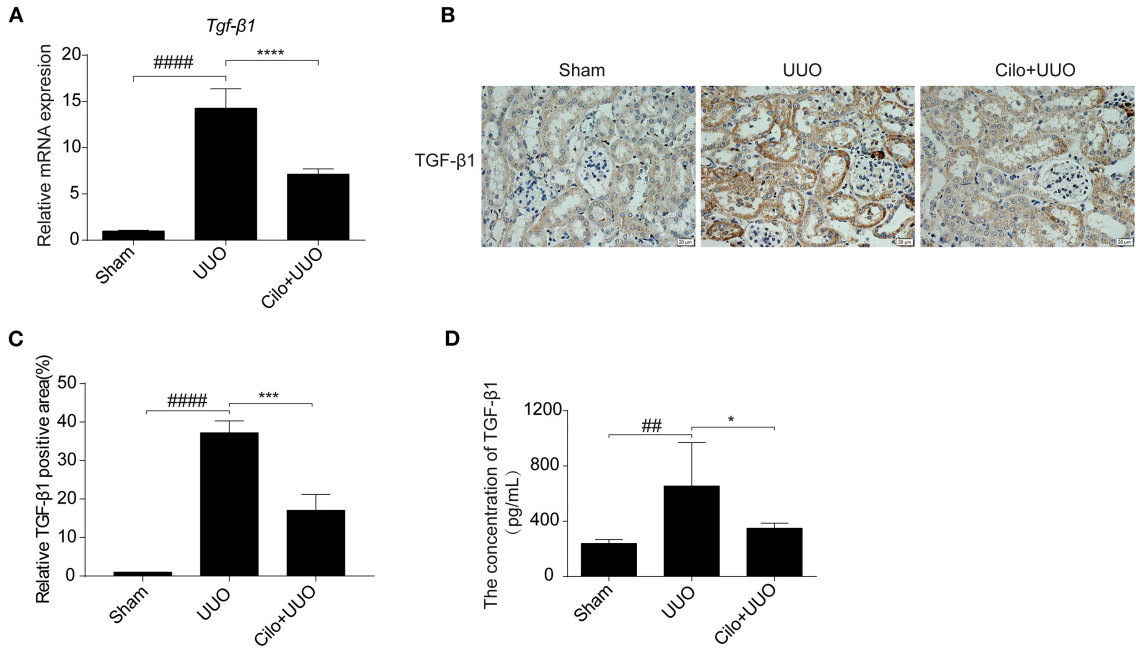

FIGURE 2 | Cilomilast treatment downregulated TGF-β1 expression induced by UUO. **(A)** qRT-PCR analyses of *TGF-β1* mRNA expression ($n = 6$). **(B,C)** Representative images and quantitative assessment of the expression and distribution of TGF-β1 in kidney tissues using immunohistochemical staining. Scale bar = 20 μm. **(D)** ELISA analysis of TGF-β1 expression in kidney tissues. The data are presented as the mean ± SEM. Statistically significant differences were determined by one-way ANOVA. $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$, $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

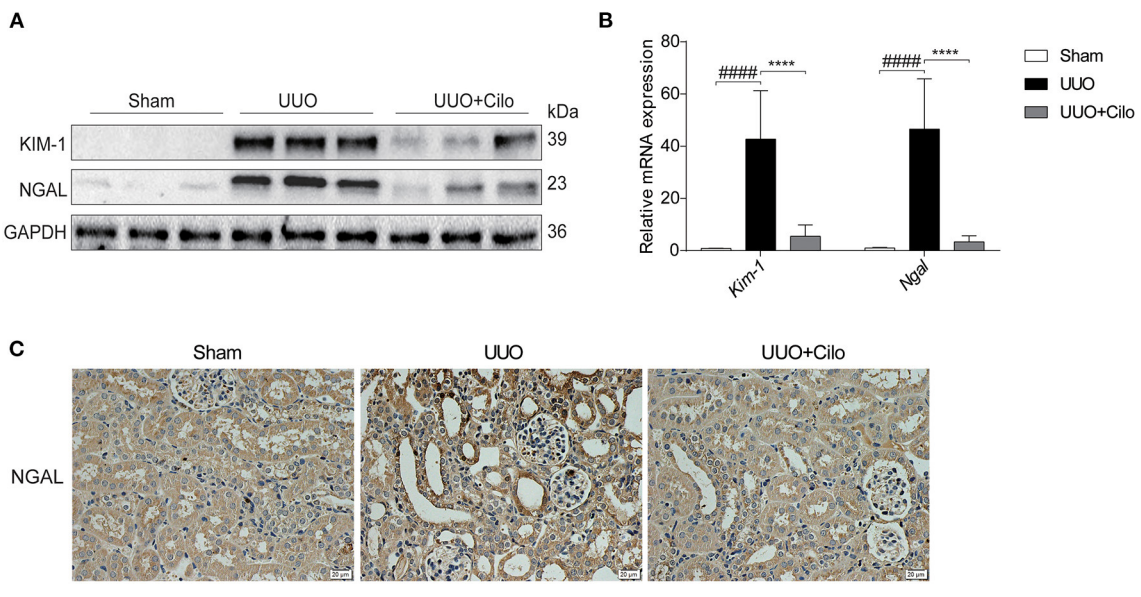

FIGURE 3 | Cilomilast treatment improved renal tubular injury induced by UUO. **(A)** Western blot analyses of renal KIM-1 and NGAL protein expression in kidney tissues. **(B)** qRT-PCR analyses of *Kim-1* and *Ngal* mRNA expression ($n = 6$). **(C)** Representative images and quantitative assessment of the expression and distribution of NGAL in kidney tissues using immunohistochemical staining. Scale bar =20 μm. The data are presented as the mean ± SEM. Statistically significant differences were determined by two-way ANOVA. $^{\#\#\#\#}P < 0.0001$, $^{****}P < 0.0001$.

(**Figure 2A**). Furthermore, immunohistochemistry staining and ELISA also showed that the expression of TGF-β1 was significantly reduced in cilomilast-treated UUO mice (**Figures 2B–D**).

Cilomilast Treatment Suppressed Renal Tubular Injury Induced by UUO

To evaluate the extent of renal damage, the expression of KIM-1 and NGAL, both markers of tubular damage, was

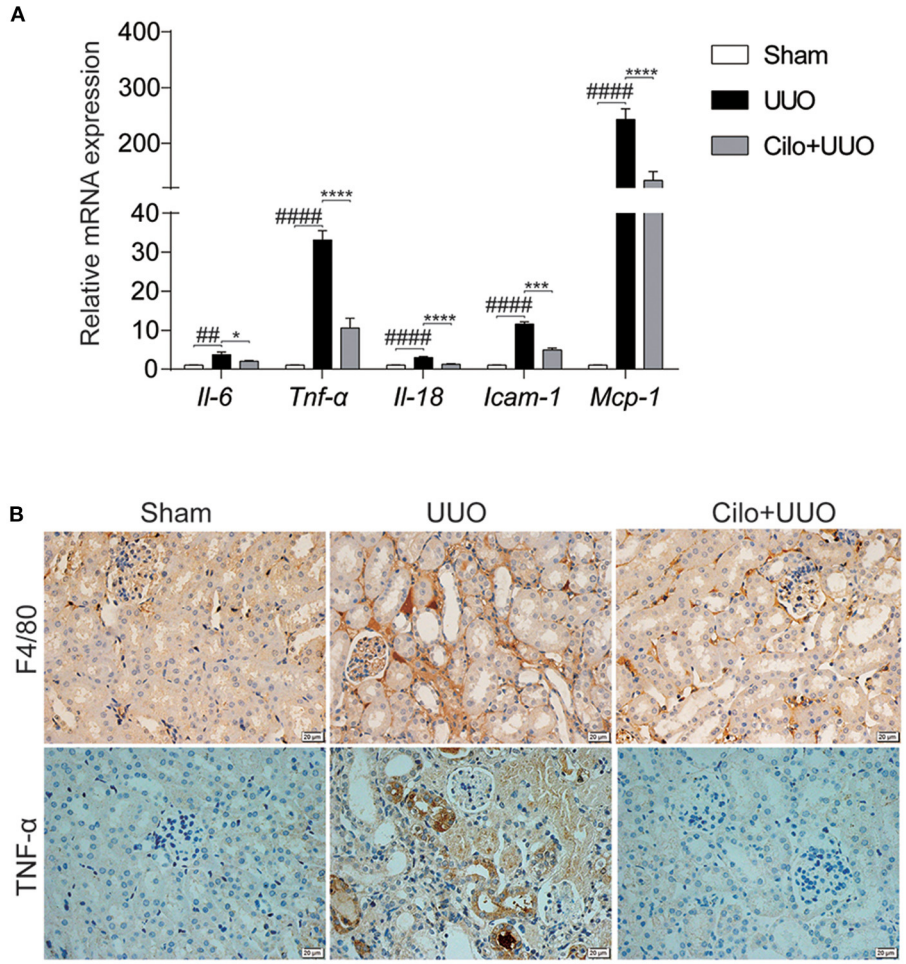

FIGURE 4 | Cilomilast improved UUO-induced inflammatory responses. **(A)** qRT-PCR analyses of *Il-6*, *Tnf-α*, *Il-18*, *Icam-1*, and *Mcp-1* mRNA expression ($n = 6$). **(B)** Representative images and quantitative assessment of the expression and distribution of F4/80 and TNF-α in kidney tissues using immunohistochemical staining. Scale bar = 20 μm. The data are presented as the mean ± SEM. Statistically significant differences were determined by two-way ANOVA. $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$, $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

measured in the kidney. As shown in **Figure 3**, KIM-1 and NGAL expression was markedly increased in the UUO group according to western blot and qRT-PCR analyses, which was attenuated by cilomilast treatment (**Figures 3A,B**). The trend of NGAL change measured by immunohistochemistry was consistent with that of the protein and mRNA levels (**Figure 3C**). These data suggested that the amelioration of tubular injury after cilomilast treatment could protect tubular integrity and attenuate subsequent pathological events.

Cilomilast Treatment Attenuated Renal Inflammation Induced by UUO

Inflammation plays a key role in the progression of renal tubulointerstitial fibrosis (29). Thus, we evaluated the inflammatory status (inflammatory cell infiltration and pro-inflammatory cytokine expression) in obstructed kidneys with or without cilomilast. As expected, the enhanced mRNA expression of *Il-6*, *Il-18*, *Tnf-α*, *Icam-1*, and *Mcp-1* was significantly blunted after cilomilast treatment (**Figure 4A**). Immunohistochemistry analysis also showed reductions in macrophage infiltration ($F4/80^{+}$) and TNF-α expression in the obstructed kidney after cilomilast therapy (**Figure 4B**). These results revealed that cilomilast could ameliorate renal inflammation in UUO.

Cilomilast Treatment Reduced the TGF-β1-Induced Profibrotic Response and Inflammation in NRK-49F Cells

To examine the cytotoxicity of cilomilast, cell viability assay was performed in cultured NRK-49F cells using a CCK8 assay kit. With cilomilast treatment at increasing concentrations from 5 to 40 μM for 24 h, we found the concentration of cilomilast at 5 μM was safe for cells (**Figure 5A**). To define the effect of cilomilast on the profibrotic response in kidney cells, we added cilomilast to NRK-49F cells stimulated with TGF-β1. As shown by qRT-PCR and western blot, cilomilast treatment inhibited

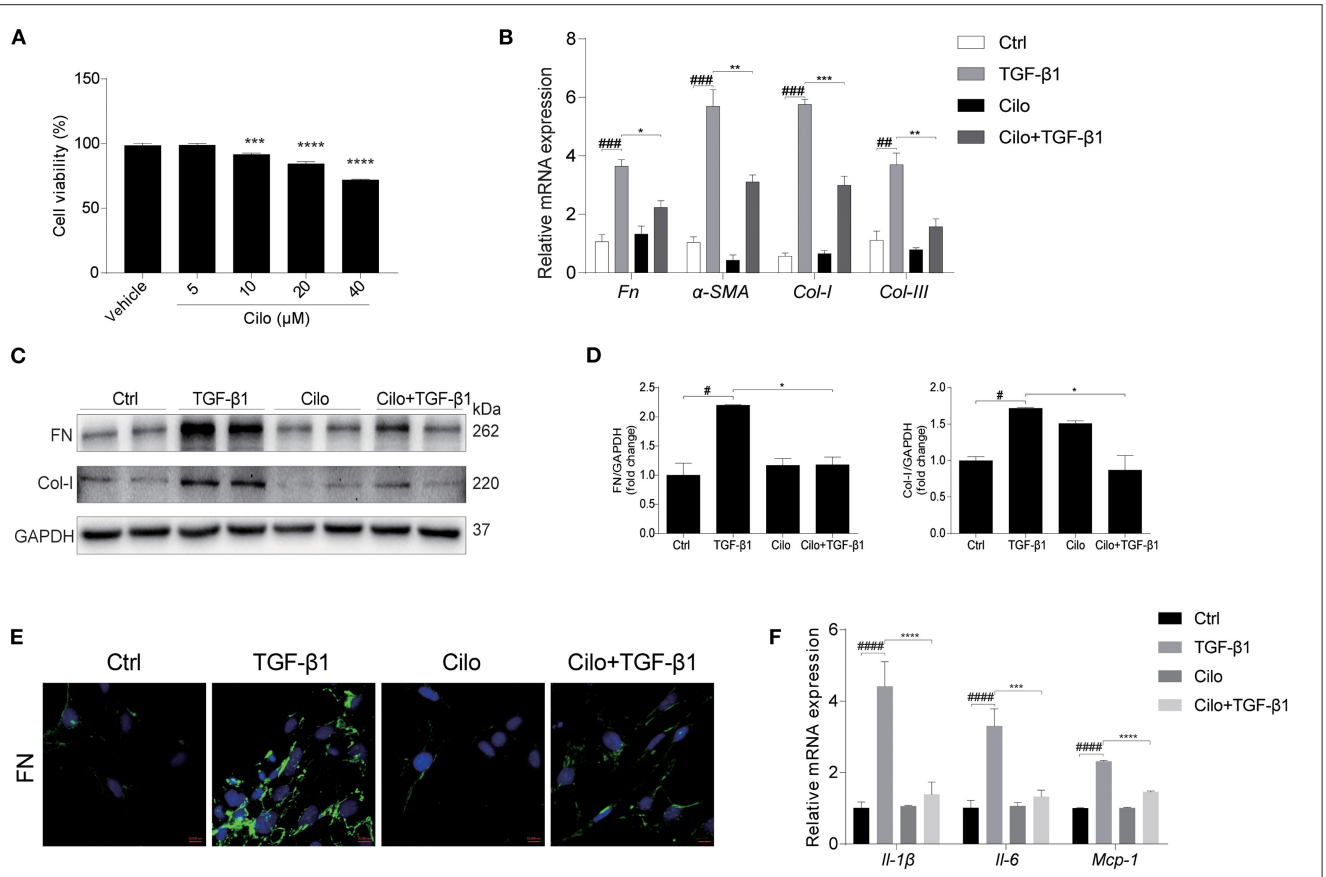

FIGURE 5 | Cilomilast reduced the TGF-β1-induced profibrotic response in NRK-49F cells. NRK-49F cells stimulated with TGF-β1 were treated with cilomilast. **(A)** Cell viability was analyzed by CCK8 assay after treatment with celastrol for 24 h at increasing concentrations from 5 μM to 40 μM. **(B)** qRT-PCR analyses of *Fn*, *Col-I*, *Col-III*, and *α-SMA* mRNA expression ($n = 3$). **(C)** Western blot analyses of FN and Col-I protein expression in NRK-49F cells. **(D)** Densitometry of the western blot results in **(B)**. **(E)** Representative images of immunofluorescence staining for FN in NRK-49F cells. **(F)** qRT-PCR analyses of *Il-1β, Il-6*, and *Mcp-1* mRNA expression ($n = 3$). The data are presented as the mean ± SEM. Statistically significant differences were determined by one-way ANOVA and two-way ANOVA. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

the expression of *Fn*, *α-SMA*, *Col-I*, and *Col-III* (**Figures 5B–D**). Immunofluorescence analysis demonstrated that the expression of FN was significantly reduced after treatment with cilomilast (**Figure 5E**). Similarly, the inflammatory response induced by TGF-β1 was also attenuated by cilomilast treatment (**Figure 5F**). These data revealed an antifibrotic role of cilomilast via the inhibition of renal fibroblast activation.

Cilomilast Treatment Inhibited the Activation of TGF-β1-Smad2/3 Signaling

TGF-β1-Smad2/3 signaling plays a critical role in renal interstitial fibrosis and inflammation (30). *In vivo*, we found cilomilast treatment inhibited the activation of Smad2/3 pathway in obstructed kidney tissues, as evidenced by decreased protein expression of TGF-β1, p-Smad2 and p-Smad3 and a significant increase in protein levels of Smad7 (**Figures 6A,B**). *In vitro*, western blot results showed that cilomilast could markedly decrease the expression of p-Smad2 and p-Smad3 in NRK-49F cells treated with TGF-β1 (**Figures 6C,D**). Furthermore, we used Smad2/3 inhibitor SB-431542 (1 μM) to explore whether cilomilast protected against TGF-β1-induced fibroblast activation through suppressing Smad2/3 pathway in this study. As shown in **Figure 6E**, when NRK-49F cells were pretreated with SB-431542 (1 μM), cilomilast failed to further ameliorate TGF-β1-induced fibroblast activation. Collectively, these results suggested that cilomilast may exert its antifibrotic effect by inhibiting the activation of TGF-β1-Smad2/3 signaling.

DISCUSSION

Renal tubulointerstitial fibrosis is the final outcome for all CKDs, leading to progression to end-stage renal failure (ESRD). To date, there are no effective therapeutic approaches in clinic, which results in a heavy socioeconomic burden. Thus, it is necessary to develop effective drugs for treating renal tubulointerstitial fibrosis. Based on the data from our study, cilomilast, a selective phosphodiesterase-4 inhibitor and phase III clinical drug, can remarkably reduce UUO-induced renal tubulointerstitial fibrosis and renal inflammation *in vivo* and *in vitro*. To the best of our knowledge, this is the first study to report that cilomilast may

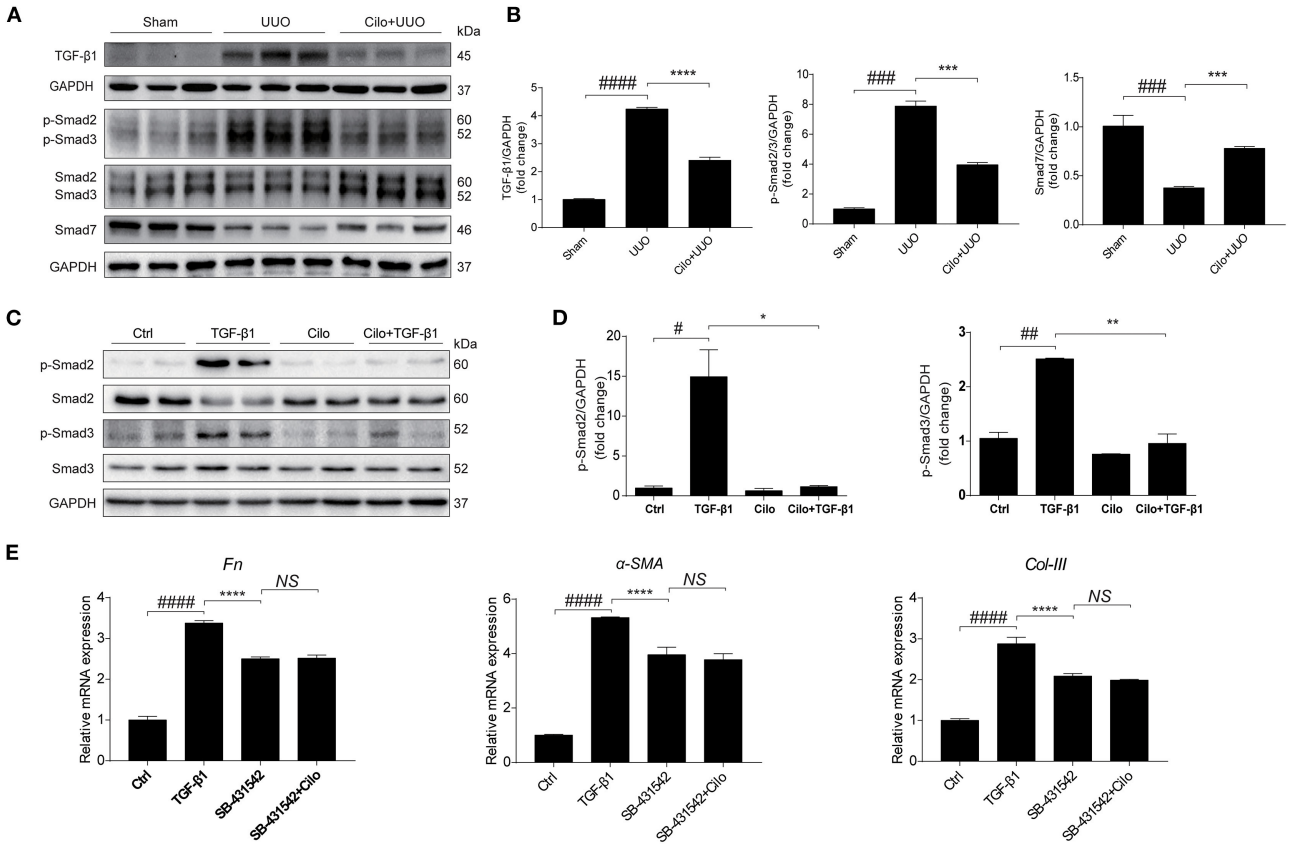

FIGURE 6 | Cilomilast treatment inhibited the activation of TGF-β1-Smad2/3 signaling. **(A)** Western blot analyses of p-Smad2, Smad2, p-Smad3, Smad3, Smad7, and TGF-β1 protein expression in kidney tissues. **(B)** Densitometry of the western blot results in **(A)**. **(C)** Western blot analyses of p-Smad2, Smad2, p-Smad3, and Smad3 protein expression in NRK-49F cells. **(D)** Densitometry of the western blot results in **(C)**. **(E)** NRK-49F cells were pretreated with SB-431542 and cilomilast, and then incubated with TGF-β1 for 24 h. qRT-PCR analyses of *Fn*, *α-SMA*, and *Col-III* mRNA expressions in NRK-49F cells. The data are presented as the mean ± SEM. Statistically significant differences were determined by one-way ANOVA. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, NS, not significant.

serve as a potent therapeutic agent for preventing the progression of renal tubulointerstitial fibrosis.

Factors that contribute to CKD progression include parenchymal cell loss, chronic inflammation, fibrosis and reduced regenerative capacity of the kidney (31). PDE4, which is a member of the PDE family, has four subtypes (PDE4A-PDE4D), and PDE4B plays an important role in inflammation (32). Cilomilast has been reported to treat cisplatin nephrotoxicity by antagonizing inflammation (12). Inflammation can lead to the progression of renal tubulointerstitial fibrosis (29). In the present study, the mRNA expression of proinflammatory factors was significantly reduced after cilomilast treatment. Immunohistology results revealed that cilomilast dramatically decreased the $F4/80^{+}$ and TNF-α expression *in vivo*. Meanwhile, we confirmed that cilomilast downregulated the expression of fibrotic markers. Thus, cilomilast, as a selective phosphodiesterase-4 inhibitor, could block the inflammation to relieve renal tubulointerstitial fibrosis to some extent. KIM-1 and NGAL are considered to be important markers for evaluating renal tubular damage (33, 34). Recent studies indicated that the expression of KIM-1 and NGAL was significantly increased in UUO mice (35, 36). Our results revealed that cilomilast reversed renal tubular injury, which could contribute to improved tubular integrity and reduced subsequent pathology, such as inflammation and fibrosis.

Excessive deposition of ECM components in the renal interstitium could result in renal tubulointerstitial fibrosis (37). Smad2/3 signaling is closely associated with renal tubulointerstitial fibrosis (38), which can promote the fibrotic response by directly facilitating the production of ECM through its binding to specific promoter regions of collagen genes and the inhibition of ECM degradation (39, 40). TGF-β1 is a potent pathogenic factor of renal fibrosis, which could promote ECM production and renal tubulointerstitial fibrosis (41). The activation of TGF-β1 triggers the nuclear localization of Smad2/3 in tubular epithelial cells and fibroblasts (42). To exert its profibrotic role in kidney disease, TGF-β1 can act by stimulating Smad2/3 to positively or negatively regulate microRNAs (43, 44). Inhibiting Smad2/3 phosphorylation during TGF-β1-mediated epithelial-mesenchymal transition attenuated kidney fibrosis (45). In the contrast, up-regulating Smad2/3-related signaling pathway may enhance the progression of CKD

(46). Indeed, Smad3-deficient mice were protected from UUO-induced renal tubulointerstitial fibrosis (47). In this study, we found that cilomilast could inhibit the expression of TGF-β1 induced by UUO and downregulated the phosphorylation levels of p-Smad2 and p-Smad3. The present study provided evidence that cilomilast has a protective effect on renal tubulointerstitial fibrosis possibly by downregulating the expression of TGF-β1, further inhibiting Smad2/3 phosphorylation.

In summary, we found that cilomilast remarkably attenuated renal tubulointerstitial fibrosis and inflammation in a CKD model of UUO. Cilomilast could decrease the expression of collagen, fibronectin and α-SMA, possibly by inhibiting TGF-β1-Smad2/3 pathway activation. Cilomilast is a selective phosphodiesterase-4 inhibitor that is currently in clinical trial for the treatment of COPD. Thus, the present study provided the rationale for further clinical trials to evaluate cilomilast in treating CKD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Nanjing Medical University Institutional Animal Care and Use Committee (registration number: IACUC-1809017).

AUTHOR CONTRIBUTIONS

XY and ZJ designed the experiment. MX, SL, XY, and JW performed the experiments and data analyses. XY, ZJ, and MX drafted the manuscript. ZJ, XY, YZ, SH, WG, and AZ revised and approved the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

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HLA-DRB1*04 as a Risk Allele to Systemic Lupus Erythematosus and Lupus Nephritis in the Malay Population of Malaysia

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Systemic lupus erythematosus (SLE) is a chronic autoimmune disease afflicting multiple organs. Lupus nephritis (LN) is a serious complication of SLE and remains a major cause of mortality and morbidity. Curative therapy remains unavailable as etiology from genetic and environmental factors is still unclear. The present study was conducted to elucidate the link between HLA-DRB1 gene polymorphisms with SLE and LN through clinical and laboratory/biological presentations in a population of Malaysian Malay females with SLE. A total of 100 Malay female SLE patients inclusive of 70 SLE patients without LN and 30 patients with LN were included in this study. HLA-DRB1 allele examination in SLE patients was performed using PCR-SSO, and the alleles' frequencies were compared with 951 publicly available datasets representing Malay healthy controls in Malaysia. Cytokines and free radical levels were detected by ELISA and bead-based multiplexed Luminex assays. The association between HLA-DRB1 alleles with clinical and serological manifestations and immune mediators was analyzed using different statistical approaches whenever applicable. Our study showed that HLA-DRB1*0405, HLA-DRB1*1502, and HLA-DRB1*1602 were associated with the increased risk of SLE while HLA-DRB1*1201 and HLADRB1*1202 alleles were associated with a lower risk of SLE development. Furthermore, HLA-DRB1*04 showed significant association to LN and arthritis while HLA-DRB1*15 was significantly associated with oral ulcer in Malay SLE patients. Association analysis of HLA-DRB1*04 with clinical and biological factors revealed that HLA-DRB1*04 was significantly associated with Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) scores, anti-nuclear antibody (ANA), C-reactive protein (CRP) in the blood, and total protein in the urine. SLE carriers with the HLA-DRB1*04 allele were significantly correlated to the increased levels of cytokines (IFN-γ, GM-CSF, IL-17F, IL-18, IL-21, and VEGF) and were significantly showing negative correlation to IL-5 and free radicals (LPO and catalase enzyme) levels compared to SLE carriers without HLA-DRB1*04 allele. The results suggested that disease severity in SLE may be determined by HLA-DRB1 alleles. The

risk of HLA-DRB1*04 allele with LN was supported by the demonstration of an intense inflammatory response in Malay SLE patients in Malaysia. More studies inclusive of a larger and multiple SLE cohorts in the future are warranted to validate these findings.

Keywords: systemic lupus erythematosus, lupus nephritis, HLA-DRB1 gene polymorphism, HLA-DRB1*04, Malaysian Malay population, cytokines and free radicals

INTRODUCTION

Systemic lupus erythematosus (SLE) is a multifactorial, chronic autoimmune disorder that involves multiple organ systems and is predisposed by immunoregulatory, hormonal, environmental, epigenetic, and genetic factors (1). SLE is predominantly characterized by the production of autoantibodies and autoreactive T cells against cytoplasmic, nuclear, and cell-surface antigens (2). Impairment in clearance of apoptotic bodies and nuclear DNA protein immune complexes leads to tissue swelling and induction of inflammation and deposition of immune complexes in multiple organs such as the lung, kidney, brain, skin, and heart, resulting in diversified clinical phenotypes of SLE (2–4). The patterns of autoimmune and clinical manifestations among SLE patients are heterogeneous, which may involve any organ systems in variable combinations, contributing to the variation in disease severity and remain a challenge for accurate diagnosis of SLE (5). Some of the common clinical features present in SLE patients consist of arthritis, mucocutaneous lesions, fever, renal involvement, serositis, hematological disorders, neuropsychiatric dysfunction, and cardiovascular diseases (6). For instance, renal involvement or lupus nephritis (LN) is the most common and severe organ complication of SLE, affecting approximately 30–60% of adults and up to 70% of children with SLE (7). This condition is often associated with high morbidity and poor survival, especially in patients who develop end-stage renal disease (ESRD) (8).

SLE is a global autoimmune disorder with a striking predisposition toward women of reproductive age compared with men. It has been reported that a peak female-to-male ratio of 12:1 is observed during childbearing years. Also, SLE can occur in the elderly and children with a narrower gender distribution (9–11). Presumably, the global incidence and prevalence rates of SLE tend to differ across different geographical regions. Studies have shown that the incidence rates of SLE worldwide range from 0.3 to 31.5 cases per 100,000 individuals while the prevalence of SLE around the world is 3.2–517.5 cases per 100,000 individuals (12). Further, existing literature also documented that racial/ethnicity variations have a huge impact on the incidence and prevalence of SLE. For example, SLE is more common in non-Caucasian populations (African, Asian, Hispanic, and Aboriginal) than in Caucasians (11, 12). Moreover, SLE in these populations exhibits higher disease activity and severity, with heightened risk of relapses and organ damage (13).

Severe disease manifestation such as LN in SLE patients remains a major cause of mortality and morbidity. Much research still needs to be carried out to understand the disease pathogenesis of LN in SLE and to provide alternative treatment. Numerous genomic studies have also highlighted the polymorphisms of various genes at different loci, in particular the major histocompatibility complex (MHC) which encodes the human leukocyte antigen (HLA), cytokines, complement proteins, and immunoglobulin-associated receptor genes and other gene variants that are predisposed to SLE (14–17). Several studies highlighted that renal involvement and severity of disease in SLE were genetically associated. HLA Class II molecules such as HLA-DRB1 and HLADQ genes were predisposed to SLE and LN where the polymorphisms in these genes were widely studied (18–20). HLA-DRB1 and HLA-DQβ1 are considered the most polymorphic with more than 1,000 alleles being discovered at present. The HLA-DR and HLA-DQ genes remain firmly in a state of linkage disequilibrium, and haplotype combinations may modulate the susceptibility to SLE and LN (21).

Ethnicity has been considered as a genetic marker in SLE. Polymorphisms in the HLA-DRB1 gene that affects susceptibility to SLE and class of LN have resulted in different conclusions, depending on the ethnic groups (22–25). Malaysia is a multiracial country consisting of three major ethnic groups, represented by Malay, Chinese, and Indian populations (26). Previous studies reported that the prevalence of SLE is higher in the Chinese population, followed by the Malay and Indian populations (27, 28). Wang and his collaborators (1997) reported that the overall 5-year and 10-year survival rates for SLE patients in Malaysia were 82 and 70%, respectively (28), whereas the mortality rate of SLE patients was 20.2% (29). Renal involvement is the highest among all clinical features in Malaysian SLE patients (28). At present, there are limited studies on HLA-DRB1 in the Malaysian SLE population (30–32). The influence of genetic polymorphisms on clinical manifestation, disease severity, and lab parameters is still unclear. Contributions from immune mediators such as cytokines and free radicals require further investigation. Therefore, the present study aimed to characterize the HLA-DRB1 gene polymorphism and disease susceptibility in Malay SLE population in Malaysia. This study also investigated the association between HLA-DRB1 gene polymorphisms and LN in SLE through clinical, serological, laboratory, and immune mediator association analysis.

MATERIALS AND METHODS

Subjects and Inclusion Criteria

A total of 100 Malay female ages between 18 to 50 years old were recruited from January 2016 to October 2017 for the purpose of this study. Patients with SLE were recruited based on the 1997 American College of Rheumatology (ACR) criteria where SLE is diagnosed based on having at least four of the 11 ACR criteria (33). SLE disease activity in SLE patients was assessed using the Systemic Lupus Erythematosus

Disease Activity Index (SLEDAI) (34). All the patients were recruited at the Nephrology Clinic Hospital Serdang, Malaysia. LN was confirmed by a nephrologist by examining the clinical pictures of renal disease/flare, and the classification follows the International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 Classification of Lupus Nephritis (35). The clinical manifestations for active nephritis consist of detecting the presence of red blood cells and casts in urine, proteinuria (>2 g), increased levels of creatinine, low complement component C3, and higher levels of anti-dsDNA antibody and erythrocyte sedimentation rate (ESR) (36). All participants gave informed consent, and the study was approved by the Medical Research and Ethics Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM), and Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (NMRR-14-1756-23234).

Clinical and Laboratory Investigations of Patients With SLE

Demographic data, SLE disease activity, and clinical and laboratory information of patients with SLE were obtained from the Hospital Serdang Information System. Clinical information includes the medical history of patients with SLE, duration of SLE disease, and assessment of clinical features and different organ manifestations. Laboratory information consists of routine blood analysis, urinalysis, and immunological investigations including anti-nuclear antibody (ANA) and anti-dsDNA detection. Other laboratory information such as complement C3, C4, and C-reactive protein (CRP) was also extracted. All the laboratory investigations were conducted by the Medical Lab Technologist of Hospital Serdang, Malaysia.

Genomic DNA Isolation and HLA-DR Genotyping

Whole-blood samples were collected from 100 Malay female SLE patients in ethylenediaminetetraacetic acid (EDTA) vacutainer tubes. DNA was extracted from whole blood using a QIAamp DNA Blood Mini extraction kit (Qiagen, Germany) in accordance to the manufacturer's instructions. Qualitative and quantitative checks on extracted DNA were performed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). Genotyping of HLA-DR in SLE patients was performed by polymerase chain reaction using sequence-specific oligonucleotides, PCR-SSO (LIFECODES DR-Typing Kit, Gen-Probe, USA) according to the manufacturer's instructions. HLA-DR allele identification was conducted using the Luminex xMAP Technology (R &D Systems, USA) with Lumines 100 IS Software and Quick Type for Life Match 2.6.1 software for Gen-Probe analysis. A total of 951 data consisting of HLA-DRB1 typing representing Malay healthy population were obtained from the Malaysian Stem Cell Registry (MSCR), Institute Medical Research (IMR), Kuala Lumpur, Malaysia (37). The data obtained serve as a control for association analysis between HLA-DR genotyping in SLE Malay female patients with clinical, laboratory, and cytokine indices.

Association of Cytokines and Free Radicals in HLA-DRB1*04 Allele Carrier and Non-carrier

A total of 28 Malay SLE patients (14 SLE patients without LN and 14 SLE patients with LN) and 28 age- and sex-matched healthy controls were used in the association analysis. Cytokines comprised IL-5, IL-17F, IL-18, IL-21, GM-CSF, IFN-γ, and vascular endothelial growth factor (VEGF), and free radicals such as lipid peroxidation (LPO) and catalase (CAT) were used in the association study with HLA-DRB1*04 allele SLE carrier and non-carrier SLE patients. Cytokines such as IL-5, IL-17F, GM-CSF, IFN-γ, and VEGF obtained from Human High Sensitivity Cytokine Premixed Performance Kit A and Kit B (R &D Systems, USA) were measured using bead-based multiplexed Luminex assays (R &D Systems, USA) according to the manufacturer's instructions. Cytokine concentrations were detected by Luminex xMAP Technology (R &D Systems, USA), and the results were expressed in pg/mL ± SEM. Concentrations of IL-18 and IL-21 were determined using ELISA kits (EIAab, China) in accordance with the manufacturer's instructions. The absorbance of each supernatants was read at 450 nm using a 96-well microplate reader (Eppendorf, Hamburg, Germany). The detection limits for both cytokines were 15.6–1,000 pg/mL. LPO and CAT activities were assessed through colorimetric measurement. The levels of LPO (Item No. 705002) and CAT (Item No. 707002) purchased from Cayman Chemicals (Ann Arbor, MI, USA) were measured following protocols provided by the manufacturer. The absorbance values of each color compounds in LPO and CAT assays were read at 480 and 540 nm, respectively, using a 96-well microplate reader (Eppendorf, Hamburg, Germany). The assay range for LPO was between 0.25 and 5 nmol while the lowest detection limit for CAT assay was 2 U/mL.

Statistical Analysis

Data analysis was performed using the SPSS statistical package version 23.0 and GraphPad Prism version 6.0 whenever applicable. The frequency of HLA-DRB1 alleles between SLE patients and healthy controls was compared using the chi-square Fisher test with two-by-two contingency tables used. The chi-square Fisher test was used for the association analysis between HLA-DRB1 alleles with clinical manifestations, disease activity (SLEDAI), and serological manifestations with Phi and Cramer's V analysis performed to assess the strength of the association. An independent t-test and linear regression were conducted to associate HLA-DRB1 alleles with laboratory findings. An independent t-test and correlation analysis were employed to associate HLA-DRB1 alleles with cytokines and free radicals. A p-value <0.05 is considered statistically significant.

RESULTS

Demographic, Disease Severity, and Clinical and Laboratory Information of Patients With SLE (With and Without LN Involvement)

A total of 100 Malaysian Malay female patients diagnosed with SLE between January 2016 and October 2017 were included in this cohort study. The demographic and clinical and laboratory findings which were significant and relevant to this SLE study cohort are depicted in **Table 1** [part of the data of this SLE study cohort was published (38)]. Among the 100 SLE patients, 70 patients (mean age 31.03 ± 0.95 years) were diagnosed without LN while 30 SLE patients (mean age 29.60 ± 1.23 years) were diagnosed with LN, as confirmed by a nephrologist in Hospital Serdang, Malaysia, by renal biopsy. Additionally, 30 SLE patients were further classified into different classes of LN as shown in **Table 1**. Majority of the SLE patients without LN were presented with mild activity (mean SLEDAI score 1.97 ± 0.15) in contrast with moderate activity (mean SLEDAI score 9.65 ± 0.57) in SLE patients with LN. SLE patients with LN were quite responsive to anti-dsDNA and ANA detection, with 100 and 93% positivity recorded, respectively, whereas only 80 and 69% positivity for anti-dsDNA and ANA, respectively, were observed in SLE patients without LN. Further analysis from immunological, blood, and urine investigations revealed that SLE patients with LN had significantly lower levels of complement proteins, C3 and C4, and higher levels of creatinine and total protein in urine when compared to SLE patients without LN.

Clinical Manifestations in SLE Patients With and Without LN

The main clinical manifestations presented in SLE patients with and without LN are shown in **Figure 1** [part of the data of this SLE study cohort was published (38)]. In general, Malay SLE patients without LN were likely presented with milder clinical complications, while Malay SLE patients with LN were more vulnerable to severe clinical manifestations. In SLE patients without LN, 58 patients developed integument disorders (oral/nasal ulcers, malar, and photosensitivity) (82.9%), 45 patients experienced headache (64.3%), 31 patients had arthritis (44.3%), 28 patients developed vasculitis (40%), 28 patients had hypertension (40%), 26 patients had alopecia (37.1%), and three patients had discoid rash (4.3%). In SLE patients with LN, all patients presented with renal disorder and hypertension (100%), whereas 27 patients developed integument disorders (oral/nasal ulcers, malar, and photosensitivity) (90%),

TABLE 1 | Demographic, clinical, and laboratory information of patients diagnosed with SLE (with and without LN).

Category	SLE (without LN)	SLE-LN (SLE with LN)	*p*-value
No of subjects (*N*)	70	30 *Classification of SLE-LN* Class I LN = 2 Class III LN = 6 Class IV LN = 9 Class V LN = 3 Class III + V LN = 8 Class IV + V LN = 2	
Mean age of SLE onset (years ± SEM)	31.03 ± 0.95 (*N* = 70)	29.60 ± 1.23 (*N* = 30)	**ns**
Disease duration (years ± SEM)	7.09 ± 0.62	7.73 ± 0.97	**ns**
SLEDAI activity score			
Mild (0–3)	1.97 ± 0.15 (*n* = 32)	N/A	
Moderate (3–12)	6.61 ± 0.35 (*n* = 28)	9.65 ± 0.57 (*n* = 17)	
Severe (>12)	13 (*n* = 2)	14.23 ± 0.32 (*n* = 13)	
Immunological profiling			
Antinuclear antibodies (ANA) (positive)	69% (*n* = 48)	93% (*n* = 28)	
Anti-dsDNA (positive)	80% (*n* = 56)	100% (*n* = 30)	
Complement (C3) (g/L) (mean ± SEM)	0.462 ± 0.056	0.036 ± 0.028	*
Complement C4 (g/L) (mean ± SEM)	0.153 ± 0.017	0.065 ± 0.010	**
C-reactive protein (CRP) (mg/L) (mean ± SEM)	8.924 ± 0.401	10.160 ± 0.724	ns
Blood profiling			
Creatinine (μmol/L ± SEM)	273.70 ± 25.990	1,535 ± 262.90	***
Urinalysis			
Total protein (Urine) (mg/dl ± SEM)	126.50 ± 7.051	322.60 ± 28.80	***

** indicates p<0.05, **indicates p<0.01, *** indicates p<0.001, ns, not significant.*

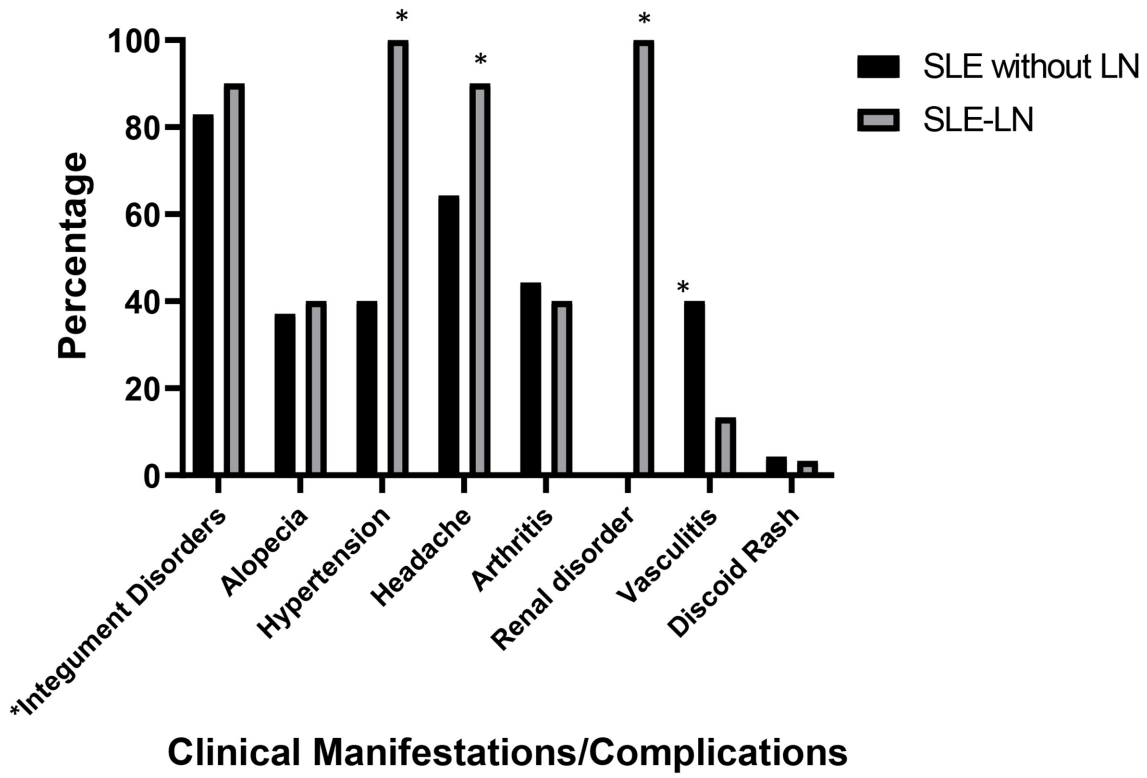

FIGURE 1 | Major clinical manifestations/complications observed in SLE patients with lupus nephritis (SLE-LN) and SLE patients without lupus nephritis (SLE without LN). *Integument disorders comprise clinical features such as oral/nasal ulcers, malar, and photosensitivity. * denotes $p < 0.05$, significant differences in the clinical manifestations between SLE patients with and without LN.

TABLE 2 | HLA-DRB1 allele carrier frequency in Malay female SLE patients and Malay healthy controls.

HLA-DRB1 allele	SLE ($2n = 200$)	Controls ($2n = 1,902$)	OR CI (95%)	*p* Fisher	p_c
HLA-DRB1*01	4 (0.02)	14 (0.007)	2.752 (0.897–8.443)	ns	ns
HLA-DRB1*03	14 (0.07)	96 (0.05)	1.416 (0.792–2.531)	ns	ns
HLA-DRB1*04	29 (0.145)	122 (0.064)	2.474 (1.603–3.820)	***	(0.001)***
HLA-DRB1*07	15 (0.075)	192 (0.101)	0.722 (0.418–1.248)	ns	ns
HLA-DRB1*08	6 (0.03)	47 (0.025)	1.221 (0.515–2.892)	ns	ns
HLA-DRB1*09	7 (0.035)	64 (0.034)	1.042 (0.471–2.305)	ns	ns
HLA-DRB1*10	1 (0.05)	37 (0.020)	0.253 (0.035–1.856)	ns	ns
HLA-DRB1*11	3 (0.015)	59 (0.031)	0.476 (0.148–1.532)	ns	ns
HLA-DRB1*12	19 (0.095)	543 (0.285)	0.263 (0.162–0.426)	***	(0.001)***
HLA-DRB1*13	6 (0.03)	65 (0.034)	0.874 (0.374–2.044)	ns	ns
HLA-DRB1*14	3 (0.015)	85 (0.045)	0.326 (0.102–1.039)	ns	ns
HLA-DRB1*15	72 (0.36)	503 (0.264)	1.565 (1.152–2.125)	**	(0.01)**
HLA-DRB1*16	21 (0.105)	75 (0.04)	2.858 (1.720–4.748)	***	(0.001)***

*SLE, systemic lupus erythematosus; HLA, human leukocyte antigen; OR, odds ratio; CI, confidence interval; * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, ns indicates no significance.*

27 patients experienced headache (90%), 12 patients had arthritis (40%), 12 patients had alopecia (40%), four patients developed vasculitis (13.3%), and only one patient had discoid rash (3.3%). Statistical analysis showed that SLE patients with LN had significant clinical presentations which include renal involvement, headache, and hypertension while SLE patients without LN showed significant clinical feature such as vasculitis disorders.

TABLE 3 | HLA-DRB1 allele carrier subtypes frequency in Malay SLE patients and Malay healthy controls.

HLA-DRB1	SLE (2*n* = 200)	Control (2*n* = 1,902)	OR CI (95%)	*P* Fisher	P_C
HLA- DR*0401	4 (0.02)	4 (0.002)	9.684 (2.403–39.023)	ns	ns
HLA- DR*0403	4 (0.02)	32(0.017)	1.192 (0.4174–3.407)	ns	ns
HLA- DR*0405	22 (0.11)	65 (0.034)	3.493 (2.103–5.801)	***	***(0.0003)
HLA- DR*1201	2 (0.01)	242 (0.127)	0.069 (0.017–0.281)	***	***(0.0002)
HLA- DR*1202	17 (0.085)	299 (0.157)	0.498 (0.299–0.831)	**	**(0.007)
HLA- DR*1401	1 (0.005)	23 (0.012)	0.411 (0.055–3.056)	ns	ns
HLA- DR*1404	2 (0.01)	56 (0.029)	0.333 (0.081–1.375)	ns	ns
HLA- DR*1501	19 (0.095)	125 (0.066)	1.492 (0.900–2.476)	ns	ns
HLA- DR*1502	52 (0.26)	345 (0.181)	1.586 (1.132–2.221)	*	*(0.014)
HLA- DR*1601	6 (0.03)	2 (0.001)	29.381 (5.8898–146.57)	ns	ns
HLA- DR*1602	15 (0.075)	72 (0.038)	2.061 (1.1579–3.668)	*	*(0.024)

*SLE, systemic lupus erythematosus; HLA, human leukocyte antigen; OR, odds ratio; CI, confidence interval; * indicates $p < 0.05$, **indicates $p < 0.01$, ***indicates $p < 0.001$, ns indicates no significance.*

HLA-DRB1 Genotyping and Allele Frequency in Malay SLE Patients

Thirteen major HLA-DRB1 alleles were identified in Malay SLE patients. The identified HLA-DRB1 alleles and their distribution are shown in **Table 2**. HLA-DRB1*15, HLA-DRB1*04, and HLA-DRβ1*16 were the most frequent and common alleles among all the HLA-DRB1 alleles identified in SLE patients with and without LN involvement, suggesting a significant role for these alleles in SLE disease. In contrast, the most frequent and common alleles identified in Malay healthy population were HLA-DRB1*15, HLA-DRB1*12, and HLA-DRB1*07 (data not shown). Our analysis also demonstrated the association of SLE disease with 11 different HLA-DRB1 alleles (**Table 3**). HLA-DRB1 alleles such as HLA-DRB1*0405 (OR: 3.493, 95% CI: 2.103–5.801, $p_c = 0.0003$), HLA-DRB1*1502 (OR = 1.586, 95% CI: 1.132–2.221, $p_c = 0.014$), and HLA-DRB1*1602 (OR = 2.061, 95% CI: 1.1579–3.668, $p < 0.05$, $p_c = 0.024$) were likely associated with the increased risk of developing SLE (with and without LN), while HLA-DRB1*1201 (OR: 0.069, 95% CI: 0.017–0.281, $p = 0.0002$) and HLA-DRB1*1202 (OR: 0.498, 95% CI: 0.299–0.831, $p = 0.007$) alleles were probably associated with a lower risk of developing SLE (with and without LN).

Association Between HLA-DRB1 Alleles With Clinical Manifestations and Disease Activity in SLE

Associations between HLA-DRB1 with clinical manifestations (based on the 11 ACR Criteria) and disease activity in SLE patients are illustrated in **Table 4**. HLA-DRB1*04 was positively associated with arthritis and renal manifestations. Additionally, HLA-DRB1*04 showed a significant and robust positive association with SLE disease activity (SLEDAI, $p = 0.01$) and ANA based on Phi and Cramer's *V* analysis. Also, HLA-DRB1*04 was significantly and positively associated with CRP level in the blood and total protein level in the urine ($p < 0.05$) (**Table 5**). Besides HLA-DRB1*04, HLA-DRβ1*15 was significantly associated with the increased risk of developing oral ulcer (OR: 5.036, 95% CI: 1.029–24.638, $p < 0.05$).

TABLE 4 | Association of the HLA-DRB1 allele with clinical manifestations and disease activity (SLEDAI) in SLE patients.

	OR (95% CI)	Chi-square Fisher	*p*-value	Phi and Cramer's *V*
HLA-DRB1*04				
Positive ANA	N/A	4.592	0.032*	0.214
Arthritis	2.870 (1.088–7.571)	4.736	0.030*	0.218
Renal	5.183 (1.896–14.169)	11.366	0.001*	0.337
SLEDAI	N/A	4.717	0.010*	0.217
HLA-DRB1*15				
Oral ulcer	5.036 (1.029–24.638)	4.697	0.030*	0.217

*SLE, systemic lupus erythematosus; HLA, human leukocyte antigen; ANA, antinuclear autoantibodies; OR, odds ratio; CI, confidence interval; * indicates p<0.05, N/A indicates data are not available.*

TABLE 5 | Association of HLA-DRB1*04 allele with laboratory investigations in Malay SLE patients.

	Independent samples *T*-test		Linear regression	
	Mean level in Allele +/- Subjects	*p*-value	Standardized Coefficient (*B*)	*p*-value
HLA- DRB1*04				
CRP (blood)	10.739/8.889	*0.031	0.232	*0.030
Total Protein (urine)	242.546/169.167	*0.022	0.320	*0.018

*SLE, systemic lupus erythematosus; HLA, human leukocyte antigen; CRP, C-reactive protein; *indicates $p < 0.05$.*

Correlation Analysis Between Cytokines and Free Radicals in SLE Carriers With HLA-DRB1*04 Allele and SLE Carriers Without HLA-DRB1*04 Allele and Healthy Controls

Figure 2 depicts the correlation between the mean levels of cytokines and free radicals in SLE carriers with HLA-DRB1*04 allele and SLE carriers without HLA-DRB1*04 allele and age- and sex-matched healthy controls. SLE patients with HLA-DRB1*04 allele were significantly correlated to the increased levels of

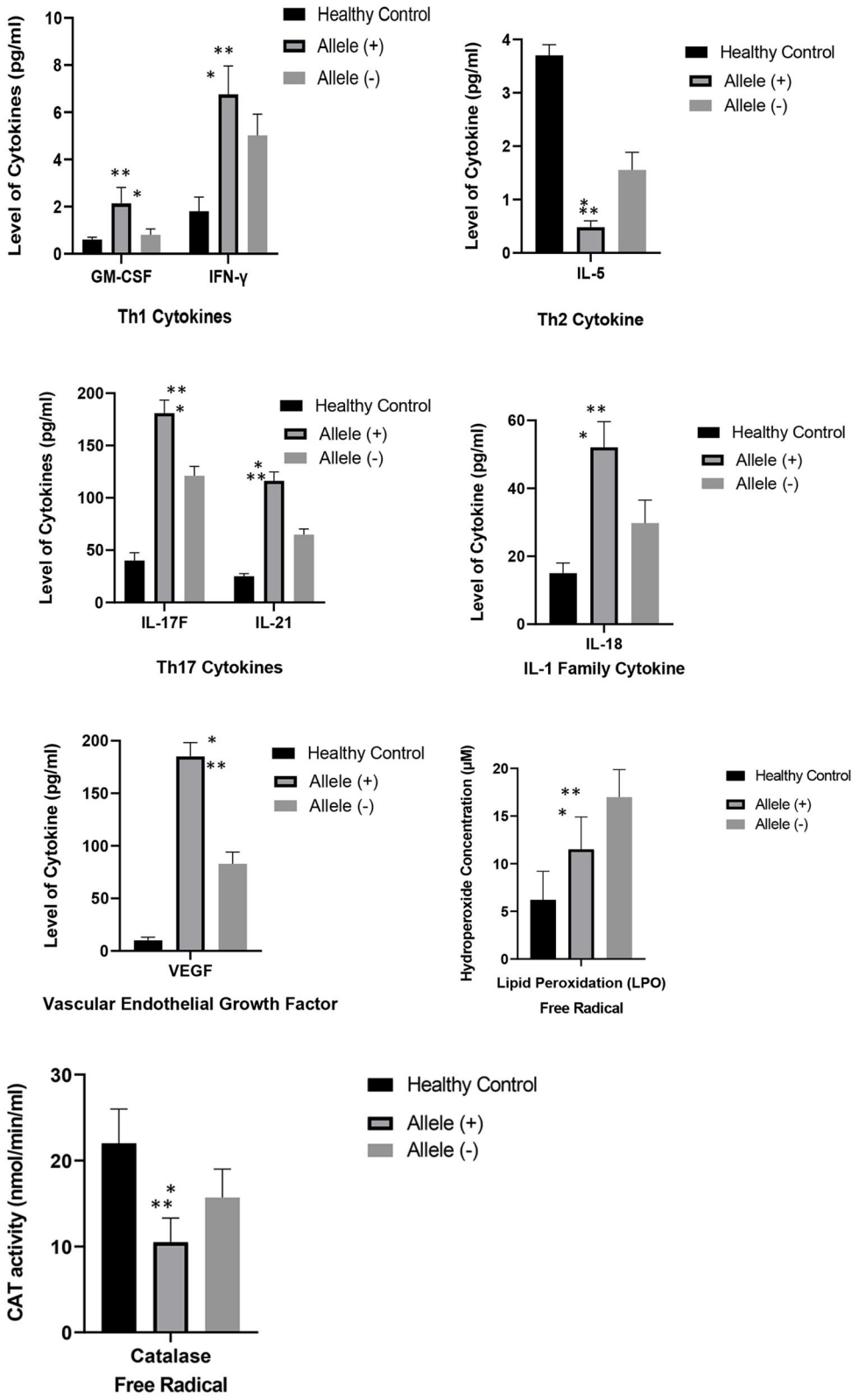

FIGURE 2 | Comparison of the mean levels of cytokines and free radicals between SLE carriers with HLA-DRB1*04 allele, SLE carriers without HLA-DRB1*04 allele and healthy controls with -age and sex-matched. Results were expressed as mean + SEM of cytokines and free radical levels in eight SLE carriers with HLA-DRB1*04

(Continued)

FIGURE 2 | allele, twenty SLE carriers without HLA-DRB1*04 allele and twenty-eight healthy controls. Keynotes: Allele (+): SLE carriers with HLA-DRB1*04; Allele (–): SLE carriers without HLA-DRB1*04. * denotes $p < 0.05$, significant differences between mean level of cytokines and free radicals between SLE carriers with and without HLA-DRB1*04 allele; ** denotes $p < 0.05$, significant differences between mean level of cytokines and free radicals between SLE carriers with HLA-DRB1*04 allele and healthy controls with -age and sex-matched.

cytokines such as IL-18, IL-21, IL-17F, IFN-γ, and GM-CSF and VEGF compared to SLE patients without HLA-DRB1*04 allele and healthy controls. Conversely, a reduction in the levels of IL-5, CAT, and LPO was significantly correlated to SLE patients with HLA-DRB1*04 allele in comparison to SLE patients without HLA-DRB1*04 allele.

DISCUSSION

The HLA complex located on the short arm of chromosome 6 (6p21.3), which encodes the MHC proteins in humans, comprises the most polymorphic gene cluster of the whole human genome and has critical roles in regulating the host immune system. Several lines of evidence showed that distinct genes of the HLA complex have significant roles in the modulation of host adaptive immunity (39). Perturbation in the presentation of antigenic peptides by HLA proteins to T cells results in the production of aberrant T-cell-mediated adaptive response, which is why different HLA genes could contribute to the pathogenesis of SLE (40). A plethora of studies have documented the association of gene polymorphisms in HLA-DRB1 allele with SLE and LN. The genetic risk toward the development of SLE and LN is further complicated by the presence of different HLA-DRB1 gene polymorphism profiles across various populations globally (18, 40–42). This warrants a call for an in-depth analysis on the association of HLA-DRB1 gene polymorphism in a specific population to determine the genetic influence in the SLE pathogenesis which could be used to explain the disparity in the clinical phenotypes presented in SLE patients. Therefore, this study was undertaken to investigate the association of HLA-DRB1 gene polymorphism in the development of SLE in Malaysian Malay SLE population. This study also attempts to identify the probable HLA-DRB1 risk alleles associated with LN development in SLE patients through association analysis encompassing clinical, laboratory, and biological factors.

HLA-DRB1 is one of the most critical susceptibility genes in SLE pathogenesis. A recently published meta-analysis that gathered all available case–control studies demonstrated that HLA-DR3 polymorphism is significantly associated with SLE in White populations while HLA-DR15 polymorphism is significantly linked with SLE in Eastern Asian populations. The analysis also reported that the frequencies of polymorphisms in the HLA-DRB1 gene in SLE patients are greatly varied across different ethnicities (43). In the context of the association between SLE and HLA-DRB1 polymorphism in Malay SLE population in Malaysia, our genotyping analysis of the HLA-DRB1 gene showed that HLA-DRB1*04, HLA-DRB1*12, HLA-DRB1*15, and HLA-DRB1*16 alleles were significantly associated with SLE development in all Malay female SLE patients (with and without LN involvement). Further analysis revealed that HLA-DRB1*0405, HLA-DRB1*1502, and HLA-DRB1*1602 could be susceptible alleles for SLE development while HLA-DRB1*1201 allele could be associated with a lower risk of SLE development. Compared to previous studies conducted in Malaysia, different HLA-DRB1 alleles associated with the risk of SLE development had been identified. For example, HLA-DR2 had been reported to be significantly associated with Malay SLE patients in Malaysia (30, 31). Indeed, HLA-DR2 has been steadily associated with SLE in both Asian and Caucasian populations (44, 45). Another study by Mohd-Yusuf et al. (32) documented that the HLA-DRB1*0701 allele could be a risk allele for SLE development, while HLA-DRB1*1201, HLA-DRB1*1202, HLA-DRB1*1203, and HLA-DRB1*1301-22 alleles might confer protection in Malay SLE patients, evident by the significant reduction in the allele frequencies of DRB1*1301-22 after Bonferroni correction (32). Collectively, studies on HLA-DRB1 gene polymorphism and SLE in different populations in Malaysia are limited, and the validity of the findings could be restricted by small sample sizes. Our findings in this study could enrich the repertoire of HLA-DRB1 alleles that are associated with SLE development in the Malay population in Malaysia. Factors such as sex disparity (46), race (47), and a larger homogenous population (43) should be considered to avoid gender bias, fluctuating effect-size estimates and genetic heterogeneity in determining the association between HLA gene polymorphisms and risk of SLE development.

LN is one of the most severe complications of SLE disease and is a crucial driver of mortality and morbidity in SLE. LN affects 40–70% of SLE patients, with the actual incidence depending on gender, age group, and ethnicity (48). In this single-cohort study, the mean age of SLE onset in SLE patients with LN was 29.60 years. The onset of SLE disease in SLE patients with LN was slightly earlier compared to SLE patients without LN (31.03 years) despite the changes being not significant (**Table 1**). Similarly, the major clinical presentation in both SLE patient groups (with and without LN) involved integument disorders (oral/nasal ulcers, malar, and photosensitivity). It has been reported that malar rash and photosensitivity are the most common clinical features in other Asian populations as well (49). One study had identified several risk factors predisposed to the development of LN and progression of renal disease to ESRD in SLE patients. These factors include disparity in ethnicity and age, presence of hypertensive condition, and homozygosity for the valine allele of FcγRIIIa (FCGR3A*GG) (50). Consistently, our study showed that SLE patients with LN were significantly presented with clinical features such as hypertension, renal disorders, and headache compared to SLE patients without LN. Hence, hypertension in SLE patients could be a probable risk factor in developing LN.

Early detection and prompt diagnosis are imperative since LN is the leading cause of morbidity and mortality in SLE. Delay in diagnosis of LN is a risk factor for the development of ESRD (51, 52). Factors that are associated with impaired renal function such as a rise in total protein concentration in urine and/or increase in serum creatine levels in the blood have been associated with LN and the occurrence of ESRD (53–55). ANA, which is a laboratory hallmark for SLE diagnosis, is also associated with LN (56). Low levels of complements C3 and C4 protein concentrations detected in SLE patients are also highly associated with LN and vasculitis in SLE patients (57). Increased serum levels of CRP in SLE patients are correlated with renal disease activity and increased risk for LN development (58). The decrement in complement levels could probably be due to an increase in immune complex deposition in the kidney, whereas an increase in CRP levels can be associated with increased levels of inflammation (59). Consistent with the aforementioned studies, our study showed that Malay SLE patients with LN had significantly higher levels of serum creatinine in the blood and total protein in urine and lower levels of complements C3 and C4 in comparison to SLE patients without LN. CRP levels in SLE patients with LN were also higher than in SLE patients without LN, although the increment was not significant.

The pathogenesis of LN is due to a loss of immune self-tolerance and subsequent polyclonal antibody activation characterized by full-house nephropathy (concurrent positive staining for IgM, IgA, IgG, C1q, and C3 by immunofluorescence) and positive ANA (60). The pathogenesis could be attributed by genetic variations in humans that encode immune-related functions. These gene variations could disrupt immune tolerance leading to generation of autoantibodies such as anti-dsDNA that might co-opt with genes that are involved in innate immune signaling to produce effector leukocytes and subsequent release of inflammatory cytokines and other autoantibodies that cause renal damage (61, 62). Some genes have been identified in the genesis of LN including HLA-DR, B lymphoid tyrosine kinase (BLK), signal transducer and activator of transcription 4 (STAT4), and toll-like receptor 9 (TLR9) (61, 63). Our association analysis demonstrated that the HLA-DRB1*04 allele in Malay SLE patients (with and without LN) was significantly associated with renal disorders and arthritis. However, previous studies showed that the HLA-DR2 allele is positively associated with renal involvement while HLA-DR8 is significantly linked to arthritis in Malay SLE patients (31). Additionally, our findings in this single cohort are also inconsistent with most of the published studies across different geographical regions. Majority of the studies documented that HLA-DRB1*15 is a risk allele (18, 64–67) while HLA-DRB1*04 is a protective allele for LN (67, 68). One source even claimed that none of the HLA-DRB1 alleles is associated with the risk of LN development among Taiwanese SLE patients (41). Also, a significant association between HLA-DR15-bearing haplotypes with LN in Saudi SLE patients is lacking (69). Although most of the studies relate HLA-DRB1*15 with LN, our study had shown that the HLA-DRB1*15 allele was significantly associated with oral ulcers in Malay SLE patients. The discrepancies in this study along with diverse association of different HLA-DRB1 alleles with LN could be contributed by genetic heterogeneity and ethnicity, leading to the complexity of different clinical manifestations in SLE patients (70). Consideration on employing genetic polymorphism in HLA-DRB1 gene as a predictor for LN in SLE remains open for debate. A genome-wide association study (GWAS) reported the association of LN with genes outside the MHC region that are more prominent than HLA-DR2 and HLA-DR3. The authors deduce that non-MHC factors may have more profound roles in promoting the development of LN and also that LN loci that influence the kidney response to the immunological aberration caused by SLE might possess higher risk to LN development (71).

The mechanistic aspects between HLA-DRB1 alleles and the risk of LN development remain elusive. Several studies illustrate an association between antibodies anti-Sm/RNP related to DR3 and antibodies against Ro to HLA-DR2 (72–74). Apart from this, Bastian et al. reported a strong association between anti-dsDNA or anti-RNP antibodies and the development of LN in patients already diagnosed with SLE in a European population (64). A comprehensive sequencing analysis of the whole MHC region of a large LN cohort showed that HLA-DRB1 amino acid 11 is one of the five functional risk variants for LN within MHC regions. These independent risk variants also suggest that the risk of development of LN could be due to aberration in peptide presentation by MHC class 1 and 2 molecules to T cells and sex hormone dysregulation (75). Another source presumed that three amino acid positions (11, 13, and 26) located at the HLA-DRB1 epitope-binding groove establish a pathogenic structure in LN patients (76).

Cytokines are small soluble mediators produced by different immune cell subsets, and tissues have shown an undisputable role in regulating the pathogenesis of SLE and severity of SLE disease. The pathogenesis of SLE lies on the imbalance between cytokines released by different T-helper cell subsets which results in immune dysregulation accompanied with elicitation of inflammatory responses and autoimmune abnormalities that cause severe tissue injuries and organ damages as seen in SLE patients (77–79). For example, overproduction of Th1- and Th17-related cytokines could promote T-cell hyperactivity and inflammation in SLE while an excess of Th2-related cytokines typically triggers B-cell hyperactivity and humoral responses (78). On the other side, free radicals and reactive oxygen species (ROS) such as superoxide dismutase (SOD), CAT, nitric oxide (NO), and LPO released by phagocytic cells during inflammation are expected to contribute to tissue injury and disease severity in SLE. The underlying mechanism could be due to deregulation of apoptosis, which leads to a delay in the clearance of apoptotic cells which stimulates the generation of autoantibodies leading to inflammation and severe organ damages (80).

The functional roles of cytokines and free radicals in SLE pathogenesis are well-elucidated. However, limited studies have associated the genetic risk of HLA-DRB1 gene polymorphism with cytokines in the development of SLE and LN. Jacob et al. (68) reported that the HLA-DRB1*04 allele confers protection against LN through high levels of TNF-α secretion (68). In this study, Malay SLE carriers with the HLA-DRB1*04 allele showed a significant association with the increasing levels of

TABLE 6 | Summary of the association of HLA-DRB1*04 allele with cytokines and free radicals (IFN-γ, GM-CSF, IL-5, IL-17F, IL-18, IL-21, VEGF, LPO, and CAT) in Malay SLE patients and the involvement of these cytokines and free radicals in the pathogenesis of SLE and LN based on the findings adapted from different previously published studies.

Association of cytokines and free radicals with HLA-DRB1*04 allele in Malaysian Malay SLE Cohort	**Involvement of cytokines and free radicals in SLE patients with and without lupus nephritis**				
	Th1 subset	**Th2 subset**	**Th17 subset**	**Other cytokines**	**Free radicals**
Significantly increased in SLE carriers with HLA-DRB1*04 allele - IFN-γ and GM-CSF (Th1 subset) - IL-17F and IL-21 (Th17 subset) - IL-18 and VEGF (other cytokines) Significantly reduced in SLE carriers with HLA-DRB1*04 allele - IL-5 (Th2 subset - LPO and CAT (Free radicals)	***IFN-γ*** - Higher level of IFN-γ is associated with SLE patients with severe nephritis and arthritis (81). - Different IFN-γ subtypes could contribute to heterogeneity in clinical manifestations in SLE (81). - Increasing level of IFN-γ is observed in SLE patients which could be potentially serve as a predictor biomarker in SLE diagnosis (82). - Increasing level of IFN-γ is observed in SLE patients that could be applied as a biomarker to assess renal activity (83). - Overexpression of IFN-γ is observed in paediatric SLE patients and IFN-γ is significantly correlated with renal manifestations and SLEDAI score (84). ***GM-CSF*** - GM-CSF induced by serum juvenile SLE significantly prevent the activation of neutrophil apoptosis and caspase (85). - Neutrophils isolated from SLE patients are more resistant towards apoptosis-inhibiting effects of GM-CSF (86). - GM-CSF secreting peripheral blood mononuclear monocytes (PBMCs) is significantly increased in SLE patients and is correlated with anti-dsDNA (87)	***IL-5*** - Increasing serum level of IL-5 is observed in SLE patients (88) - Absence of significant differences between patients with SLE-LN and SLE without LN (88). - Low level of IL-5 is observed in SLE patients and without significant difference between SLE patients and controls (89) - Overexpression of IL-5 is observed in SLE patients with skin lesions (90)	***IL-17*** - Increasing serum level of IL-17A in SLE patients with no significant differences is observed between patients with SLE-LN and SLE without LN (88). - Polymorphism of IL-17F rs763780 A/G is associated with susceptibility to SLE in Polish population (91). - IL-17F level is significantly higher in SLE patients which suggests the role of IL-17F in inflammation and angiogenesis (92). - Reduction in the expression of IL17F is observed in SLE T cells in which the reduction is independent of epigenetic pattern activation (93). - Increased in IL-17A/IL-17F ratio may intensify the pro-inflammatory phenotype of SLE (93). ***IL-21*** - The expression of IL-21 is increased in SLE patients. - IL-21 is positively correlated with SLEDAI score, C3 and erythrocyte sedimentation rate (ES) (94). - Lower serum level of IL-21 is detected in SLE patients compared to healthy controls along with the absence of significant difference in IL-21 level between SLE patients with and without LN.	***IL-18*** - IL-18 is significantly increased in active SLE patients and is correlated with SLEDAI score (95). - IL-18 could be a risk predictor of active renal SLE disease (95). - IL-18 showed higher sensitivity and specificity than C3 and anti-dsDNA in predicting active renal and active non-renal SLE cases (95). - Increasing serum level of IL-18 is observed in SLE patients (96). - IL-18 is potentially associated with active renal disease in SLE disease (96). - Higher serum levels of IL-18 is observed in lupus nephritis Class (III, IV and V) than lupus nephritis Class (I and II) (97) - IL-18 is significantly correlated with creatinine and activity index of renal biopsies (97). - IL-18 could inform the extent of renal injury and serve as a potential biomarker to distinguish between the histologic classes of subclinical lupus nephritis (97). - IL-18 level is significantly higher in SLE patients with LN than without LN (98). - IL-18 could be a potential biomarker to assess renal disease in SLE patients (98). - IL-18 is significantly associated with SLEDAI score, proteinuria, renal disease score and disease activity (98). ***VEGF*** - Increasing serum level of VEGF is observed in SLE patients with and without anti-phospholipid syndrome (99).	***LPO*** - Significant differences in the plasma LPO level (measured by the reaction of tiobarbituric acid-reactive substances (TBARS)) is observed between SLE patients with active nephritis, inactive nephritis and non-nephritis (100). - LPO could be one of the potential biomarkers in measuring disease activity of LN (100). - Elevation of LPO, by means of higher malondialdehyde (MDA) level is observed in SLE patients and is associated with disease activity, in particular alopecia and nephritis (101). - Significantly increased in LPO activity, indicated by increased in MDA level is observed in SLE patients (102). - LPO (MDA level) is significantly correlated with SLEDAI score, IFN-γ and IL-12 (102). - Robust positive correlation of MDA and IFN-γ with SLEDAI score suggests the involvement of LPO and pro-inflammatory cytokine in SLE pathogenesis (102). - LPO level, by means of higher MDA level is elevated in SLE patients and is associated with arterial and renal manifestations (103). ***CAT*** - No significant difference is observed in CAT levels between SLE patients with active nephritis, inactive nephritis and non-nephritis (100). - CAT level is significantly lower in SLE patients compared to healthy controls (104)

(Continued)

TABLE 6 | Continued

Association of cytokines and free radicals with HLA-DRB1*04 allele in Malaysian Malay SLE Cohort	Involvement of cytokines and free radicals in SLE patients with and without lupus nephritis				
	Th1 subset	**Th2 subset**	**Th17 subset**	**Other cytokines**	**Free radicals**
			- IL-21 is significantly associated with anemia (105). - Increasing level of IL-21 is observed in PBMCs of SLE patients (106). - IL-21 may synergise with TLR-9 signalling in the production of plasma B cells (106). - Expansion of circulating $CD4^+$ T-cells producing IL-21 is observed in SLE patients (107). - IL-21 produced by distinct cellular $CD4^+$ T-cell subsets are correlated with T and B cell subsets alterations in SLE (107).	- VEGF is significantly associated with SLEDAI score (99). - Higher level of VEGF expression is significantly associated with SLE patients with LN (108). - Increasing serum level of VEGF is observed in SLE patients with LN (109). - Significant higher level of VEGF is observed in active nephritis than quiescent nephritis (109). - Mild to moderate expression of VEGF is observed in paediatric cases of SLE with lupus nephritis (110).	- CAT level is significantly associated with Arabs, ages ≥40 and SLEDAI score <6 in SLE (104). - Increasing level of CAT is observed in SLE patients (111). - Increasing levels of IgG antibodies (Ab) against CAT is observed in SLE patients (111). - Primary cause of oxidative stress in SLE is due to excessive free radical production rather than impaired CAT activity (111).

cytokines including IFN-γ, GM-CSF, IL-17F, IL-18, IL-21, and VEGF and a significant negative association with IL-5, LPO, and CAT enzymes. We have performed a literature search and provide a brief summary on the cytokines and free radicals (IFN-γ, GM-CSF, IL-5, IL-17F, IL-18, IL-21, VEGF, LPO and CAT) that are significantly correlated with HLA-DRB1*04 allele in Malay SLE patients together with the evidence gathered from a number of previously published studies that depicted the involvement of these cytokines and free radicals in SLE and LN pathogenesis (**Table 6**). Among all the mediators that are significantly associated with HLA-DRB1*04 alelle, IFN-γ, IL-18, VEGF and LPO warrant further investigations as these immune mediators are robustly linked to the pathogenesis of LN in SLE patients (**Table 6**). Some studies even suggested that IL-18 (95, 97, 98), IFN-γ (81–83), LPO (100) and VEGF (108, 109) could serve as host biomarkers in assessing the renal disease activity and/or discerning between SLE patients with and without lupus nephritis.

Taken together, we surmise that the genetic risk of HLA-DRB1*04 for LN could be assessed through different serological manifestations including ANA, CRP, SLEDAI score, and increased proteinuria. We also speculate that intense inflammatory responses regulated by high levels of Th1 (IFN-γ and GM-CSF) and Th17 cytokines (IL-17F and IL-21) along with decreased levels of Th2 cytokine (IL-5) and free radicals (LPO and CAT) contribute to the development of LN in SLE. The expression of these cytokines is more prominent in SLE carriers with the HLA-DRB1*04 allele. Nevertheless, further studies are deemed necessary to unravel the actual mechanism inclusive of genetic, environmental, and biological determinants that drives the development of LN in SLE patients. This will eventually pave a path to uncover the genesis of LN and its pathogenesis and facilitate the identification of predictive biomarkers in the evaluation of disease activity and treatment intervention.

In conclusion, there are some limitations in our study. The findings from this study were solely based on 100 Malay female SLE patients with the HLA genotype, and the association analyses with clinical, serological, and laboratory manifestations were compared with the publicly available dataset. A larger SLE cohort considering sex and ethnicity is required to untangle the genetic basis of SLE and LN in a multiracial country like Malaysia. The environmental, sociodemographic, and epigenetic influences should be carefully assessed as well in SLE. Despite these limitations in our study, at least in the Malay SLE cohort in Malaysia, the HLA-DRB1*04 allele could be associated with susceptibility to SLE and LN. We agree that more biological studies are needed to validate and to confirm these associations and to explain discrepancies in different populations. Further, the significant correlation of HLA-DRB1*04 allele with specific cytokines and free radicals suggests that specific HLA molecules may significantly influence cytokine responses to antigenic stimulation and immune outcomes. These cytokines could serve as a panel of biosignature to assess the disease severity and biomarker in a specific population.

DATA AVAILABILITY STATEMENT

The data presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Research and Ethics Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM) and Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia (NMRR-14-1756-23234). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS carried out the whole SLE study and the analysis of the data. VKC analyzed data, wrote and reviewed the first draft and final version of the manuscript. SA-N and MAb were involved in the conception, funding acquisition, designation of the study, supervision, and analysis of the data and made substantial contributions to the analyses and review of the manuscript. MAr was involved in the supervision and analysis of the data. All authors read and approved the final version of the manuscript.

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REVIEW
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Interleukin-18 in Inflammatory Kidney Disease

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Interleukin (IL)-18, a member of the IL-1 superfamily, is a pro-inflammatory cytokine that is structurally similar to IL-1β. IL-18 promotes the production of interferon gamma (IFN-γ) and strongly induces a Th1 response. IL-18 drives the same myeloid differentiation factor 88 (MyD88)/nuclear factor kappa B (NF-κB) signaling pathway as IL-1β. In physiological conditions, IL-18 is regulated by the endogenous inhibitor IL-18 binding protein (IL-18BP), and the activity of IL-18 is balanced. It is reported that in several inflammatory diseases, the IL-18 activity is unbalanced, and IL-18 neutralization by IL-18BP is insufficient. IL-18 acts synergistically with IL-12 to induce the production of IFN-γ as a Th1 cytokine, and IL-18 acts alone to induce the production of Th2 cytokines such as IL-4 and IL-13. In addition, IL-18 alone enhances natural killer (NK) cell activity and FAS ligand expression. The biological and pathological roles of IL-18 have been studied in many diseases. Here we review the knowledge regarding IL-18 signaling and the role of IL-18 in inflammatory kidney diseases. Findings on renal injury in coronavirus disease 2019 (COVID-19) and its association with IL-18 will also be presented.

Keywords: IL-18, inflammatory kidney disease, inflammation, IL-1, COVID-19

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INTRODUCTION

Inflammation is a defense mechanism that is caused by harmful stimuli and conditions such as infection and tissue injury (1). Innate immunity is the host's first line of defense against pathogens and is activated by pattern recognition receptors (PRRs). PRRs recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) that are common to pathogens. There are several classes of PRRs, including Toll-like receptors (TLRs), C-type lectin receptors, nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene-I-like receptors (RLRs), and absent in melanoma 2 (AIM2)-like receptors. The inflammasome, a multiprotein complex formed intracellularly in response to PAMPs and DAMPs, converts procaspase-1 to active caspase-1 and induces pro-inflammatory cytokines such as interleukin 1beta (IL-1β) and IL-18 (2).

The NLR family member leucine rich repeat and pyrin domain containing 3 (NLRP3) forms the NLRP3 inflammasome together with the adapter molecules apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and procaspase-1 and activates caspase-1 to processes IL-1β and IL-18 to the bioactive mature form (3, 4). The NLRP3 inflammasome has been implicated in the pathogenesis of many diseases, including microbial pathogens, inflammatory diseases, cancer, and metabolic and autoimmune disorders (3, 4), and it has also been implicated in various kidney diseases (5). A member of the IL-1 superfamily, IL-18 is a pro-inflammatory cytokine that is structurally similar to IL-1β (6, 7). IL-18 promotes the production of interferon gamma (IFN-γ) and strongly induces a Th1 response (8).

In recent years, the biological and pathological roles of IL-18 have been studied in many diseases. Inflammation underlies the pathogenesis of many acute or chronic kidney diseases, and IL-18 plays an important role. This paper focuses on the roles of IL-18 in inflammatory kidney diseases. We review the current knowledge regarding IL-18 signaling, and we outline the existing evidence about the roles of IL-18 in inflammatory kidney diseases. We are in the midst of an epidemic of coronavirus disease 2019 (COVID-19). Findings on renal injury in cases of COVID-19 and its association with IL-18 will also be presented.

THE PRODUCTION AND PROCESSING OF IL-18

IL-18 was initially identified as IFN-γ-inducing factor (IGIF) in sera from *Propionibacterium acnes*-primed and lipopolysaccharide (LPS)-challenged mice (9–11). Although IL-18 and IL-1β share only about 17% sequence homology, they have a common β-pleated sheet structure (12, 13). IL-18 is produced by macrophages, dendritic cells, epithelial cells, keratinocytes, chondrocytes, osteoblasts, synovial fibroblasts, and adrenal cortex cells, and it plays an important role in inflammatory pathology (7, 13, 14). In the kidney, the predominant source of IL-18 production is tubular epithelial cells (15–17). IL-18 gene expression may be enhanced by stimulation with microbe products such as LPS and by cytokines such as IFN-α/β/γ and TNF-α (13, 18, 19). IL-18 is stored intracellularly as a biologically inactive 24-kDa precursor (pro-IL-18), similar to IL-1β, and is secreted extracellularly as the 18-kDa bioactive mature molecule after being cleaved by caspase-1. Nitric oxide suppresses the secretion of IL-1β and IL-18 by inhibiting caspase-1 (20). An inhibitor of mammalian target of rapamycin (mTOR), rapamycin is widely used as an autophagy inducer (21). The induction of autophagy by rapamycin can suppress the production and secretion of IL-1β and IL-18 and limit excessive inflammation (21).

IL-18 RECEPTOR AND SIGNAL TRANSDUCTION

The IL-18 signaling pathway is illustrated in **Figure 1**. IL-18 recognizes a heterodimeric receptor that consists of IL-18 receptor (R) α- and β-chains (22). IL-18Rα, also known as IL-1 receptor-related protein (IL-1Rrp), binds specifically to the extracellular IL-18 at the cell surface. However, its affinity is low. IL-18Rβ (i.e., accessory protein-like [AcPL]) is recruited to form a high affinity binding and activate intracellular signaling pathway (23, 24). IL-18R is expressed in most types of cells, including T cells, natural killer (NK) cells, macrophages, dendritic cells, neutrophils, basophils, mast cells, endothelial cells, and smooth muscle cells (25–32). The diversity of productive and receptor-expressing cells is linked to the functional diversity of IL-18.

Like IL-1R, IL-18R contains a Toll/IL-1 receptor (TIR) domain in the intracellular region that is shared with TLRs, and signaling into the cell is mediated by myeloid differentiation factor 88 (MyD88) (33–35). MyD88 is a well-known adaptor molecule for TLRs and IL-1R. The activation of IL-18R results in the recruitment of MyD88 to the TIR and anchors IL-1 receptor-associated kinase (IRAK) (36). Phosphorylated IRAK dissociates from the complex and binds to tumor necrosis factor receptor-associated factor 6 (TRAF6), which in turn phosphorylates nuclear factor kappa B (NF-κB)-induced kinase (NIK) (37). This results in the activation of I kappa B (IκB) kinase (IKK). The phosphorylation of IκB by IKK leads to the ubiquitination and degradation of IκB (38). NF-κB is then able to migrate into the nucleus and initiate the transcription of target genes such as IFN-γ (39).

Although the major signaling pathway of IL-18 is NF-κB signaling, it has been reported that stimulation by IL-18 strongly promotes the tyrosine phosphorylation of STAT3 and the mitogen-activated protein kinases (MAPKs) p44erk-1 and p42erk-2 in human NK cell lines (40). In murine T cells, IL-18 induced the activation of the lymphocyte-specific tyrosine protein kinase p56lck and p42 MAPK (41).

IL-18 BINDING PROTEIN

As with IL-1, the activity of IL-18 is regulated by the endogenous inhibitor IL-18 binding protein (IL-18BP). IL-18BPa, the major splice variant of IL-18BP, is present in excess concentrations compared to IL-18 in the serum of healthy individuals and it binds with high affinity to IL-18 to neutralize its activity (42–44). IL-18BP inhibits the binding of IL-18 to the IL-18 receptor and inhibits the production of IFN-γ (**Figure 1**). IFN-γ has been reported to mediate the gene expression of IL-18BPa in non-leukocytic cells (45). IL-18 activity is regulated by a negative feedback mechanism mediated by IL-18BPa induced by IFN-γ. It is thus likely that IL-18BPa functions as a "shut off" signal to stop the excessive inflammatory response by IL-18 (44). The expression of IL-18BP is regulated mainly at the transcriptional level, and signal transducer and activator of transcription 1 (STAT1) and CCAAT/enhancer binding protein β (C/EBPβ) have been reported to be important transcription factors in the regulation of IL-18BP gene promoter activity (46, 47).

The activity of IL-18 is balanced by the presence of IL-18BP. Serum IL-18BP levels are significantly elevated in sepsis and other inflammatory diseases (42–44, 48, 49). Patients with granulomatosis with polyangiitis (i.e., Wegener's granulomatosis) and those with systemic lupus erythematosus showed elevated serum levels of IL-18 as well as IL-18BP, but the levels of IL-18BP were not sufficient to neutralize IL-18, and the levels of free IL-18 were higher than those of healthy subjects (42, 48).

Exogenous IL-18BP may be useful as a novel therapeutic agent for diseases involving IL-18 (42–44, 48, 49). A phase II clinical trial was conducted in patients with adult-onset Still's disease (50); the administration of tadekinig, a recombinant IL-18BP, was observed to reduce the patients' serum C-reactive protein and ferritin levels and improve their clinical symptoms. A Phase III clinical trial of tadekinig is currently underway in patients suffering from pediatric monogenic auto-inflammatory diseases and harboring deleterious mutations of NLRC4 and XIAP (NCT03512314).

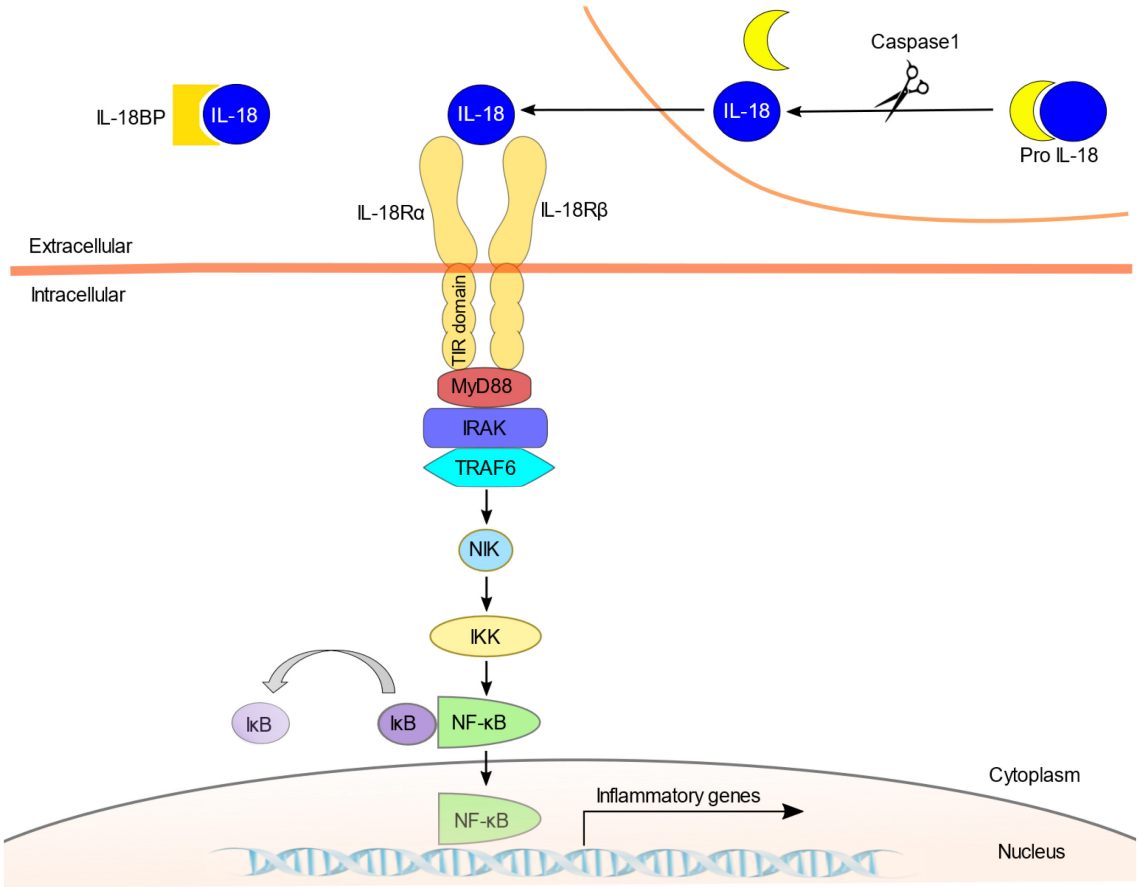

FIGURE 1 | The IL-18 signaling pathway. IL-18 is stored intracellularly as biologically inactive pro-IL-18 and is secreted extracellularly as the bioactive mature molecule after being cleaved by caspase-1. IL-18 is regulated by the endogenous inhibitor IL-18BP. Since IL-18BP has high affinity for IL-18, IL-18BP binds preferentially to IL-18 and inhibits binding to the IL-18R. IL-18 first binds to the IL-18Rα; however, this binding is low-affinity, and the IL-18Rβ chain is recruited to form a high-affinity heterodimeric complex. The activation of IL-18R recruits MyD88 to the TIR domain and anchors IRAK. Phosphorylated IRAK activates TRAF6, and which in turn phosphorylates NIK. This is followed by the activation of IKK and finally NF-κB, which initiates the transcription of target genes such as IFN-γ.

PHYSIOLOGICAL FUNCTIONS OF IL-18

IL-18 was originally discovered as a factor that induces IFN-γ from Th1 cells. The most important role of IL-18 in the immune system is the induction of the production of IFN-γ by Th1 cells. IL-18 acts synergistically with IL-12 to induce a potent Th1 response (51–53), and IL-18 plays an important role in the host's defense mechanism against infections caused by pathogens such as bacteria, viruses, fungi, and protozoa (13). In concert with IL-12, IL-18 also induces the production of IFN-γ by NK cells, B cells, and macrophages (51, 54, 55). Although IL-18 induces IFN-γ production as a Th1 cytokine by co-stimulation with IL-12, IL-18 acts alone as a Th2 cytokine (13). Basophils and mast cells derived from bone marrow cells cultured with IL-3 for 10 days expressed IL-18Rα (56). IL-3 is involved in the differentiation of mouse bone marrow cells into basophils and mast cells (57). Basophils produced both of the Th2 cytokines IL-4 and IL-13 in response to stimulation with IL-3 + IL-18 (56). Although mast cells did not produce IL-4, they produced IL-13 in response to stimulation with IL-3 + IL-18 (56). The administration of IL-18 together with IL-12 inhibits both the production of IgE and the productions of IL-4 and IL-13 by basophils and mast cells in an IFN-γ-dependent manner (58). On the other hand, the administration of IL-18 alone has been reported to induce IgE production by B cells (59). IL-4 and IL-13 are involved in the production of IgE and the differentiation and proliferation of eosinophils and are important in the formation of allergic pathologies such as bronchial asthma and atopic dermatitis (60). These findings suggest that IL-18 may be involved in allergic inflammation.

In addition, IL-18 has been reported to up-regulate Fas ligand (FasL) expression in NK cells and induce apoptosis in Fas-positive target cells (61, 62). In NK cells, IL-18 also enhances perforin-mediated cytotoxic activity (32). The activation of NK cells suggested that IL-18 may be associated with tumor immune responses (13, 63). It has been reported that treatment with IL-18 in combination with the B7-1 costimulatory molecule resulted in the regression of melanoma with increased NK

TABLE 1 | IL-18 in renal disease models.

Disease Model	Intervention	Outcome	References
AKI Model			
IRI	IL-18 BP	Protective	(68)
IRI	IL-18-deficient	Protective	(69)
LPS	IL-18Rα-deficient	Protective	(70)
Cisplatin	Anti-IL-18 antibodies	Not protective	(71)
Cisplatin	Overexpression of IL-18BP	Not protective	(71)
Cisplatin	IL-18-deficient	Protective	(72)
Cisplatin	IL-18Rα-deficient	Detrimental	(73)
CKD Model			
Anti-GBM GN	IL-18-deficient	Protective	(74)
Immune complex GN	IL-18Rα-deficient	Protective	(75)
LN (MRL/lpr)	IL-18	Detrimental	(76)
LN (MRL/lpr)	IL-18Rα-deficient	Protective	(77)
LN (MRL/lpr)	IL-18-deficient	Protective	(78)
LN (MRL/lpr)	Anti-IL-18 autoantibodies (IL-18 vaccination)	Protective	(79)
UUO	Overexpression of IL-18BP	Protective	(80)
UUO	IL-18Rα-deficient	Protective	(81)

IL, interleukin; IRI, ischemia-reperfusion injury; BP, binding protein; LPS, lipopolysaccharide; GBM, glomerular basement membrane; GN, glomerulonephritis; LN, lupus nephritis; UUO, unilateral ureteral obstruction.

cell infiltration at the tumor tissue (64). In addition to the above-described activities, IL-18 induces the production of granulocyte/macrophage colony-stimulating factor (GM-CSF) and the expression of adhesion molecules. In co-culture with osteoblasts and hematopoietic cells, IL-18 inhibited the formation of osteoclastlike cells *via* the production of GM-CSF (65). IL-18 enhanced the expression of intercellular adhesion molecule-1 (ICAM-1) in human myelomonocytic cell lines (66), and ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells and rheumatoid arthritis synovial fibroblasts (67). As described above, IL-18 has various pro-inflammatory effects besides the induction of IFN-γ production, and IL-18 may be associated with various pathologies such as infections, allergic diseases, and tumor immunity.

IL-18 AND INFLAMMATORY KIDNEY DISEASE

Inflammation underlies the pathogenesis of many renal diseases, including acute kidney injury (AKI) and chronic kidney disease (CKD), and the role of IL-18 in inflammation has been reported in many experimental animal models (summarized in **Table 1**). In clinical practice, IL-18 is expected to be useful in the diagnosis of diseases and the estimation of disease severity and prognosis.

IL-18 as a Biomarker for AKI

Urinary IL-18 has been reported to be increased in patients with acute tubular necrosis after kidney transplantation (82, 83). The urinary level of IL-18 is expected to be an early diagnostic marker of acute kidney injury (AKI), and many clinical trials have been conducted. A meta-analysis summarizing reports after cardiac surgery showed that the sensitivity and specificity values of urinary IL-18 as a biomarker for the diagnosis of AKI were 0.58 and 0.75, respectively (84). The area under the receiver operating characteristic curve (AUROC) of urinary IL-18 levels predictive of AKI was 0.70 (84). In another meta-analysis, the AUROC was similar at 0.77 (85). Urinary IL-18 is a biomarker of AKI with moderate diagnostic value. Although it does not reliably predict the development of AKI, urinary IL-18 has been reported to be useful to predict clinical outcomes including mortality and dialysis in a heterogeneous intensive care unit (ICU) population (86).

It has been reported that urinary IL-18 levels in patients with AKI after cardiopulmonary bypass increased over the first 4–6 h, peaked in 12 h, and remained elevated up to 48 h after surgery (87, 88). This elevation of urinary IL-18 in AKI is slower than that observed in urinary neutrophil gelatinase-associated lipocalin (87, 88). The urinary IL-18 level in patients with acute tubular necrosis has been shown to be significantly elevated compared to patients with urinary tract infections, pre-renal acute renal failure, chronic kidney disease, and nephrotic syndrome (82). However, because urinary IL-18 is also elevated in septic patients (86, 89), caution should be exercised when using the urinary IL-18 level for the diagnosis of AKI.

Ischemic Renal Disease

Ischemia-reperfusion injury (IRI) in the kidney is used as a model of AKI. An AKI caused as a result of IRI involves both innate and acquired immune responses (90). In an IRI mouse model, the plasma and renal IL-18 levels were shown to be significantly increased after IRI stress (68, 69). Compared to wild-type mice, IL-18-deficient mice were protected from IRI and showed better renal function, less tubular damage, less neutrophil and macrophage infiltration, and less expression of downstream inflammatory mediators of IL-18 (69). In a mouse model of IRI, treatment with IL-18BP, an IL-18 inhibitor, showed a renal-protective effect (69). Treatment with IL-18 BP has also been shown to reduce the levels of profibrotic molecules in the kidneys of mice after IRI and to inhibit the progression of IRI-induced renal fibrosis (68). Because caspase-1 activates IL-18, caspase-1-deficient mice are also protected against ischemic acute renal failure (15). Although IL-18 is produced by various types of cells, it has been reported that cells of bone marrow origin play a more important role than intrinsic kidney cells in the renal damage caused by IRI (69). Blocking IL-18 signaling may be protective against IRI-induced AKI.

LPS-Induced AKI

We reported the role of IL-18 in LPS-induced AKI in IL-18Rα-deficient mice (70). In CD4+ T cells derived from splenocytes, the mRNA expressions of IL-18 and IL-18Rα were significantly increased after LPS injection. The IL-18Rα-deficient mice showed lower blood urea nitrogen (BUN) levels, a higher survival rate, and reduced levels of pro-inflammatory cytokines such as IL-18 and IFN-γ compared to wild-type mice. Glomerular CD4+ T cells and interstitial macrophage infiltration were reduced in the kidneys of the IL-18Rα-deficient mice. IL-18R-mediated

signaling pathways may plays critical roles in these cells in the pathogenesis of LPS-induced AKI.

Cisplatin-Induced AKI

In vitro, cisplatin induces the apoptosis or necrosis of renal tubules (91). Cisplatin administration increases serum and renal levels of IL-18 (71). However, methods to inhibit IL-18 using IL-18 antiserum or transgenic mice that overproduce IL-18BP did not protect against cisplatin-induced AKI (71). On the other hand, IL-18-deficient mice have been reported to be protected from AKI, and the exogenous supplementation of recombinant IL-18 prior to cisplatin administration caused AKI (72). In our study using IL-18Rα-deficient mice, the inhibition of IL-18 signaling did not result in a favorable effect. We observed that compared to wild-type mice, the IL-18Rα-deficient mice had worse renal function and downregulated expressions of suppressor of cytokine signaling (SOCS) 1 and SOCS3 in the spleen and kidney (73). The inhibition of cytokine signaling by the members of the SOCS family constitutes a major negative feedback mechanism to prevent runaway inflammation. SOCS1 reduces the impact of cytokines by inhibiting JAK kinases and several other mechanisms (92). Although the mechanism is not clear, we speculate that IL-18Rα may induce an anti-inflammatory response by affecting the expressions of the cytokine signaling inhibitors SOCS1 and SOCS3 in addition to the inflammatory response. In summary, the inhibition of IL-18 may not be sufficient for the prevention of cisplatin-induced AKI. The effect of IL-18 on cisplatin-induced AKI appears to vary between mouse models, and further research is needed.

Glomerulonephritis

Neutrophils play an important role in the pathogenesis of antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV). Neutrophils require priming for subsequent ANCA-induced activation, and IL-18 is thought to be important for neutrophil priming in AAV, as is tumor necrosis factor-alpha (TNF-α) (93, 94). *In vitro*, IL-18 can prime neutrophils, and it enhances superoxide production by cells after ANCA binding (93, 94). AAV patients have higher serum IL-18 concentrations compared to healthy controls (95). In renal biopsies from AAV patients, IL-18-positive cells were found in podocytes in the glomerulus and in myofibroblasts, distal tubular epithelial cells, and infiltrating macrophages in the interstitium (94).

In patients with IgA nephropathy, serum IL-18 levels have been reported to correlate significantly with urinary protein excretion, serum creatinine, and the estimated glomerular filtration rate (eGFR) (96). In addition, patients with high IL-18 levels at baseline were shown to have worse renal function during the follow-up period (96). The serum IL-18 level may predict the reduction of renal function in patients with IgA nephropathy. In renal biopsies of IgA nephropathy patients, the expression levels of IL-18 were positively correlated with both the infiltration of inflammatory cells into the interstitium and the extent of proteinuria (74).

A role of IL-18 has been reported in several experimental animal models of glomerulonephritis. In a model of anti-glomerular basement membrane nephritis in mice, IL-18-deficient mice had reduced leukocyte infiltration in the glomeruli and interstitium (97). Based on our findings obtained with a bovine serum albumin glomerulonephritis mouse model, we reported that IL-18Rα-deficient mice showed a significant reduction of proteinuria, renal pathological findings including glomerular IgG and C3 deposits, and leukocyte infiltrates compared to control mice (75). Thus, in experiments with several animal models, the suppression of IL-18 signaling has been shown to be protective against glomerulonephritis.

Lupus Nephritis

Lupus nephritis (LN) is a frequent and severe organ lesion associated with systemic lupus erythematosus (SLE) (98). IL-18 has been implicated in the pathogenesis of SLE, based on studies in mice and humans. MRL/lpr mice, which develop spontaneous lupus-like autoimmune disease, had higher levels of serum IL-18 compared to controls (76), and the mice treated with IL-18 developed accelerated proteinuria, glomerulonephritis and vasculitis (76). We reported that IL-18Rα-deficient MRL/lpr mice survived longer than IL-18α-intact MRL/lpr mice, and we observed significant reductions in glomerular IgG deposition, proteinuria, and serum anti-DNA antibodies in the IL-18Rα-deficient MRL/lpr mice (77). Similarly, some other groups have reported improved survival and proteinuria in IL-18-deficient and IL-18-vaccinated MRL/lpr mice (78, 79).

High serum IL-18 levels have been reported in patients with SLE (99, 100). Patients with active renal disease also have higher serum levels of IL-18 than those without renal activity (99, 100). It was reported that in the serum of LN patients, not only IL-18 but also IL-18BP, which neutralizes the activity of IL-18, are significantly increased, but the IL-18/IL-18BP ratio is also increased (101). This imbalance between IL-18 and IL-18BP may be involved in the pathogenesis of LN (101).

Serum IL-18 levels correlate with the disease activity of SLE (99, 100, 102) and are also associated with the severity of LN (100, 103, 104). Several studies indicated that the IL-18 value in the serum or glomeruli of patients with class IV LN was increased compared to the LN class III and V patients. Thus, IL-18 may be useful for the identification of LN in SLE patients and for estimating the severity of LN.

Diabetic Nephropathy

Inflammatory cytokines play an important role in the development and progression of diabetic nephropathy (105). In clinical studies, elevated plasma and urinary IL-18 levels were associated with diabetic nephropathy, and IL-18 was observed to be a predictive marker for the development of diabetic nephropathy in diabetic patients and to be associated with the progression of renal dysfunction (106–108). Serum and urinary IL-18 levels correlate with the degree of urinary albumin excretion (107, 108). Elevated serum and urinary IL-18 levels in diabetic patients may be a risk factor for the development of diabetic nephropathy. In kidney tissue of diabetic nephropathy patients, IL-18 is overexpressed in tubular epithelial cells, which

may occur *via* the activation of the MAPK pathways induced by transforming growth factor-beta (TGF-β) (109). Treatment that blocks IL-18 signaling may be a new approach in the treatment of diabetic nephropathy.

Obstructive Nephropathy

Renal interstitial fibrosis is a common and important lesion in the process of various progressive renal diseases that progress to renal atrophy. Unilateral ureteral obstruction (UUO) is an important model for studying the mechanisms of renal fibrosis and evaluating the potential therapeutic approaches (110). In UUO model mice, it was reported that serum IL-18 levels were elevated and the renal IL-18 and IL-18R expressions were enhanced after a UUO operation (16, 17, 80, 81). We reported that compared to wild-type mice, IL-18Rα-deficient mice had reduced tubular cell apoptosis and suppressed renal interstitial fibrosis after UUO (81). Similarly, transgenic mice with neutralized IL-18 activity also show reduced fibrosis (80).

In general, TGF-β is a mediator that plays a central role in renal fibrosis (111). Interestingly, in our previous study, there was no significant difference in the expression of renal TGF-β between IL-18Rα-deficient and wild-type mice (81). IL-18 may be involved in renal interstitial fibrosis by a mechanism that is independent of TGF-β (80, 81). *In vitro*, FasL expression in human proximal tubular cells has been reported to be enhanced by IL-18 exposure, and IL-18 may stimulate proapoptotic signaling through a FasL-dependent mechanism and affect obstructive nephropathy (112). In addition, TLR4 signaling may affect IL-18-mediated profibrotic effects (113, 114). Experiments using these mouse models suggested that (i) IL-18 signaling plays an important role in renal interstitial fibrosis during renal obstruction, and (ii) the inhibition of IL-18 acts protectively against fibrosis.

COVID-19

COVID-19, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and first detected in the city of Wuhan in China's Hubei Province, has become a global pandemic. In response to SARS-CoV-2 infection, the human body produces pro-inflammatory cytokines such as IL-1β, IL-6, IL-7, IL-8, TNF-α, granulocyte-colony stimulating factor (G-CSF), interferon gamma-induced protein 10 (IP-10), monocyte chemotactic protein (MCP)-1, and MCP-3 (115–117). COVID-19 produces an excessive inflammation "cytokine storm" that leads to acute respiratory distress syndrome (ARDS) and the failure of multiple organs including kidney.

The incidence of AKI in COVID-19 patients is higher than that in non-COVID-19 patients, and AKI associated with COVID-19 has been shown to be independently associated with an almost 4-fold higher odds of death than AKI associated with other acute illnesses (118). However, in general, critically ill patients with ARDS and AKI have many complications that can induce acute tubulointerstitial injury, and the causal relationship between coronavirus infection and AKI remains unclear. Factors that may contribute to the development of AKI in COVID-19 patients include direct viral infections, the cytokine storm, drug treatments, hemodynamic instability, and advancing hypercoagulable state. In a renal histological analysis of samples from autopsies of 26 patients who died of COVID-19-induced respiratory failure, clinical signs of renal injury, including increased serum creatinine and/or new-onset proteinuria, were observed in only nine of the 26 patients (34.6%), while mild to severe acute tubular injury was observed in all 26 patients (119). Three patients had pigmented tubular casts, three had segmental glomerular fibrin thrombi and two had focal segmental glomerulosclerosis. In seven patients, electron microscopy showed coronavirus-like particles in the tubular epithelium and podocytes. The SARS-CoV-2 virus uses angiotensin-converting enzyme 2 (ACE2) as a receptor for host cell entry. In the kidney, ACE2 is predominantly expressed in proximal tubules and is also present in podocytes and endothelial and smooth muscle cells of vessels (120). The finding that SARS-CoV-2 infects these cells (119) may indicate that the virus causes renal injury directly. However, it has been reported that multivesicular bodies (MVBs) mimicking SARS-CoV-2 are found in podocytes of COVID-19-negative patients, and it has not yet been established whether SARS-CoV-2 truly causes direct kidney injury (121).

Many cytokines are involved in the pathogenesis of COVID-19, and IL-18 may also be relevant (122, 123). Serum IL-18 levels have been shown to correlate with serum IL-6 levels, with inflammatory markers such as C-reactive protein and ferritin, and with markers of organ injury such as creatinine, liver enzymes, and troponin (122). It has also been reported that serum IL-18 levels on admission are higher in COVID-19 patients requiring mechanical ventilation and lethal cases (123). IL-18 may be related to the severity of COVID-19. The appropriate control of pro-inflammatory cytokines, including IL-18, may be a therapeutic option for managing the complications caused by the cytokine storm in COVID-19. There are currently no clinical trials examining IL-18 signaling. On the other hand, the effect of the humanized anti-IL-6 receptor antibody tocilizumab on COVID-19 has been reported. Several open-label trials and non-randomized case series reported positive effects of tocilizumab on COVID-19 (124); however, phase III clinical trials did not show efficacy of tocilizumab for preventing intubation or death in moderately ill hospitalized patients with COVID-19 (125). Although the regulation of IL-18 signaling may be a potential therapeutic target for COVID-19, the suppression of IL-18 signaling alone may not be sufficient to control the disease, as many cytokines are involved in the severity of COVID-19.

CONCLUSION

IL-18 belongs to the IL-1 superfamily and drives the same MyD88/NF-κB signaling pathway as IL-1β. IL-18 is a pro-inflammatory cytokine that induces IFN-γ production and has a variety of other functions, including the enhancement of NK cell activity and up-regulation of FasL expression. IL-18 appears to regulate inflammation at multiple checkpoints. Pre-clinical and clinical studies have obtained interesting results in many circumstances in which IL-18 is associated with an increased inflammatory infiltrate and more severe kidney lesions.

These results suggest that IL-18 may play an important role in the pathology of inflammatory kidney diseases, and they raise expectations that IL-18 may be a potential therapeutic target. However, there is a lack of clinical studies targeting IL-18 in inflammatory renal disease. In addition, the role and signaling of IL-18 in inflammatory kidney disease are not fully understood. It remains unknown whether IL-18 is clearly implicated in disease pathogeneses. In experimental animal models, IL-18-deficiency, anti-IL-18 antibodies, IL-18R-deficiency, and IL-18BP all regulate IL-18 signaling, and in many cases their effects are protective for the kidneys. Some conflicting results suggest that their respective signaling pathways, effects on cytokines, etc. may not be identical. Studies that will further elucidate IL-18 signaling are important for understanding the pathogenesis of inflammatory kidney disease and for therapeutic applications.

AUTHOR CONTRIBUTIONS

YH drafted the manuscript. YN edited the manuscript. Both authors contributed to the manuscript's revision and have read and approved the submitted version.

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Novel Predictors and Risk Score of Treatment Failure in Peritoneal Dialysis-Related Peritonitis

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Objective: Peritonitis is a severe complication in peritoneal dialysis (PD). This study was performed to identify predictors and establish a risk score for treatment failure in peritonitis patients.

Methods: A single-center, retrospective observational study was conducted. The basic demographic characteristics, clinical and laboratory data of all patients with peritonitis during the study period were documented and analyzed. Multivariate logistic regression was applied to examine independent predictors of treatment failure, and a risk prediction score was established.

Results: Three hundred fourteen episodes experienced by 241 patients were included in the final analysis. Logistic regression analysis indicated that PD duration (OR 1.017; P 0.005), fibrinogen (OR 1.327; P 0.021), high-density lipoprotein (OR 0.443; P 0.032), fungal infection (OR 63.413; $P < 0.001$), intestinal obstruction (OR 5.186, P 0.007), and diabetes mellitus (OR 2.451; P 0.018), hemodialysis history (OR 2.804, P 0.006) were independent predictors of treatment failure. The risk prediction score system showed a good calibration ($P > 0.05$) and discrimination (AUROC 0.80, $P < 0.001$).

Conclusions: Fibrinogen, PD duration, fungal infection, hemodialysis history, concurrent intestinal obstruction, or diabetes mellitus were independent risk factors for a poor peritonitis outcome, while the high-density lipoprotein was a protective factor. This novel risk prediction score system may be used to predict a high risk of treatment failure effectively.

Keywords: predictors, treatment failure, catheter removal, risk score, peritoneal dialysis associated peritonitis

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INTRODUCTION

Peritoneal dialysis (PD) is one of the main renal replacement treatments for patients with end-stage renal diseases (ESRD), accounting for about 11% of global dialysis patients (1). It provides a similar or better survival outcome vs, hemodialysis (HD) (2) and is more cost-effective. However, this therapy utilization has decreased recently in some countries due to shortage of high-quality evidence for prevention of peritonitis, poor control of dialysis center infections, and relatively high technique failure rate (3, 4).

PD-associated peritonitis (PDAP), as one of the most severe complications contributing to substantial morbidity and mortality of PD patients (5), was reported to be a significant cause of technical failure in PD patients (6), responsible for about 22% catheter removal, 18% transfer to HD, and 2–6% death (7). Furthermore, ongoing PDAP and an inadequate response to treatment can lead to extended hospitalization time, increased health care costs and damage on peritoneal structure and function (4, 8); once the treatment fails and the catheter is removed, only a small part of patients resume PD therapy (8). Despite the guidelines of PDAP, there are still considerably baffling variations in treatment outcomes of peritonitis in many centers and countries (4).

Recently the emphasis has also been put on improving peritonitis outcome and lowering the incidence of peritonitis. Early identification of risk factors predicting the poor outcome helps guide early treatment strategies and ameliorate prognosis. Although studies have reported the predictors of PDAP outcome previously, plenty of conflicting results were presented. PD duration (9), serum albumin (10), and concurrent DM (11) were observed to have prognostic value for peritonitis outcome in some studies, while some others thought these factors could not predict the treatment outcome (12). Therefore, further investigations are needed to explore these potential predictive factors.

In this study, risk factors of treatment failure of PDAP episodes were identified and turned into a novel risk score system, which may help predict poor outcome of PDAP and guide early interventions in these episodes.

MATERIALS AND METHODS

Study Population

The single-center, retrospective observational study was carried out at the PD center of the West China Hospital, Sichuan University. Data regarding all peritonitis episodes from December 2014 to July 2018 were collected by reviewing case records. All patients received continuous ambulatory peritoneal dialysis (CAPD) using lactate-buffered glucose dialysis solution through Tenckhoff PD catheters with a twin-bag connection system (Baxter Healthcare, Guangzhou, China). The exclusion criteria included (1) patients with a previous kidney transplant; (2) episodes without bacterial cultures or missing data; (3) patients have received PD treatment for <1 month. All PD patients have accepted the established PD training curriculum conducted by nursing staff with appropriate qualifications and experience regularly. Patients were instructed to contact the hospital upon the appearance of cloudy effluent or digestive tract symptoms of unknown origin. When peritonitis was suspected, dialysate was collected for cell count and classification, gram staining and bacterial culture. Eight to ten milliliters of dialysate effluent were collected at the bedside into 2 (aerobic and anaerobic) blood-culture bottles, the culture would prolong to 5 days unless a positive signal was obtained within 48 h; another about 50 milliliters dialysate were gathered in a sterile tube for gram staining and blood agar culture after centrifugation of 3,500 rpm for 15 min, the culture of agar plates will last for 3 days under the aerobic and anaerobic circumstances unless organisms are detected.

This study was approved by the Medical Ethics Committee of West China Hospital of Sichuan University, Sichuan, China, on January 13, 2019, and written informed consent was obtained from each patient. This study was registered at the Thai Clinical Trials Registry (http://www.clinicaltrials.in.th/index.php?tp=regtrials&menu=trialsearch&smenu=fulltext&task=search&task2=view1&id=3339).

Diagnosis

PDAP was diagnosed when at least two of the following manifestations were met: (1) abdominal pain and/or cloudy dialysate effluent; (2) dialysate effluent white cell count >100/ul or $>0.1 \times 10^9$/L with >50% polymorphonuclear cells, and (3) positive dialysate culture (5). Intestinal obstruction was considered if abdominal pain and/or distention, nausea/vomiting or cease of exhaust and defecation occurred and if it was combined with bowel dilation and extensive accumulation of gas and liquid (Gas-fluid levels in radiology).

Treatment and Outcomes

The standard antibiotic protocol was used according to ISPD guidelines (5). Generally, initial therapy for PDAP should cover both gram-positive (G^+) and gram-negative (G^-) organisms, including first-generation cephalosporin or vancomycin combined with third-generation cephalosporin or aminoglycoside, and the regimen was modified according to the culture results and drug sensitivity.

Treatment success was defined as complete resolution of peritonitis (WBC count of <100/ul in the dialysate effluent with a relief of clinical manifestations) without the need for catheter removal; treatment failure included catheter removal or death. Catheter removal was considered in patients with refractory peritonitis (failure of the PD effluent to clear up after 5 days of appropriate antibiotic treatment), refractory exit-site or severe tunnel infection, or deterioration of the clinical condition as judged by the physician. Peritonitis-associated death was defined as death within 4 weeks of peritonitis, death with active peritonitis, or any death during hospitalization for a peritonitis episode (5).

Basic Demographic, Clinical and Laboratory Data

Potential predictors were identified based on a comprehensive literature review and clinical experiences of nephrologists. These include basic demographic characteristics, such as gender, age, comorbidities, Charlson comorbidity index (13), etiology of ESRD, duration of PD, mean arterial pressure, history of antibiotic usage and history of HD; laboratory data including white blood cells (WBC), neutrophils, hemoglobin (Hb), serum albumin, creatinine (Cr), uric acid (UA), high-density lipoprotein (HDL) etc. that tested within 1 week of the diagnosis of peritonitis and causative organisms.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp, Armonk, NY, USA). All data were expressed as mean ± standard deviation (SD) for normally distributed data, median values with their interquartile range for skewed data and numbers (n) with percentage (%) for categorical variables. Data were analyzed by using the Chi-square test or Fisher's exact test for categorical variables, Student's *t*-test for normally distributed data and Wilcoxon rank sum test for continuous skewed variables. Variables whose $P < 0.1$ in the univariate analysis were selected for the final logistic regression to examine the independent risk factors of treatment failure. The stepwise procedure (forward: LR) was used to isolate the predictors. Odds ratios and 95% confidence intervals (CI) were calculated, and a two-tailed $P < 0.05$ was considered statistically significant. Data with <10% missingness were supplemented by the expectation-maximization (EM) method. Data with more than 20% of the values missingness were excluded from the final analysis.

A risk prediction score was converted for ease of application in clinical practice; To develop a simple integer-based point score for each variable, each β coefficient was divided by the model's minimum coefficient value and rounded up to the nearest integer to assign a score (14). To identify the cutoff values and evaluate the discrimination of the risk score, the area under the receiver operating characteristics curve (AUROC) was measured. Calibration was estimated by the Hosmer-Lemeshow goodness-of- fit test (15). To address the robustness of the risk score system, additional analysis was performed by excluding peritonitis episodes with: (1) age>65; (2) albumin>38 g/L;(3) culture negative; (4) intestinal obstruction; (5) subsequent peritonitis.

RESULTS

Peritonitis Characteristics

There were 359 PDAP episodes in 265 patients during the study period. Twenty three episodes without bacteria culture, 21 episodes whose peritoneal dialysis duration was <1 month, one peritonitis with renal transplantation history and four episodes with missing data were excluded. Finally, a total of 314 episodes experienced by 241 patients were included in the final analysis (shown in **Figure 1**). The detailed data of patient baseline demographic characteristics are listed in **Table 1**, and causative PDAP organisms are listed in **Table 2**.

Clinical Features of PDAP Categorized by Treatment Outcomes

Among the 314 PDAP episodes included in the current study, treatment success was achieved in 249 (79.3%) episodes, and the remaining 65 (20.7%) episodes (two deaths and 63 catheter removals) resulted in treatment failure. Univariate analysis showed that variables associated with treatment failure included a higher level of peripheral WBC, neutrophils, TG, Fib, PT, and a lower level of Alb, CHOL, HDL, LDL ($P < 0.05$). Furthermore, patients with longer PD duration ($P = 0.003$), subsequent peritonitis ($P = 0.018$), or complicated with DM ($P = 0.04$) or intestinal obstruction ($P = 0.002$) were found to have a higher risk of treatment failure.

Details of the organisms responsible for the 314 peritonitis episodes are shown in **Table 2**. For causative organisms, 141 (44.9%) episodes were culture positive, of which 89 (28.3%) were due to G^+ organisms, with *Coagulase negative staphylococcus* being the most common organism (64%), followed by *Streptococcus mitis* (12.4%) and *Staphylococcus aureus* (10.11%). 37 (11.8%) episodes were due to G^- organisms, of which *Escherichia coli* (45.9%) was the most common, followed by *Pseudomonas aeruginosa* (10.8%) and *Corynebacterium spp* (8.1%); 4 (1.3); and 11 (3.5%) peritonitis episodes infected multiple bacteria and fungus, respectively. The remaining 173 (55.1%) episodes are negative cultures.

To eliminate the interference of fungus for its high rate of poor outcomes (5), fungal peritonitis was excluded when we analyzed the effect of other bacteria on treatment outcome. Culture-negative peritonitis was found to have a significantly lower catheter removal rate than in culture-positive episodes ($p = 0.026$). A significant difference was observed in the percentages of G^- bacteria between treatment failure and success groups ($P = 0.016$), while the differences were not seen in episodes caused by G^+ bacteria. Among gram-positive and gram-negative organisms, no single bacteria was found to have an association with treatment failure.

The Predictors of Treatment Failure in PDAP

Logistic regression suggested that HDL, Fibrinogen, PD duration, fungal infection, intestinal obstruction, DM, HD history were demonstrated to be independent predictors of treatment failure in peritonitis (**Table 3**). Specifically, the odds of treatment failure increased to 1.327 with every 1 g/L Fib increment ($P = 0.021$), 5.186, 2.451 and 2.804 in patients with intestinal obstruction ($P = 0.007$), DM ($P = 0.018$) and history of HD ($P = 0.006$), respectively, compared to those without these complications. In terms of the relationship between PD duration and treatment outcomes, the distribution of treatment outcomes of the 314 peritonitis episodes by dialysis duration was shown in **Figure 2**. The proportion of catheter removal or death rose over the PD time. In the regression model, the risk of treatment failure increased to 1.017 with every 1 month longer (95%CI 1.005 to 1.028; $P = 0.005$) (**Table 3**).

Risk Score

Episodes of fungus peritonitis ($n = 11$) were excluded in the final risk score system because of the high rate of catheter removal and it is an indication of catheter removal in ISPD guideline. **Table 4** shows the β regression coefficient and the risk score of each predictor, the total score ranges from 0 to 8 points. The expected probability of treatment failure calibrated well with the observed probability ($P > 0.05$, shown in **Figure 3A**); the area under the receiver operating characteristic of the 303 episodes is 0.80 (95% CI 0.74–0.86, $P < 0.0001$) (shown in **Figure 3B**), >0.7, showing a good discrimination capability. The optimal cutoff value for risk

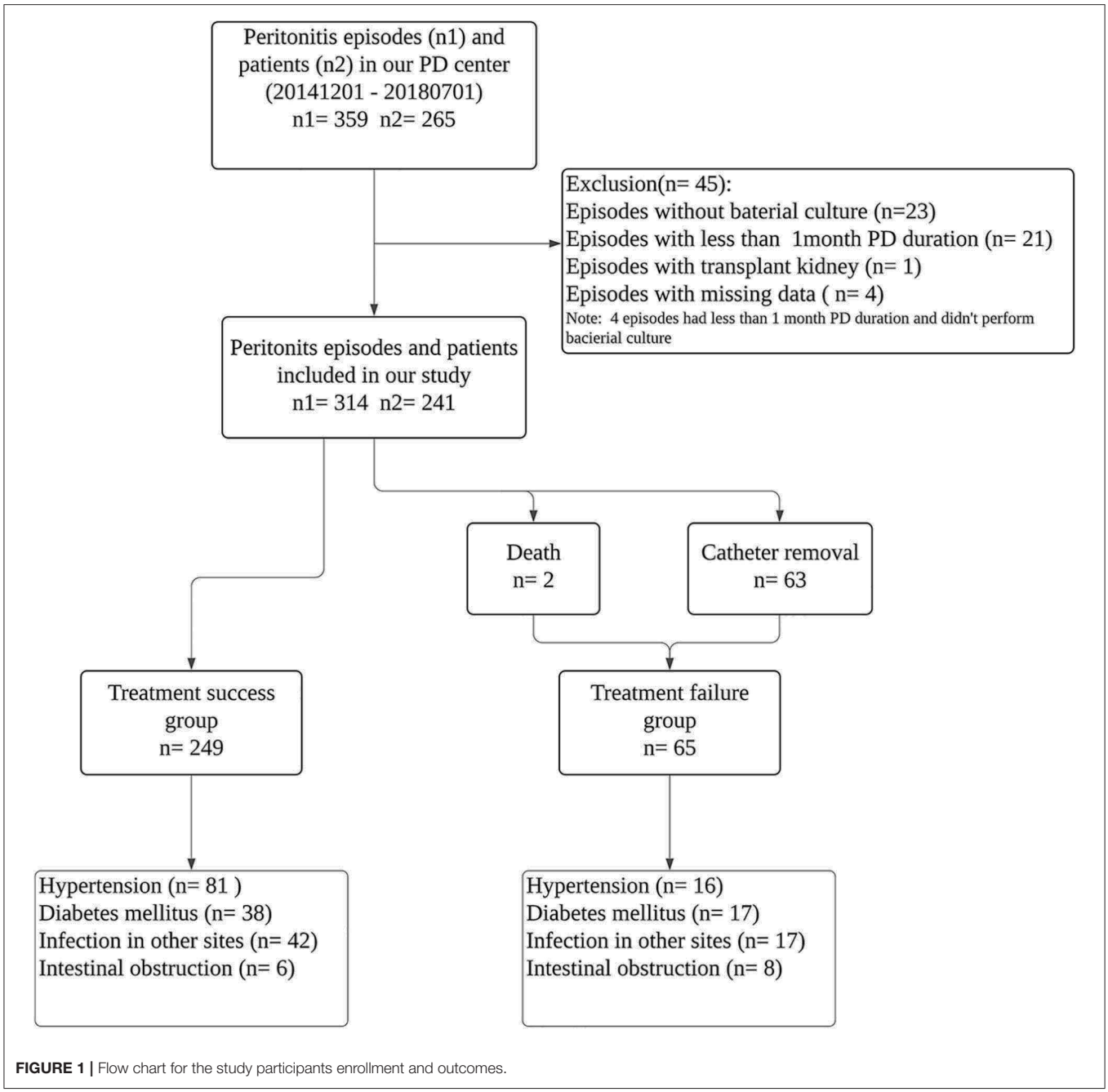

FIGURE 1 | Flow chart for the study participants enrollment and outcomes.

score was 3.5 points (sensitivity 78%, specificity 71%, Youden's index 0.491), indicating that a patient with a score of 4 or more had a high risk of treatment failure when experiencing a PDAP episode.

Subgroup and Sensitivity Analysis

Supplementary Table 1 shows the characteristics of episodes complicated with ileus, which revealed that patients with a higher level of fibrinogen, neutrophils, and those infected by gram-negative organisms, especially the *E. coli*., taking up 88.9%, had a higher possibility of concurrent intestinal obstruction.

Logistic regression analysis was performed again after exclusion of fungal peritonitis and subsequent peritonitis, the results were comparable (**Supplementary Table 2**), indicating the good robustness of these predictors.

For risk score system, stratification of age, albumin, and exclusion of culture-negative, intestinal obstruction did not change the prediction model performances, the discrimination ranged from 0.74 to 0.80, and all *P*-values of Hosmer-Lemeshow statistic were >0.05 (**Supplementary Table 3**), indicating the good robustness of the risk score system.

TABLE 1 | The Basic demographic and clinical characteristics, laboratory data by treatment outcomes.

Values	Total episodes (n = 314)	Success group (n = 249)	Failure group (n = 65)	P
Demographic and clinical characteristics				
Male (n, %)	163 (51.9)	127 (51)	36 (55.4)	NS
Age at the onset of PD (year)	47.64 ± 13.42	50.49 ± 13.63	51.58 ± 13.41	NS
Age at the peritonitis (year)	50.56 ± 13.51	50.37 ± 13.67	51.29 ± 12.99	NS
PD duration (month)	28 (11–59)	25 (9–54)	39 (17.5–67.5)	0.003
Mean arterial pressure (MAP, mmHg)	102.69 ± 18.48	102.24 ± 18.4	103.74 ± 19.38	NS
History of antibiotics usage	89 (28.3)	71 (28.5)	18 (27.7)	NS
History of hemodialysis	57 (18.2)	40 (16.1)	17 (26.6%)	0.052
Etiology of ESRD				
Chronic glomerulonephritis	179 (57)	145 (58.2)	34 (52.3)	NS
Diabetic nephropathy	49 (15.6)	33 (13.3)	16 (24.6)	0.034
Immunologic nephropathy	15 (4.8)	12 (4.8)	3 (4.6)	NS
Others	71 (22.6)	59 (23.7)	12 (18.5)	NS
Comorbidities (n, %)				
Hypertension	97 (30.9)	81 (32.5)	16 (24.6)	NS
Diabetes mellitus	55 (17.5)	38 (15.3)	17 (26.2)	0.04
Infections in other sites	59 (18.8)	42 (16.9)	17 (26.2)	NS
Intestinal obstruction	14 (4.5)	6 (2.4)	8 (12.3)	0.002[a]
Charlson comorbidity index	3 (2–5)	3 (2–5)	4 (2–5)	NS
Laboratory data				
White blood cell (WBC, 10^9/L)	6.88 (5.44–9.13)	6.75 (5.31–9.02)	7.58 (6.08–10.21)	0.034
Neutrophils (N, %)	0.77 (0.69–0.85)	0.76 (0.68–0.84)	0.8 (0.74–0.87)	0.005
Hemoglobin (Hb, g/L)	93.94 ± 19.63	95 ± 19.41	89.84 ± 20.11	NS
Albumin (Alb. g/L)	30.09 ± 6.02	30.62 ± 6.01	28.08 ± 5.63	0.002
Creatinine (Cr, umol/L)	778 (615.25–999.75)	773 (610–991)	823 (635.96–1056)	NS
Uric acid (UA, umol/L)	354.6 (304.63–412.93)	356 (309–412.95)	343 (290.6–423)	NS
Cholesterol (CHOL, mmol/L)	4.09 (3.48–4.81)	4.13 (3.55–4.92)	3.79 (3.33–4.42)	0.01
Triglyceride (TG, mmol/L)	1.33 (0.99–1.89)	1.3 (0.93–1.82)	1.49 (1.09–2.36)	0.024
High density lipoprotein (HDL, mmol/L)	1.15 ± 0.48	1.20 ± 0.49	0.95 ± 0.37	<0.001
Low density lipoprotein (LDL, mmol/L)	2.17 (1.67–2.85)	2.21 (1.75–2.89)	2.02 (1.43–2.38)	0.008
Fibrinogen (Fib, g/L)	5.25 ± 1.34	5.08 ± 1.30	5.89 ± 1.32	<0.001
Prothrombin time (PT, s)	12.22 (11.5–13)	12.2 (11.5–12.9)	12.52 (11.78–13.80)	0.021
Parathyroid hormone (PTH, pmol/L)	20.13 (9.64–39.52)	20.41 (10.6–40.23)	20.12 (6.96–36.30)	NS
Initial peritonitis (n, %)	208 (66.2)	173 (69.5)	35 (53.8)	0.018

NS, not significant; ESRD, end stage of renal failure; eGFR, estimated glomerular filtrate rate; [a]Fisher's exact test.

DISCUSSION

In this retrospective study of 314 PDAP episodes in 3.6 years, we found that high fibrinogen level, long PD duration, fungal peritonitis, HD history, intestinal obstruction or DM were independent predictors of a poor treatment outcome. In contrast, HDL seemed to be a protective factor in these patients. To facilitate the clinical application, we developed a novel risk score system based on our findings and found that PDAP patients with risk score of four or more were in high risk of treatment failure. Sensitivity analysis indicated good robustness of these predictors and a risk score system.

Our study provides new implications and directions in predicting adverse outcomes in peritonitis; these novel predictors may be incorporated into a large prediction model (15). All these risk factors are easily obtained with low cost from medical history records and routine biochemical examination to be of practical clinical value. According to the risk stratification, physicians may estimate the severity of peritonitis early, and then give the appropriate care and treatment strategy. For patients with a score ≥4, more comprehensive testing and assessment, more closely monitor, timely adjustments of antibiotics, and early nephrology care as well as other expert health care services should be considered.

The association of HD history and prognosis of peritonitis in PD patients has has never been detected in previous researches. Some patients in our center have HD history for some reasons; some begin HD unaware of PD as a treatment choice for

TABLE 2 | Causative organisms of the 314 peritonitis episodes.

Causative organisms	Total episodes ($n = 314$)	Success group ($n = 248$)	Failure group ($n = 55$)	*P*
Culture negative (*n*, %)[a]	173 (55.1)	149 (60.1)	24 (43.6)	0.026
Gram positive[a] (*n*, %)	89 (28.3)	72 (29)	17 (30.9)	NS
Coagulase-negative staphylococci[b]	57 (64)	45 (62.5)	12 (70.6)	NS
Streptococcus mitis[b]	11 (12.4)	11 (15.3)	0 (0)	NS
Staphylococcus aureus[b]	9 (10.11)	6 (8.3)	3 (17.6)	NS[c]
Enterococcus spp	6 (6.7)	5 (6.9)	1 (5.9)	NS[c]
Others[b]	6 (6.7)	5 (6.9)	1 (5.9)	NS[c]
Gram-negative (*n*, %)[a]	37 (11.8)	25 (10.1)	12 (21.8)	0.016
Escherichia coli[b]	17 (45.9)	10 (40)	7 (58.3)	NS
Pseudomonas aeruginosa[b]	4 (10.8)	2 (8)	2 (16.7)	NS[c]
Corynebacterium spp[b]	3 (8.1)	3 (12)	0 (0)	NS[c]
Klebsiella spp[b]	2 (5.4)	2 (8)	0 (0)	NS[c]
Others[b]	11 (29.7)	8 (32)	3 (27.3)	NS[c]
Polymicrobial[a]	4 (1.3)	2 (0.8)	2 (3.6)	NS[c]
Fungus	11 (3.5)	1 (0.4)	10 (15.4)	<0.001[c]

Fungus peritonitis was excluded when analyzing the other bacterial organisms.
[a]*The number and percentage of causative organisms examined to all the episodes.*
[b]*The number and percentage of specific organism to related causative organisms examined.*
[c]*Fisher's exact test.*

TABLE 3 | Multivariate logistic regression model on prediction of the peritonitis treatment failure.

Values	*P*	OR	95% CI
High density lipoprotein	0.032	0.443	0.211–0.993
Fibrinogen	0.021	1.327	1.043–1.687
PD duration/year	0.005	1.017	1.005–1.028
Intestinal obstruction	0.007	5.186	1.575–17.069
Diabetes mellitus	0.018	2.451	1.167–5.148
Fungal peritonitis	<0.001	63.413	7.421–541.845
Hemodialysis history	0.006	2.804	1.342–5.860

OR, odds ratio; CI, confidence intervals.

ESRD, some transfer to PD under the consideration of the financial situation, living condition or HD complications etc. In the current study, the HD history is demonstrated to be a marked predictor of technical failure in peritonitis episodes ($P = 0.006$, 0R 2.804). No studies to date have assessed the possible drivers, but a higher rate of technique failure is also found in patients who transfer from HD to PD in several studies (16, 17). These patients may have difficulties in adapting the manual management of PD after getting used to HD; this may be a contributor. Furthermore, psychosocial factors may also affect; more psychosocial supports than other patients when starting dialysis at home are needed, given that acute dialysis initiation could raise feelings of anxiety and reduce the patient's confidence in home therapy (17).

Previously, albumin was detected to be a predictor of treatment failure in PDAP (18), partly because of malnutrition; the albumin level was associated with technique failure in the univariate analysis in this study, but not a predictor in the multivariate logistic regression analysis. Controversial results have been reported about the effect of DM on PDAP outcome. Some studies revealed that DM (9, 10) could not predict the peritonitis outcome. However, Krishnan et al. (9) excluded peritonitis with culture-negative infection, and the indication of catheter loss in a study conducted by Yang CY was not clear (10). In the present analysis, DM is recognized as a risk factor of treatment failure of PDAP, similar to previous research (11). It was reported that DM could impair peritoneal defense (19), which may adversely affect the prognosis of PDAP; in addition, as long-term exposure to glucose dialysate may cause dysfunction of the peritoneum, which impairs the peritoneal immunologic reactions against bacteria and increases the severity of peritonitis (20); furthermore, some patients with DM are complicated with diabetic retinopathy, PD needs to manage manually at home; this probably results in operation errors, which may be a contributor to technique failure. Longer PD duration was identified as a predictor of worse clinical outcome of PDAP ($P = 0.005$), in line with a previous study (9). Our results underscore the importance of timely management and meticulous care of peritonitis in patients with DM or long PD duration for favorable prognosis.

Notably, fibrinogen concentration was recognized as a predictor of treatment failure in peritonitis patients in our cohort. Although fibrin deposition may ameliorate systematic inflammation by preventing bacterial spread (21), fibrin mesh formed from extensive exudation and deposition of fibrin can facilitate bacteria's proliferation and prevent them from host phagocytic cells attack and bactericidal effects of antibiotics (22, 23). Additionally, fibrin exudation and deposition may lead to fibrous capsulation and paralytic intestinal obstruction (24), as

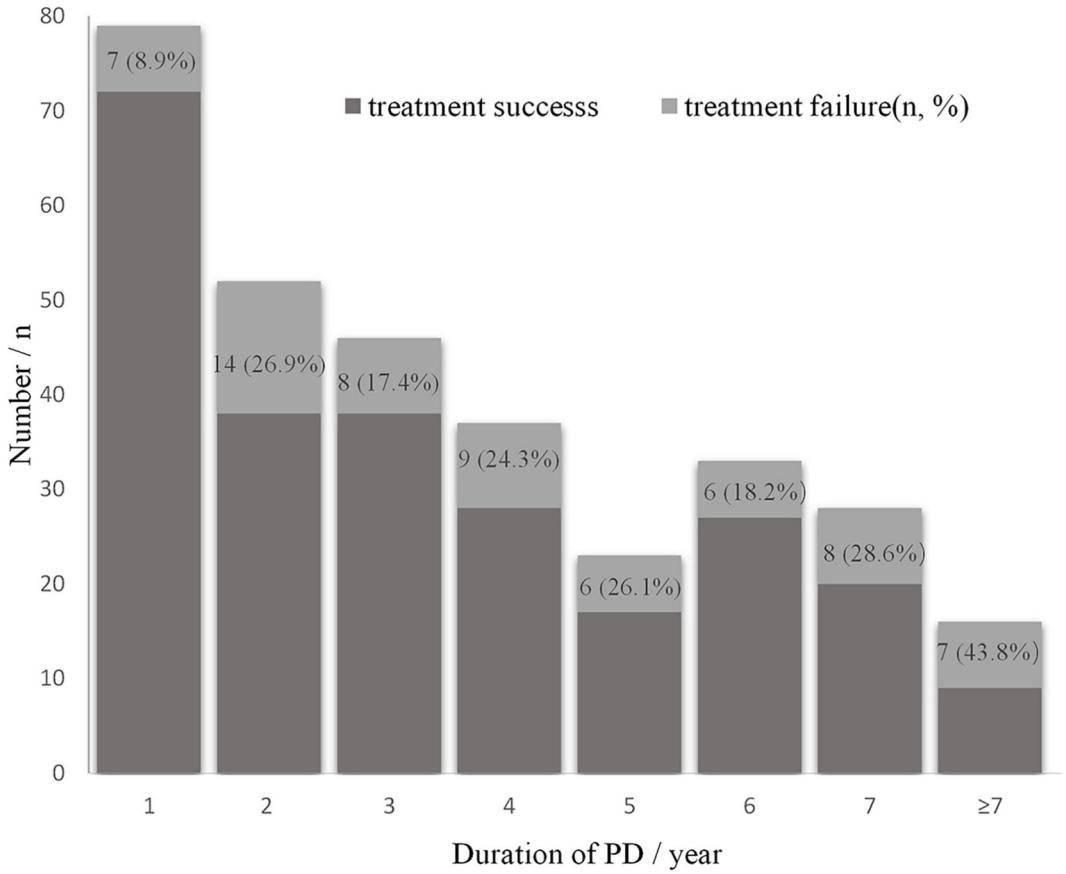

FIGURE 2 | Distribution of treatment outcomes by peritoneal dialysis time.

our study observed, PDAP patients with intestinal obstruction seemed to have increased Fib level (**Supplementary Table 1**); Intestinal obstruction has been proved to be linked with technical failure in fungal (25) and bacterial PDAP (26) previously. Our results further confirmed these findings. For one reason, ileus could result in bowel immobilization and dysfunction of nutrients absorption; for another, ileus has a higher possibility of infections with gram-negative organisms, especially *E. coli* (**Supplementary Table 1**), which may lead to more severe peritonitis compared to other organisms (27). The exact mechanism needs to be studied further.

We also found that patients in the treatment failure group are characterized as lower HDL concentrations. As far as we know, this is the first study to reveal the effect of dyslipidemia on the peritonitis treatment outcome. HDL can exert antioxidant and anti-inflammatory activity by inhibiting oxidative stress and the formation of pro-inflammatory oxidized lipids; meanwhile, HDL can attenuate systematic inflammation by increased scavenging of endotoxin (28), this anti-inflammatory and antioxidant effect of HDL may play a crucially positive role in the prognosis of peritonitis. Notably, HDL in ESRD patients has been reported to have impaired antioxidant and anti-inflammatory effect and has even a pro-oxidant and pro-inflammatory effect because of functional and structural abnormalities (28, 29). Therefore, worse outcomes in dialysis patients were reported to be associated with HDL increment (30). There are many controversies, and uncertainties in the relationship between lipid disorders and peritoneal dialysis, the mechanisms of the effects of dyslipidemia on the prognosis of peritonitis require further investigation.

This study has several limitations. Firstly, this is a retrospective, single-center research, which may lead to bias in our findings. Secondly, considering that the external validation is not performed, the generalization of risk prediction score was restricted. Also, negative culture rate (55.1%) is high compared to the 15% rate suggested by the IPSD (5). It might be due to the use of antibiotics before sample collection. Many patients in our center live in villages or cities far away from our hospital, antibiotics have been prescribed to them by local doctors; furthermore, even though the culture protocol conforms to the IPSD guideline recommendations (5), methods that could improve the rate of culture-positive such as usage of rapid blood-culture bottle kits or lysis centrifugation technique should be considered. Nevertheless, the distribution of causative organisms in our study is following previously reported studies, and the empiric therapy has satisfactory outcomes with 80% rate of treatment success; besides, our findings are independent

TABLE 4 | ß coefficient and corresponding risk score developed from risk prediction model.

Risk factors	Adjusted OR (95% CI)	*P*-value	ß-coefficients	Transformed score	Assigned score
HDL ≤ 1.5mmol/L					
No	1.0 (Reference)	-	-	0	0
Yes	4.78 (1.35–16.97)	0.015	1.565	1.79	2
Fibrinogen ≥ 5.25 g/L					
No	1.0 (Reference)	-	-	0	0
Yes	2.83 (1.39–5.79)	0.004	1.042	1.19	1
Diabetes mellitus					
No	1.0 (Reference)	-	-	0	0
Yes	2.40 (1.13–5.12)	0.024	0.875	1	1
Intestinal obstruction					
No	1.0 (Reference)	-	-	0	0
Yes	6.05 (1.76–20.74)	0.004	1.800	2.06	2
Hemodialysis history					
No	1.0 (Reference)	-	-	0	0
Yes	2.88 (1.37–6.05)	0.005	1.057	1.21	1
PD duration ≥ 32 month					
No	1.0 (Reference)	-	-	0	0
Yes	2.51 (1.23–5.14)	0.011	0.922	1.05	1

HDL, High density lipoprotein. Fungal peritonitis were excluded.

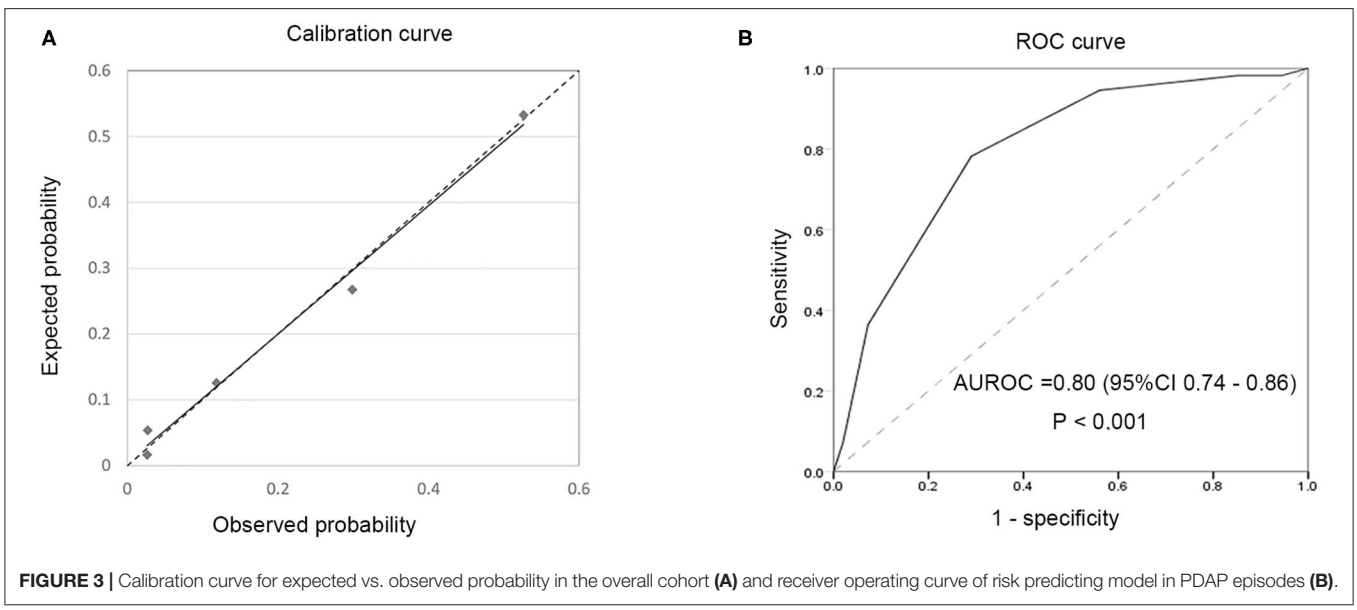

FIGURE 3 | Calibration curve for expected vs. observed probability in the overall cohort **(A)** and receiver operating curve of risk predicting model in PDAP episodes **(B)**.

of the type of organisms, demonstrating that these findings can apply to some PD cohorts elsewhere. Finally, all patients in our study are Chinese and perform CAPD; the generalizing these findings may be limited to Asian and PD patients with CAPD modality. Even though all patients in our center take CAPD for financial considerations rather than automated peritoneal dialysis (APD), which is more commonly used in developed countries, such as the US (31), no significant differences are found in technique survival between CAPD and APD as reported by a few large representative cohort studies (31–34).

In conclusion, high Fib level, long PD duration, fungal infection, HD history, concurrent intestinal obstruction or DM were found to be independent predictors of treatment failure of PDAP, while HDL seems to be a protective factor in patients with peritonitis. A novel risk score system could be employed to predict the risk of treatment failure of PDAP easily.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

XL, AQ, YT, and WQ: conception and design. XL, AQ, HZ, XH, SC, and SW: administrative support. XL, AQ, HZ, YT, and WQ: collection and assembly of data. XL, AQ, HZ, XH, SC, SW, YT, and WQ: data analysis and interpretation. XL, AQ, HZ, XH, SC, YT, and WQ: manuscript writing. XL, AQ, HZ, XH, SC, SW, YT, and WQ: final approval of manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.639744/full#supplementary-material

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Erythropoietin Attenuates Experimental Contrast-Induced Nephrology: A Role for the Janus Kinase 2/Signal Transducer and Activator of Transcription 3 Signaling Pathway

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The aim of the present study was to investigate the effect of erythropoietin (EPO) on contrast-induced nephrology (CIN) *in vivo* and *in vitro*. Male C57BL/6J mice were divided into four groups: control, CIN (iohexol 6.0 g/kg), EPO (3,000 IU/kg), and CIN+EPO. Hematoxylin and eosin (H&E) staining and biochemical index analyses were performed to evaluate renal injury. The cellular proliferation rate was detected using the Cell Counting Kit-8 (CCK-8) assay. In addition, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and flow cytometric assay were used to assess the apoptosis of tissue and cells, respectively. Renal protein expression associated with apoptosis, pyroptosis, and signaling pathways was determined by Western blot (WB) assays for tissues and cells. The results showed that EPO significantly decreased serum creatinine, blood urea nitrogen, and cystatin C levels and alleviated renal histological changes *in vivo*. The protein levels of Janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) signaling pathway components were overexpressed in the EPO treatment group. Furthermore, EPO suppressed the cell apoptosis and pyroptosis; decreased the protein levels of cleaved caspase-3, Bax, gasdermin D (GSDMD), and caspase-1; and enhanced the expression of Bcl-2. In summary, EPO could exert renoprotective effect by activating the JAK2/STAT3 signaling pathway, which may be a novel potential therapy for the treatment of CIN in the clinic.

Keywords: erythropietin, contrast-induced nephrology, JAK2, stat3, apoptosis

INTRODUCTION

Contrast-induced nephrology (CIN), as the name indicates, is an acute kidney injury (AKI) caused by endovascular application of contrast medium (CM) when excluding other causative factors. CIN has been discovered as the third most common cause of hospital-acquired AKI (1). The incidence of CIN ranges from 10 to 30% according to different studied populations and diagnostic criteria (1–4).

The underlying mechanisms of CIN are complicated and not yet completely expounded. Current studies suggest that the occurrence of CIN is mainly associated with the following mechanisms: hypoxia damage to the renal parenchyma, direct toxicity of contrast media, oxidative stress, immunizing inflammatory response, cell pyroptosis, and apoptosis (5, 6).

Erythropoietin (EPO) is an endogenous cell factor excreted by the kidney and is regarded initially as a hematopoietic factor. Recombinant human EPO has played a part in the treatment of renal anemia in patients with end-stage renal disease (ESRD) or chronic kidney disease. More recently, the protective effect of EPO on multiple tissues, including the kidney, has been reported (7–9).

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway, as an essential downstream mediator of a variety of metabolically relevant hormones, including EPO, is one of the major signaling pathways of cell metabolism and apoptosis (10). The JAK family includes JAK1/2/3 and TYK2, and the STAT family consists of STAT1/2/3/4, STAT5A/B, and STAT6, which have been confirmed in mammals. JAK1/2 and STAT3/5/6 are involved in the process of AKI and have been shown in experimental AKI or ischemia–reperfusion injury models (11–13).

Therefore, we hypothesized that EPO could also attenuate renal injury caused by CM. In this study, we provide evidence that systemically administered EPO could attenuate CIN by enhancing the JAK2/STAT3 signaling pathway.

MATERIALS AND METHODS

Animals and Contrast-Induced Acute Kidney Injury Model

The Animal Experimentation Ethics Committees of West China Medical Center and Institutes of Animal Science approved this study. Healthy C57BL/6J mice (male, 8–10 weeks old, weighing 20–26 g) were purchased from the Experimental Animal Center at the West China Medical Center of Sichuan University; housed in a specific pathogen-free, temperature-controlled environment; and kept on a 12-h light/dark cycle with standardized feeding and drinking. The experimental mice were randomly divided into four groups ($n = 18$ in each group) as follows: control group, EPO group, CIN group, and EPO+CIN group. The mouse model of CIN was a modified version of a previously described model (14, 15). In brief, after 7 days of acclimation to the experimental area and 24 h of water deprivation, mice were injected with indomethacin (10 mg/kg; MedChemExpress, NJ, USA) and N^G-nitro-L-arginine methyl ester (10 mg/kg; MedChemExpress, NJ, USA) intraperitoneally before iohexol (6.0 g/kg organically bound iodine, General Pharmaceutical Co., Ltd., Shanghai, China). Normal saline was injected at each time point in the control group. To explore the possible protection of EPO against CIN, recombinant human EPO (3,000 IU/kg) or saline was administered subcutaneously 1 h before the indomethacin injection. At 6, 24, and 48 h after CM injection, the animals were euthanized using a 10% chloral hydrate to collect blood and kidney samples for various examinations.

Renal Function

Serum creatinine (Scr), blood urea nitrogen (BUN), and cystatin C (CysC), serving as indicators of kidney function, were measured by enzymatic methods using the respective assay kits (Mindray, Shenzhen, China).

Kidney Histology

After fixation in 10% formalin for 48 h, kidneys were dehydrated in a graded ethyl alcohol series, embedded in paraffin, and then cut into 4-μm sections. Tissue sections were subjected to hematoxylin and eosin (H&E) staining for morphologic analysis. H&E-stained tissue slices were observed by optical microscopy at ×200 magnification, and 10 different fields of renal interstitium from each group were randomly selected for semiquantitative analysis. According to a previous study (16), the semiquantitative scoring criteria of renal tubular histopathology focused on tubular cell swelling and vacuolation were as follows: 0, no abnormalities; 1+, changes affecting <25% of the sample; 2+, changes affecting 25–50%; 3+, changes affecting 50–75%; and 4+, changes affecting more than 75%.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to detect cell apoptosis, and sections were stained using the DeadEnd™ Fluorometric Apoptosis Detection Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The number of TUNEL-positive tubular cells was counted under an upright fluorescence microscope at ×400 magnification.

Cell Culture and Treatment

Human kidney proximal tubular epithelial cells (HK-2 cells) were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM/F-12 medium supplemented with 10% fetal bovine serum. Cells were maintained in a humidified incubator at 37°C (5% CO_2). Cells were pretreated with different concentrations (25, 50, and 100 IU/ml) of EPO for 1 h and then treated with iohexol (75 mgI/ml) to mimic CIN *in vitro*. Cells maintained in normal medium were used as controls.

Annexin V/Propidium Iodide Assay

The apoptosis rate was analyzed using an Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (4A Biotech Co. Ltd, Beijing, China). Briefly, cells were seeded into 6-well plates (1.5×10^5 cells/well) and cultured in medium with free serum for 24 h. After treatment with EPO (100 IU/ml) and iohexol (75 mgI/ml), cells were harvested and washed twice with phosphate-buffered saline (PBS) and then resuspended in binding buffer mixed with Annexin V-FITC reagent and PI reagent according to the manufacturer's protocol. Apoptotic cells were measured by flow cytometry (Olympus, IX71).

Cell Counting Kit-8 Assay

Cell viability was determined by a Cell Counting Kit-8 (CCK-8) assay kit (Us Everbright Inc., CA, USA) according to the

manufacturer's protocol. HK-2 cells were seeded into 96-well plates at a density of 1.0×10^4 cells/well. After treatment with or without EPO and iohexol, cells were incubated with 10 μl of CCK-8 solution in each well at 37°C for 2 h. Then, we measured the absorbance at 450 nm and calculated the optical density.

Western Blotting

Total proteins of cultured HK-2 cells and kidneys of the mice were extracted with cell lysis buffer supplemented with protease and phosphatase inhibitor cocktails. The concentration of proteins was detected with a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts

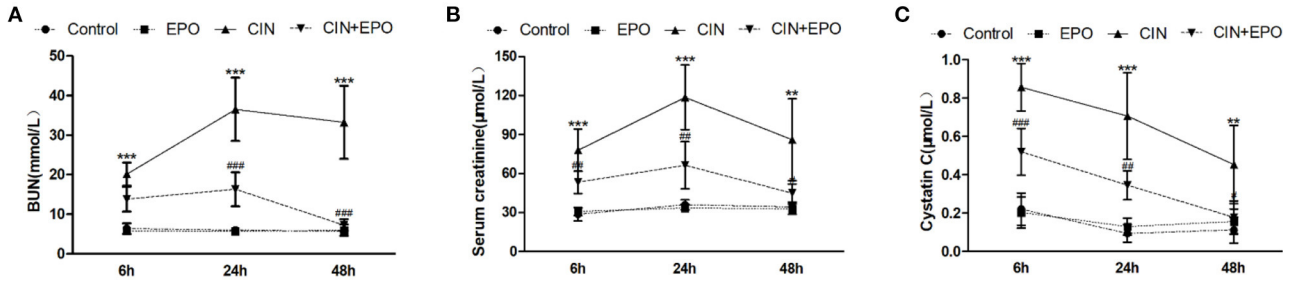

FIGURE 1 | Effect of EPO administration in renal functional parameters during CIN. **(A)** BUN (mmol/L), **(B)** serum creatinine (μmol/L), and **(C)** cystatin C (μmol/L). Values are the mean ± SD ($n = 6$ mice/each group). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. the control groups; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ vs. the CIN groups. BUN, blood urea nitrogen; EPO, erythropoietin; CIN, contrast-induced nephrology.

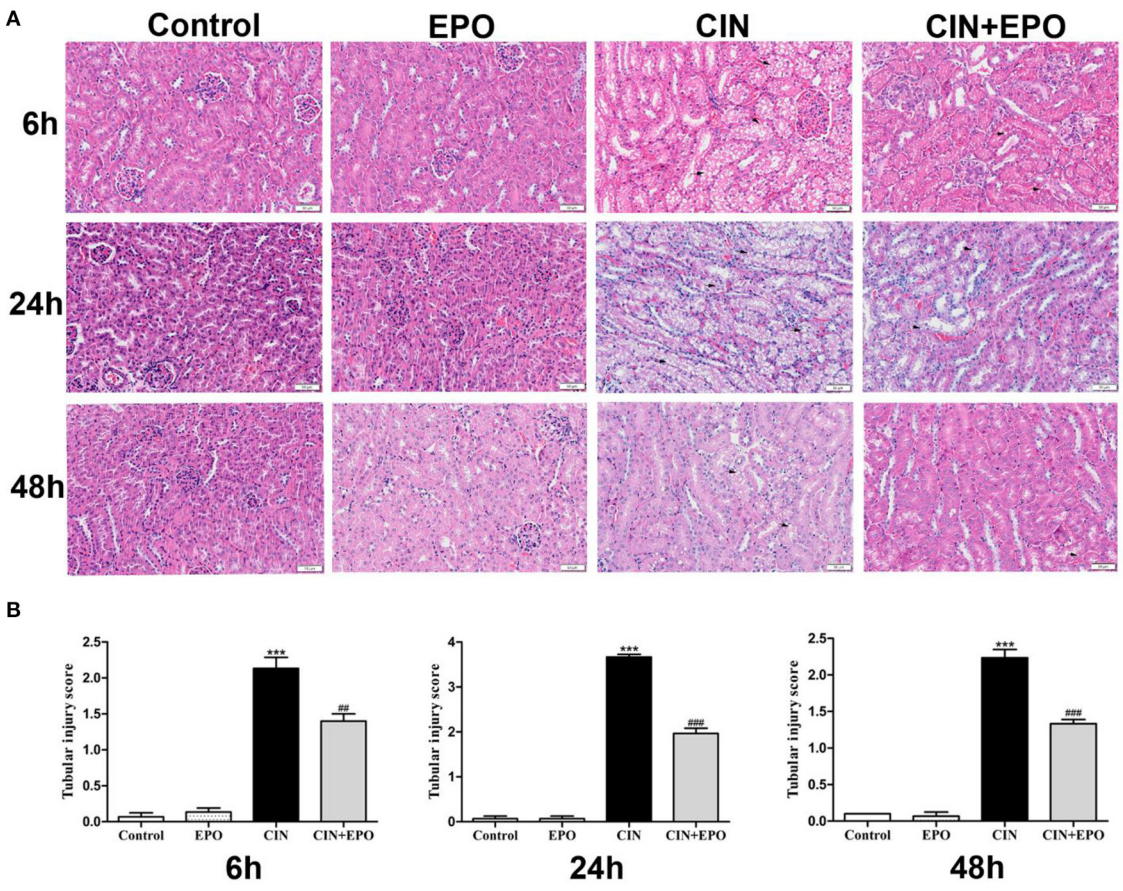

FIGURE 2 | Effect of EPO administration in tubular injury score in CIN. **(A)** In the four groups, representative images of kidney tissues were stained with H&E at various time points (magnification ×200). **(B)** The average renal tubular injury scores in the four groups at various time points. $^{***}P < 0.001$ vs. the control groups. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the CIN groups. EPO, erythropoietin; CIN, contrast-induced nephrology.

of proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. After being blocked with 5% skim milk for 1 h, the membranes were incubated overnight at 4°C with antibodies directed against total STAT3 and phospho-STAT3-Tyr705 (1:2,000, Cell Signaling Technology), total JAK2 (1:1,000, Cell Signaling Technology), phospho-JAK2-Tyr1007/1008 (1:2,000, Abcam), cleaved caspase-3 (1:1,000, Cell Signaling Technology), Bax (1:2,000, ProteinTech), Bcl-2(1:1,000, ProteinTech), gasdermin D (GSDMD, 1:1,000, Abcam), and caspase-1 (1:2,000, Huabio). The next day, the membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence substrate and quantified by ImageJ software ver. 1.8.

Statistical Analysis

All data are presented as the means ± standard deviations (SDs). Differences between the groups were determined using one-way ANOVA. Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA). The results were recognized as statistically significant when $P < 0.05$.

RESULT

Erythropoietin Ameliorates Renal Dysfunction and Tissue Damage in Contrast-Induced Nephrology

The CIN model was confirmed by the elevation of biochemical parameters of renal dysfunction and histopathology evaluation. As shown in **Figure 1A**, serum BUN was significantly increased in the CIN group at all time points compared with the control group and reached maximum levels at 24 h in the study. Moreover, compared with the control group, the levels of Scr (**Figure 1B**) and cystatin C (**Figure 1C**) were dramatically increased, reaching maximum levels at 24 and 6 h, respectively. High levels of BUN, Scr, and CysC caused by CM were obviously reduced by pretreatment with EPO.

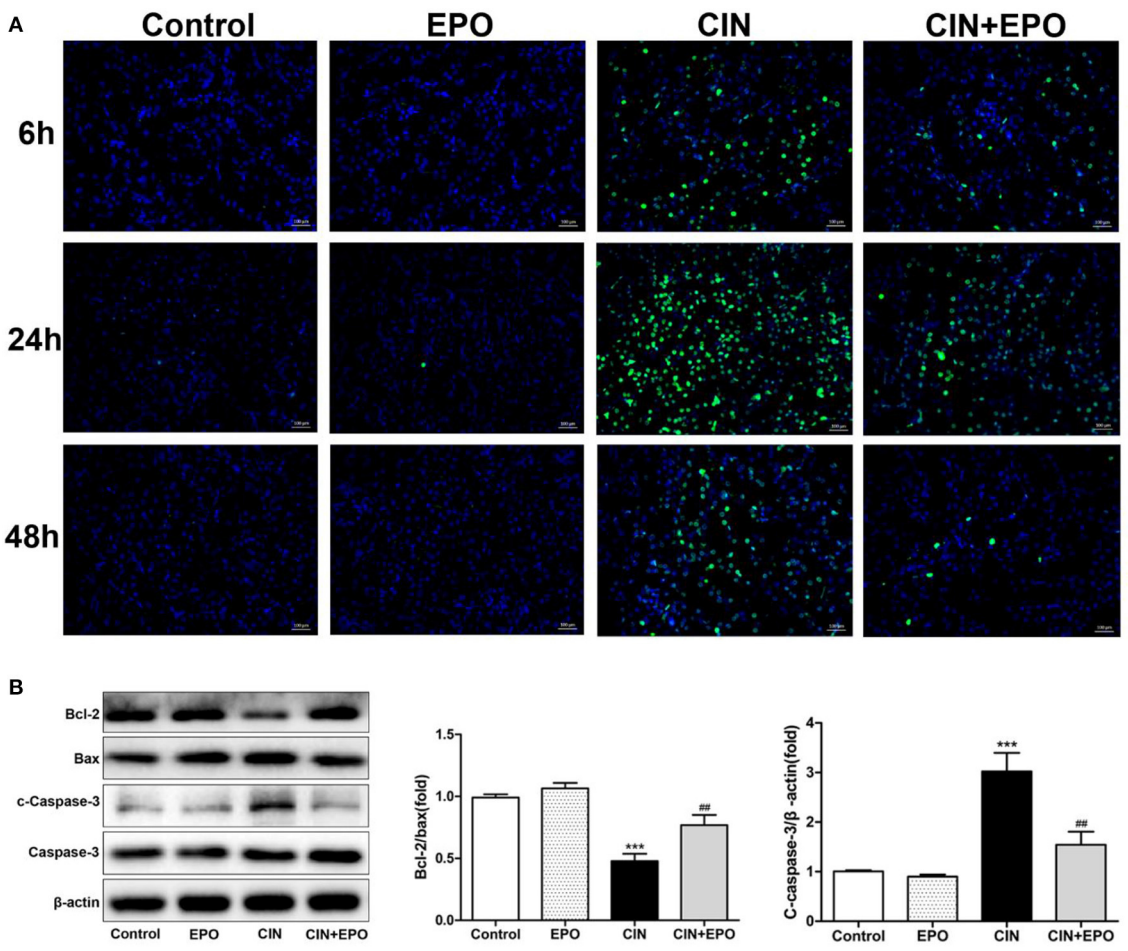

FIGURE 3 | Effect of EPO on apoptosis of renal tissue in CIN. **(A)** TUNEL staining was used to assess the kidney cell apoptosis in the presence and absence of EPO. Original magnification, × 400. **(B)** Apoptosis-related proteins, including Bax, cleaved caspase-3, and Bcl-2, were determined by Western blot assay. Each bar represents the mean ± SD calculated from three independent experiments. $^{***}P < 0.001$ vs. the control groups; $^{\#\#}P < 0.01$ vs. the CIN groups. EPO, erythropoietin; CIN, contrast-induced nephrology; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

Erythropoietin Reduces Histopathological Changes in the Kidneys of Contrast-Induced Nephrology Mice

Renal histological changes were assessed using H&E staining. As shown in **Figure 2A**, extensive tubular injury, characterized by tubular cell swelling and vacuolation, was observed in the CIN group and reached a peak at 24 h. Compared with the CIN group, the average renal tubular injury scores in the CIN+EPO group were significantly ameliorated across time points (**Figure 2B**).

Erythropoietin Alleviates Apoptosis in Renal Contrast-Induced Nephrology

Emerging evidence has demonstrated that apoptosis is involved in the development of CIN. Our results from the TUNEL assay are shown in **Figure 3A**. The CIN group showed a significant increase in the apoptosis rate, which was decreased by EPO pretreatment. The peak values of cell apoptosis by observation of green-stained cells in the field of vision were also observed at 24 h. In addition, the production of apoptosis-related proteins was determined by Western blot (WB) assay at 24 h post-CM. The results revealed that the levels of Bax and cleaved caspase-3 significantly decreased while the levels of Bcl-2 obviously increased in the CIN group, and they returned with the administration of EPO (**Figure 3B**).

Erythropoietin Preconditioning Alleviates the Inhibition of HK-2 Cell Proliferation Induced by Contrast Medium

As shown in **Figure 4**, after a 24-h incubation period with CM, the proliferation rate of the CIN group was much lower than that of the control group. With pretreatment with EPO at a concentration of 50 or 100 IU/ml, especially the latter, the proliferation rate was found to be higher than that in the CIN group, while 25 IU/ml EPO failed to enhance the proliferation rate.

Erythropoietin Inhibited Apoptosis and Pyroptosis of Tubular Epithelial Cells in Response to Contrast Medium

Cell apoptosis and pyroptosis rate were evaluated by Annexin V-FITC/PI apoptosis detection. Apoptosis protein levels were detected by WB. The results of cell apoptosis and pyroptosis rate are shown in **Figure 5A**. In contrast to the control group, the CIN group's apoptotic and pyroptosis rates increased at 6 h, peaked at 24 h, and then gradually decreased. Pretreatment with EPO attenuated early cell apoptosis at all time points and inhibited late cell apoptosis and pyroptosis at any other time point except 6 h. The expression levels of apoptosis proteins at 24 h post-CM were in accord with the corresponding apoptotic rate, while Bcl-2, an anti-apoptotic effector, was decreased in the CIN group and recovered with EPO treatment (**Figure 5B**).

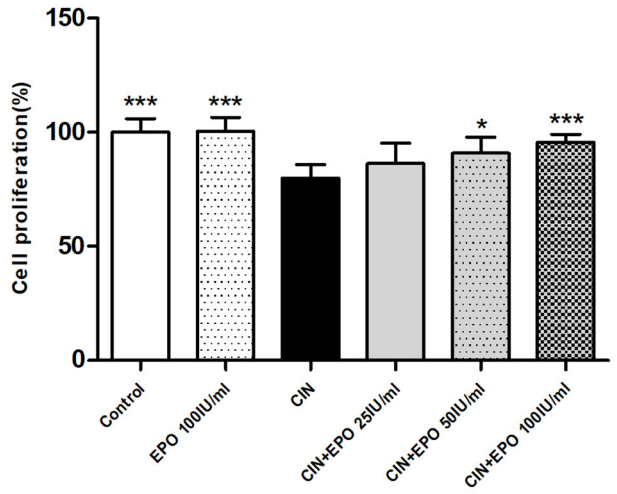

FIGURE 4 | Change of proliferation rate in each group. Cell Counting Kit-8 assay was used to determine the proliferation rate. Data are expressed as mean ± S.D. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. the CIN group. EPO, erythropoietin; CIN, contrast-induced nephrology.

Erythropoietin Ameliorated Contrast-Induced Nephrology-Associated Pyroptosis

Considering that pyroptosis may play a role in the development of CIN, we further explored the expression of GSDMD and caspase-1. After a 24-h stimulation with CM, the levels of GSDMD and caspase-1 of the CIN group were increased statistically than those of the control group. Notably, the presence of EPO can ameliorate cell pyroptosis of mouse suffering CIN (**Figure 6A**). The similar expression of GSDMD and caspase-1 was detected in a cell model (**Figure 6B**). Taken together, our data suggested that the EPO can attenuate pyroptosis in CIN.

Erythropoietin May Protect Against Contrast-Induced Injury via the Janus Kinase/Signal Transducer and Activator of Transcription Signaling Pathway *in vitro* and *in vivo*

Next, we explored the possible underlying mechanism associated with the regulatory effect of EPO on CIN. We measured the total and phosphorylated protein levels of JAK2 and STAT3 by WB in kidney tissue (**Figure 7A**). The results revealed that the expression of p-JAK2 and p-STAT3 was clearly enhanced at 24 h after EPO treatment. Furthermore, similar expression of these proteins was detected in the corresponding group of HK-2 cells (**Figure 7B**). In conclusion, JAK2/STAT3 signaling pathway activity played a role in alleviating CIN with EPO.

DISCUSSION

The emergence of CIN leads to increased mortality, prolonged hospitalization, and higher expenses (17). In addition to

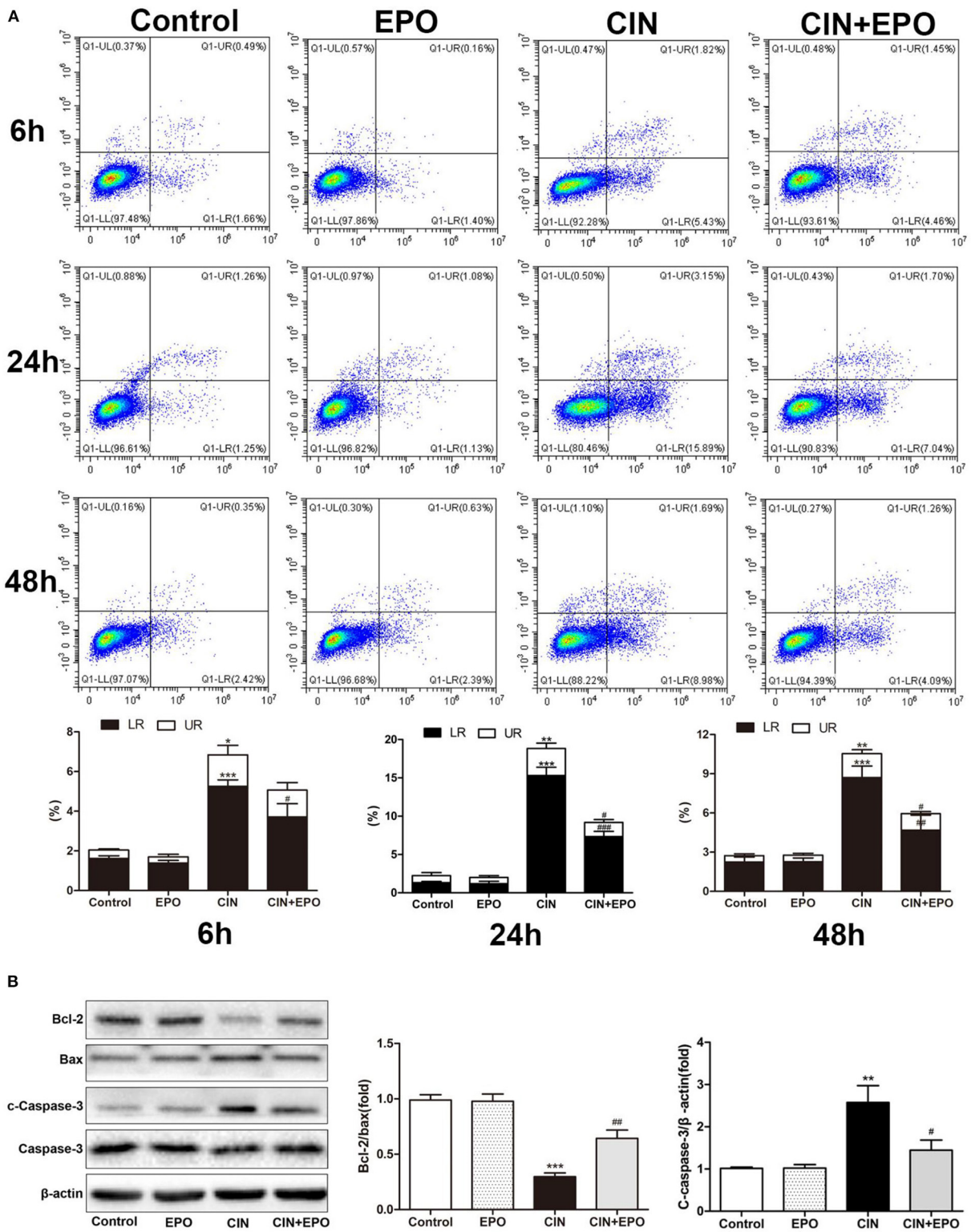

FIGURE 5 | Effect of EPO on apoptosis induced by contrast in HK-2 cells. **(A)** Annexin V/PI assay was used to assess the cell apoptosis and pyroptosis in the presence and absence of EPO. **(B)** Apoptosis-related proteins, including Bax, cleaved caspase-3, and Bcl-2, were determined by Western blot assay. Each bar represents the mean ± SD calculated from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the CIN group. EPO, erythropoietin; CIN, contrast-induced nephrology; PI, propidium iodide.

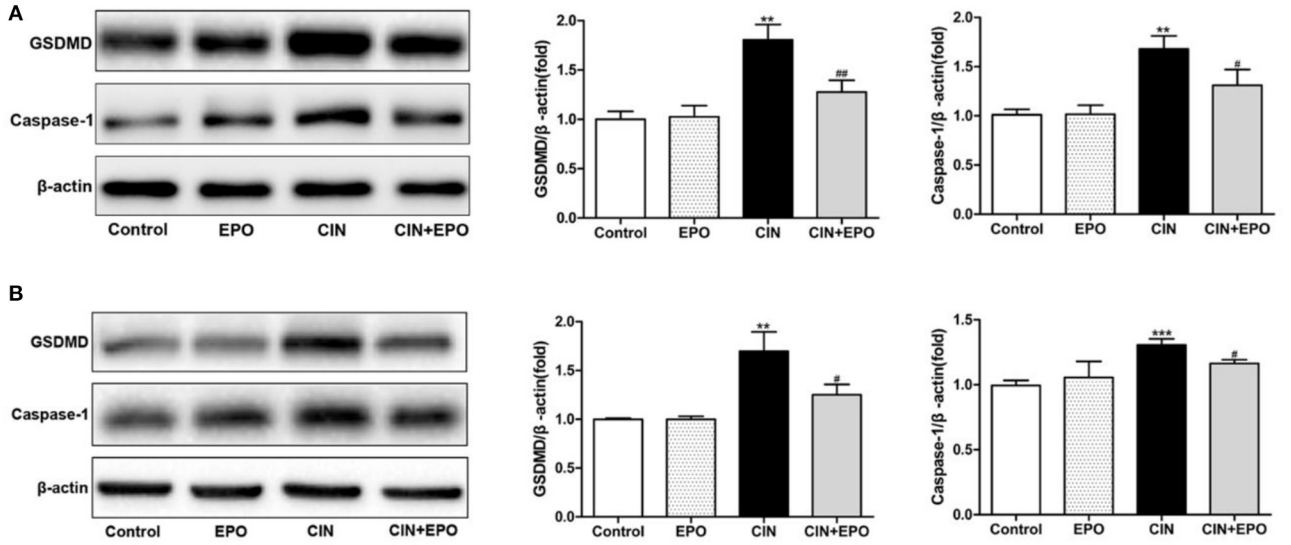

FIGURE 6 | Effect of EPO on protein levels of GSDMD and caspase-1 in mice **(A)** and HK-2 cells **(B)**. Values are expressed as the mean ± standard deviation ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the CIN group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. the CIN group. EPO, erythropoietin; CIN, contrast-induced nephrology; GSDMD, gasdermin D.

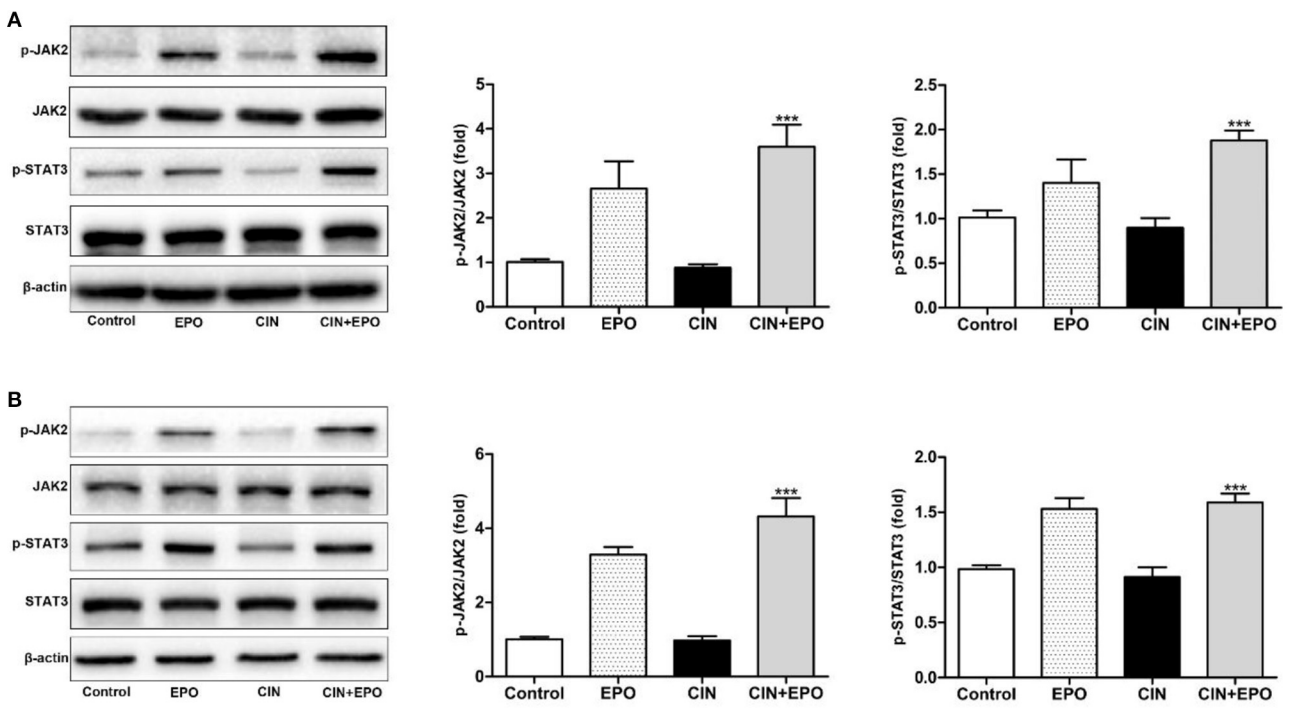

FIGURE 7 | Effect of EPO on protein levels of the JAK2/STAT3 signaling pathway components induced by contrast in mice **(A)** and HK-2 cells **(B)**. Values are expressed as the mean ± standard deviation ($n = 3$). $^{***}P < 0.001$ vs. the CIN group. p-JAK2, phosphorylated Janus kinase 2; p-STAT3, phosphorylated signal transducer and activator of transcription 3; EPO, erythropoietin; CIN, contrast-induced nephrology.

hydration, there is no special treatment widely used in the clinic to prevent CIN. Therefore, searching for effective treatments to prevent CIN and improve prognosis is crucial. EPO may protect multiple organs and tissues, such as the brain, heart, liver, lung, and kidney, and has been confirmed in experimental models or clinical trials (8, 9, 18–20). Meanwhile, studies did not report a higher risk of adverse events, such as symptomatic thrombosis, deep thrombophlebitis, myocardial

infarction, stroke, and hypertension, which were caused by the EPO intervention (21–23). Otherwise, the use of EPO for the prevention and treatment of AKI in patients is still controversial. Prophylactic administration of EPO has a role in preventing AKI, reducing cardiac complications and lowering the incidence of prolonged vasopressor dependence in patients suffering cardiovascular surgery (7, 24, 25). Contrary to the former, a clinical trial concentrating on patients in intensive care units or undergoing complex cardiac surgery demonstrated that intravenous administration of EPO did not provide renal protection to patients with an increased risk of developing AKI (22, 23, 26). The timing of administration and risk stratification of patients may play a role in exerting EPO's renoprotection effect. Therefore, the controversy between different clinical trials requires more fundamental studies focused on the underlying mechanisms of EPO administration in AKI.

In our mouse and cell models of CIN, EPO pretreatment potently repaired histological injury of the kidney and reduced biochemical parameters of renal dysfunction. Meanwhile, EPO markedly alleviated apoptosis, shown as decreasing protein expression of Bax and cleaved caspase-3 and increasing level of Bcl-2. The presence of EPO also decreased the protein expression of GSDMD and caspase-1, which are involved in the occurrence of pyroptosis. Moreover, upregulation of JAK2 and STAT3 phosphorylation played a key role in these processes. These results suggested that EPO may attenuate CIN renal injury by suppressing apoptosis and pyroptosis, which depends on the JAK2/STAT3 signaling axis. To our knowledge, this is the first report documenting that EPO can ameliorate renal injury in experimental models of CIN through the JAK2/STAT3 signaling pathway.

At present, the pathogenesis involved in CIN has been researched widely. Several experimental studies have shown that the production of reactive oxygen species (ROS) or hypoxia-induced factor (HIF), markers of hypoxia, is enhanced after the administration of CM and then either directly or indirectly mediates cellular apoptosis and necrosis (27, 28). Quintavalle et al. revealed that increased ROS triggered the activation of Jun N-terminal kinases (JNK) and p38 stress kinases and then led to cell apoptosis (29). Our results suggested that CM leads to the apoptosis of renal tubular epithelial cells by enhancing the activation of caspase-3 and breaking the balance between Bcl-2 and Bax. GSDMD forms plasma membrane pores to cause pyroptotic cell death and the release of inflammatory factor in AKI (6). Tubular epithelial cell pyroptosis mediated by caspase-1 and caspase-11 has been confirmed in the process of CIN (16, 30). Our result is consistent with the previous study. Therefore, we concluded that cell apoptosis and pyroptosis play important roles in the process of CIN.

EPO renoprotection involves the attenuation of apoptosis, hypoxia, and inflammation and promotes microvascular cell survival and/or angiogenesis (31, 32). Moreover, EPO reduced macrophage infiltration and enhanced the phenotypic switch toward M2 macrophages in an experimental model of rhabdomyolysis-induced AKI (11, 33). Nevertheless, the relevant processes at the cellular and molecular levels were not completely illustrated in CIN. Previous studies have proven that EPO can prevent renal dysfunction caused by CM, alleviate renal morphologic tubular injuries, and decrease apoptosis by activating the JAK2/STAT5 signaling pathway (12). Consistent with previous discoveries, caspase-3, Bcl-2, and Bax were involved in the apoptosis process in CIN. Meanwhile, pathological and functional injury was improved by EPO. In our study, CM could directly induce cytotoxic effects, while EPO could reactivate cells. Indeed, we also found that EPO alleviated the renal injury caused by CM in a concentration- and time-dependent manner.

A mounting body of evidence has revealed the involvement of the JAK/STAT signaling pathway in various diseases (10, 13, 20, 34). To the best of our knowledge, no studies have investigated the role of JAK2/STAT3 signaling in CIN. It was reported that the phosphorylated activation of JAK2/STAT3 played a part in the induction of apoptosis, autophagy, inflammation, and oxidative stress and that the activation of the JAK2-STAT3 signaling pathway protected tissue against acute injury in some studies (35–37). In contrast, the renoprotective effect of curcumin in AKI caused by lipopolysaccharide (LPS) or severe acute pancreatitis may be associated with inflammation reduction mediated by suppression of JAK2/STAT3 signaling pathway (38, 39). In our study, increased phosphorylation of JAK2/STAT3 after EPO treatment alleviated CIN by ameliorating cell apoptosis and pyroptosis *in vivo* and *in vitro*. In the future, the effects of EPO on apoptosis and pyroptosis should be explored further to distinguish more treatment targets and promote the clinical application of EPO for CIN.

CONCLUSION

In conclusion, the findings of this study suggest that EPO protects the kidney against histological injury and reduces biochemical parameters of renal dysfunction in CIN. Concerning the underlying mechanism, the findings indicate that EPO ameliorated apoptosis and pyroptosis via activation of the JAK2/STAT3 signaling pathway. The findings of this study are promising, and they need to be confirmed in human patients to explore the possibility of using EPO as a therapeutic agent for CIN.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the West China Hospital of Sichuan, China.

AUTHOR CONTRIBUTIONS

JY and XW performed the experiments. JY was a major contributor to the writing of the manuscript. JZ and LY were

responsible for the experimental design and statistical analysis. LJ, SW, and XC participated in the experiments and data analysis. All the authors have read and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.634882/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Rituximab Therapy for Primary Membranous Nephropathy in a Chinese Cohort

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Background: Rituximab has become one of the first-line therapies for the treatment of moderate and high-risk primary membranous nephropathy (pMN). We retrospectively reviewed 95 patients with pMN who received rituximab therapy and focused on the therapeutic effects and safety of this therapy in a Chinese cohort.

Methods: Ninety-five consecutive patients with pMN diagnosed by kidney biopsy received rituximab and were followed up for >6 months. Four weekly doses of rituximab (375 mg/m^2) was adopted as the initial administration. Repeated single infusions were administrated to maintain B cell depletion levels of <5 cells/mL.

Results: A total of 91 patients completed rituximab therapy with the total dose of 2.4 (2.0, 3.0) g; 64/78 (82.1%) patients achieved anti-PLA2R antibody depletion in 6.0 (1.0, 12.0) months; 53/91 (58.2%) patients achieved clinical remission in 12.0 (6.0, 24.0) months, including complete remission in 18.7% of patients and partial remission in 39.6% of patients. Multivariate logistic regression analysis showed that severe proteinuria (OR = 1.22, $P = 0.006$) and the persistent positivity of anti-PLA2R antibodies (OR = 9.00, $P = 0.002$) were independent risk factors for no-remission. The remission rate of rituximab as an initial therapy was higher than rituximab as an alternative therapy (73.1 vs. 52.3%, $P = 0.038$). Lastly, 45 adverse events occurred in 37 patients, but only one patient withdrew from treatment due to severe pulmonary infection.

Conclusion: Rituximab is a safe and effective treatment option for Chinese patients with pMN, especially as an initial therapy.

Keywords: primary membranous nephropathy, CD20 monoclonal antibody, rituximab, anti-PLA2R antibody, first line treatment

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INTRODUCTION

Primary membranous nephropathy (pMN) is an autoimmune kidney disease and the most common cause of adult nephrotic syndrome (1, 2). The M-type phospholipase A2 receptor (PLA2R) expressed on podocytes is the most representative *in situ* antigen, and anti-PLA2R IgG4 antibodies are detected in nearly 70% of patients (2, 3), which bind to the antigen and form a subepithelial immune complex.

Nearly 30% of patients with pMN are able to achieve spontaneous remission; however, remission is less likely to occur in moderate and high-risk patients, namely patients with massive proteinuria, with or without normal and stable kidney function (4, 5). For those patients, cyclophosphamide combined with corticosteroids, or calcineurin inhibitors (cyclosporine or tacrolimus) have been widely administrated (1). With the advances in our understanding of the pathogenesis of pMN, there has been increased interest in rituximab, a monoclonal antibody against the CD20 antigen found on the surface of B cells. Remuzzi et al. (6) reported the first clinical application of rituximab for pMN treatment in 2002, showing the superiority of rituximab therapy to traditional immunosuppressive regimens in the short term. In 2012, Ruggenenti et al. (7) reported a 65% remission rate in 7.1 months, and a similar remission rate was observed in the GEMRITUX study. In addition, the authors of the GEMRITUX study found a higher rate of anti-PLA2R antibody depletion (56%) compared to the non-immunosuppressive treatment (4%) (8). In the MENTOR study, rituximab showed superiority in its therapeutic effect compared to cyclosporine (9). In the latest version of the KDIGO clinical practice guidelines (2020) on glomerular diseases, rituximab has been listed as a first-line therapy for moderate and high-risk patients with pMN (4).

Race has a certain influence on the pathogenesis and treatment of pMN. Xie et al. (10) mapped the human leukocyte antigen (HLA) locus, and reported that DRB1*1501 is the major risk allele in Asians, DQA1*0501 in Europeans, and DRB1*0301 in both races, suggesting that T cells may activate the PLA2R pathway on diverse epitopes. Additionally, Zhang et al. (11) proposed another mechanism of pMN pathogenesis in Chinese patients, namely environmental pollution. Long-term exposure to PM2.5 (particulate matter with diameter $\leq$ 2.5 microns in the atmosphere, also known as lung accessible particles) is a major risk factor for pMN, and the study by Zhang et al. showed that when the PM2.5 concentration is higher than 70 g/m^3, the incidence rate of pMN is directly proportional to PM2.5 concentration. Therefore, it is necessary to study the effect of rituximab on Chinese patients with pMN because of its specificity shown above.

In our study, rituximab therapy was administrated to a cohort of Chinese patients with pMN as an initial or alternative therapy, between January 2013 and 2020. To our knowledge, this is the largest-scale retrospective study of rituximab treatment in Asian patients with pMN.

METHODS

Participants

A total of 95 consecutive patients treated at Peking University First Hospital from January 2013 to 2020 were retrospectively reviewed and fulfilled the following criteria: (i) biopsy-proven pMN; (ii) received rituximab as an initial or alternative therapy; and (iii) were not accompanied by chronic infectious diseases that affect immunosuppressive therapy, such as tuberculosis and acquired immune deficiency syndrome (AIDS). Clinical data were collected from the patient's medical records.

Rituximab Treatment and Follow-Up

Four weekly doses of rituximab therapy (375 mg/m^2) was adopted as the initial administration. The clinicians adjusted the dosage and/or frequency based on the individual characteristics of the patients, such as kidney function. Rituximab was infused as previously described (10). CD19+ B lymphocyte depletion was defined as <5 cells/mL and was evaluated at each follow-up. After the initial administration, repeated infusions of rituximab were administrated at 375 mg/m^2 for single usage, once B cell levels reached >5 cells/mL within a few days.

All the patients underwent a series of follow-up appointments after initial rituximab administration at month 0, 1, 3, 6, and at subsequent 6-month intervals until the endpoint. The endpoint was end-stage renal disease (ESRD) or death. Laboratory evaluations, including 24-h proteinuria, serum albumin, creatinine, eGFR, anti-PLA2R antibodies, and circulating B cell amount, were performed at baseline and at every visit. Adverse events related to rituximab were evaluated during drug infusion and the entire follow-up period.

Treatment Responses and Renal Outcomes

To evaluate therapeutic responses, complete remission was defined as proteinuria <0.3 g/24 h, and partial remission was defined as proteinuria <3.5/24 h or a reduction of >50% from baseline, with improvement or normalization of serum albumin concentration, and stable or elevated <30% from baseline of serum creatinine. Patients who did not reach remission were considered non-responders. The recurrence of proteinuria >3.5 g/24 h after a period of remission was regarded as relapse (9, 12). To evaluate kidney outcomes, the primary endpoint was ESRD with eGFR < 15 mL/min/1.73 m^2 or receiving dialysis, and the secondary endpoint was the elevation of serum creatinine or a >50% reduction of eGFR from baseline (12).

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 23.0 (IBM, New York, USA). Data following a non-normal distribution were presented as median [interquartile range (IQR)]. For Data following a normal distribution, quantitative and semi-quantitative data were expressed as mean $\pm$ standard deviation (SD). A *t*-test was used to assess differences between quantitative data and Kruskal–Wallis test or Mann–Whitney *U*-test was used for semi-quantitative data. Qualitative data were expressed as amount (percentage) and a chi-squared test or one-way variation analysis (ANOVA) were used to assess the differences. All probabilities were two-sided and a $p < 0.05$ was considered statistically significant. Logistic regression and Cox regression analyses were performed to confirm potential risk or protection factors of treatment responses.

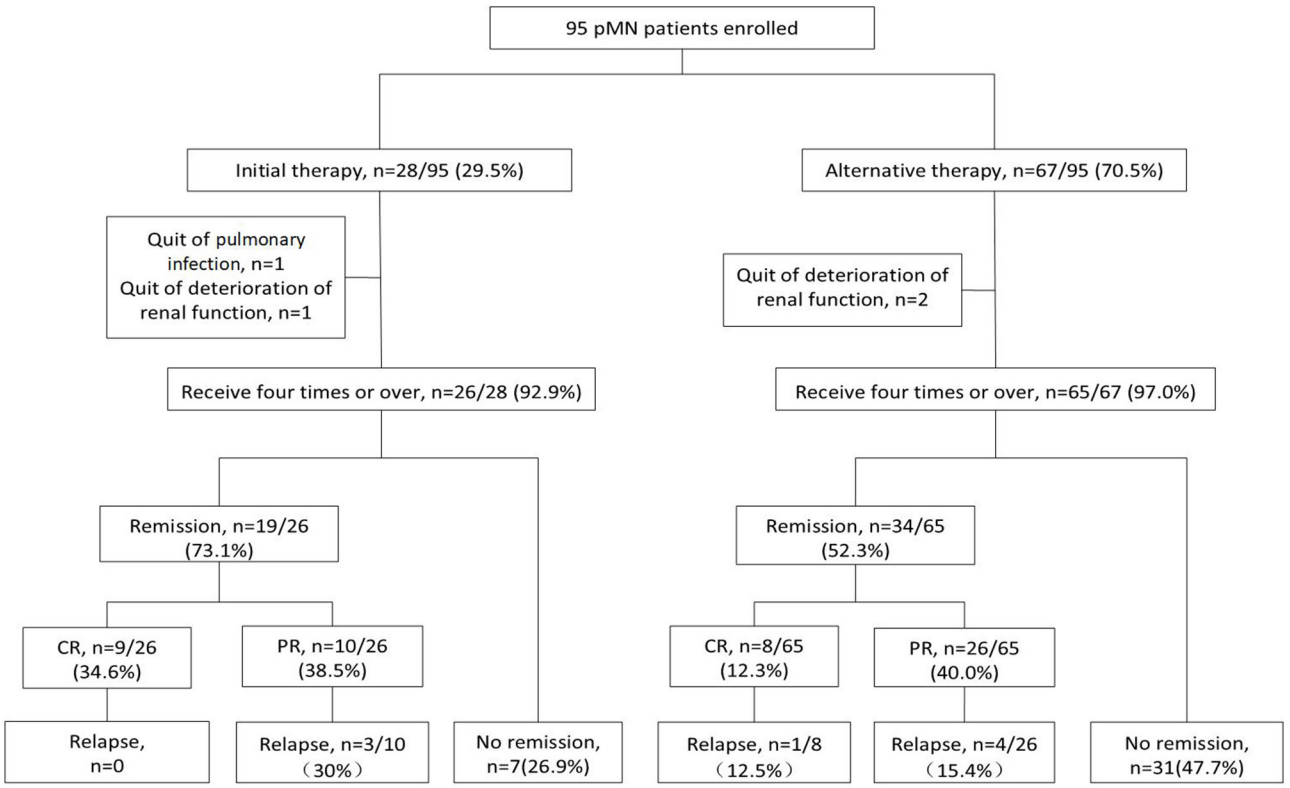

FIGURE 1 | Flow chart of the patients with pMN receiving rituximab therapy. There were 95 PMN patients enrolled, with 28 patients received rituximab as initial therapy and 67 as alternative therapy. There were 91 (95.8%) patients who accomplish the first administration of four times weekly usage and the remaining 4 (4.21%) patients did not. One patient discontinued treatment due to severe pulmonary infection, and three patients progressed into ESRD before the fourth infusion of rituximab. The remission rate was 73.1% (34.6% CR and 38.5% PR) in initial group and 52.3% (12.3% CR and 40.0% PR) in alternative group. 3/10 (30%) PR patients relapsed in initial group. 1/8 (12.5%) CR patients and 4/26 (15.4%) PR patients relapsed in alternative group. CR, complete remission; PR, partial remission.

RESULTS

Patients

There were 95 patients with pMN who received rituximab treatment as an initial or alternative therapy (**Figure 1**). The clinical and pathological characteristics of the patients at baseline is presented in **Table 1**. There were 83 male patients and 12 female patients, with a median age of 47 (30, 59) years old. The level of proteinuria was 9.6 (5.7, 13.4) g/24 h, serum albumin was 21.7 (18.9, 28.1) g/L, and eGFR was 63.8 (39.4, 93.9) mL/min/1.73 m^2. There were 82 (86.3%) patients positive for anti-PLA2R antibodies (>20 U/mL), with a median level of 122.5 (47.0, 309.0) U/mL. All patients underwent kidney biopsy and were diagnosed with pMN. Granular deposits of IgG were observed in all patients, with a median staining intensity of 3+, and the staining intensities of IgG1, IgG2, IgG3, and IgG4 were 2+, 1+, 1+, and 3+, respectively. Additionally, 93.5% of the patients had stage I or stage II membranous injury, 6.5% had stage III, and no patient had stage IV.

A total of 28 patients received rituximab as initial therapy and 67 as alternative therapy (**Figure 1**). Following diagnosis by percutaneous renal biopsy, the alternative therapy group was administered 2 (1–6) courses of immunosuppressant therapy before rituximab treatment, including cyclosporine combined with steroids in 53 (76.8%) patients, cyclophosphamide combined with steroids in 43 (62.3%) patients, tacrolimus in 36 (52.2%) patients, mycophenolate mofetil in 23 (33.3%) patients, and leflunomide in 6 (8.7%) patients. Among the 67 patients in the alternative therapy group, 35 (52.2%) achieved remission and the remaining 32 (47.8%) had no response.

In general, the alternative therapy group was younger and presented with more severe condition, both of which predicted worse responses (**Table 1**). The initial therapy group were older [51.0 (32.3, 67.5) vs. 45.0 (28.0, 58.0) years, $p = 0.020$], and presented with lower levels of proteinuria [7.3 (4.6, 12.2) vs. 10.6 (6.3, 15.0) g/24 h, $p < 0.001$], higher levels of serum albumin [23.2 (19.8, 30.1) vs. 20.8 (17.8, 28.1), $p = 0.006$], and higher levels of anti-PLA2R antibodies [243.0 (73.0, 460.0) vs. 94.0 (39.0, 213.0), $p = 0.003$], compared to the alternative therapy group. Gender, anti-PLA2R antibody positivity, serum creatinine, eGFR, and pathological features were comparable between the two groups ($p > 0.05$).

Rituximab Dosage

There were 91 (95.8%) patients who received four or more rituximab infusions, with a total dose of 2.4 (2.0, 3.0) g. The remaining 4 (4.21%) patients did not complete the

TABLE 1 | Baseline characteristics of patients with pMN included in this study.

Parameters*	Total ($n = 95$)	Initial therapy ($n = 28$)	Alternative therapy ($n = 67$)	$P^{\S}$
Gender [male, *n* (%)]	83 (86.5%)	22 (75.0%)	61 (91.0%)	0.057
Age (years)	47.0 (30.0, 59.0)	51.0 (32.3, 67.5)	45.0 (28.0, 58.0)	**0.020**
Proteinuria (g/24 h)	9.6 (5.7, 13.4)	7.3 (4.6, 12.2)	10.6 (6.3, 15.0)	**<0.001**
Serum albumin (g/L)	21.7 (18.9, 28.1)	23.2 (19.8, 30.1)	20.8 (17.8, 28.1)	**0.006**
Anti-PLA2R antibody positivity, *n* (%)	82 (86.3%)	27 (96.4%)	55 (82.1%)	0.427
Anti-PLA2R antibodies (U/mL)	122.5 (47.0, 309.0)	243.0 (73.0, 460.0)	94.0 (39.0, 213.0)	**0.003**
Serum creatinine (μmol/L)	110.7 (85.3, 161.0)	88.1 (64.6, 127.4)	131.6 (92.5, 180.0)	0.078
eGFR† (mL/min/1.73 m^2)	63.8 (39.4, 93.9)	84.8 (53.5, 108.5)	52.6 (37.6, 87.2)	0.066
Pathological features				
PLA2R staining (0–4+)	1.8 (1.0, 2.0)	1.3 (1.0, 2.0)	2.0 (1.0, 2.0)	0.507
IgG deposit (0–4+)	3.0 (3.0, 4.0)	3.3 (2.5, 4.0)	3.0 (3.0, 3.0)	0.290
Stages of membranous injury	2.0 (1.0, 2.0)	2.0 (1.0, 2.0)	2.0 (1.0, 2.0)	0.807

**Median (IQR). §Comparisons between initial therapy group and alternative therapy group. †eGFR, estimated glomerular filtration rate. The bold values represents P < 0.05.*

TABLE 2 | Details of rituximab therapy and follow-up.

Parameters	Total ($n = 95$)	Initial therapy ($n = 28$)	Alternative therapy ($n = 67$)	*P*
Rituximab treatments†	$n = 91$	$n = 26$	$n = 65$	
Total dose (g)	2.4 (2.0, 3.0)	2.4 (2.4, 2.8)	2.4 (2.0, 3.0)	0.604
Infusion times (mean ± SD)	4.7 ± 1.3	4.2 ± 0.6	4.9 ± 1.4	**0.021**
Treatment responses				
Remission, *n* (%)	53 (58.2%)	19 (73.1%)	34 (52.3%)	**0.038**
CR/PR	17/36	9/10	8/26	0.173
Relapse, *n* (%)	8/53 (15.1%)	3/19 (15.8%)	5/34 (14.7%)	0.577
No response, *n* (%)	38 (41.8%)	7 (26.9%)	31 (47.7%)	**0.038**
Follow-up time (months)	24.0 (7.5, 36.0)	18.0 (6.0, 24.0)	24.0 (12.0, 36.0)	0.065

†91 patients who received rituximab for four times or over. The bold values represents P < 0.05.

first administration of four doses (**Figure 1**). One patient discontinued treatment due to severe pulmonary infection, and three patients progressed into ESRD before the fourth infusion of rituximab. Compared to the initial therapy group, the alternative therapy group required more rituximab infusions (4.9 ± 1.4 vs. 4.2 ± 0.6, $P = 0.021$), and achieved a lower remission rate (52.3 vs. 73.1%, $P = 0.038$; **Table 2**).

Treatment Responses

Immunological Responses

In the 91 patients who received at least four infusions of rituximab, 78 (85.7%) patients had positive anti-PLA2R antibodies at baseline, with a median level of 120.2 (45.1, 306.4) U/mL. After rituximab therapy, 64/78 (82.1%) patients achieved immunological remission with anti-PLA2R antibody depletion (<20 U/mL) in 6.0 (1.0, 12.0) months. Among the antibody-deleption group, 48/64 (75.0%) patients achieved clinical remission, with 17 achieving complete remission and 31 achieving partial remission. There were 14 (17.9%) patients who maintained positive anti-PLA2R antibodies during the entire follow-up period, although their B cell levels were maintained at <5/mL. Among these 14 patients, only 2 (14.3%) patients achieved clinical remission. There was a significant difference in remission rate between antibody-deleption and antibody-undeleption group ($P < 0.001$). During follow-up, antibodies reoccurred in 12/64 (18.8%) patients, in which 9/12 (75.0%) patients were non-responders, 2 (16.7%) patients relapsed, and 1 (8.3%) patient remained in partial remission.

Clinical Responses

During the follow-up period of 24.0 (7.5, 36.0) months, clinical remission was achieved in 53/91 (58.2%) patients at 12.0 (6.0, 24.0) months, including 17 (18.7%) with complete remission and 36 (39.6%) with partial remission. Anti-PLA2R antibodies ($P = 0.037$) and proteinuria ($P < 0.001$) decreased in all patients receiving rituximab therapy, especially in responders. eGFR remained stable in responders and decreased significantly in non-responders ($P < 0.001$). The initial therapy group had a higher remission rate compared to the alternative therapy group [19/26 (73.1%) vs. 34/65 (52.3%), $P = 0.038$] and achieved remission sooner [12.0 (6.0, 18.0) vs. 15.0 (6.0, 24.0) months] (**Figure 2**).

For the initial therapy group, the persistent positivity of anti-PLA2R antibodies (OR = 45.00, 95% CI = 3.35–603.99, and $P = 0.004$) was the only risk factor to treatment failure. For the alternative therapy group, univariate logistic regression analysis showed that higher levels of proteinuria (OR = 1.18, 95% CI = 1.06–1.31, and $P = 0.002$), higher levels of anti-PLA2R antibodies (OR = 1.01, 95% CI = 1.00–1.01, and $P = 0.024$), and the persistent positivity of anti-PLA2R antibody (OR = 11.02, 95% CI = 2.78–43.75, and $P = 0.001$) were risk factors to no remission. In contrast, older age (OR = 0.96, 95% CI = 0.93–0.99, and $P = 0.016$) and a higher concentration of serum albumin (OR = 0.89, 95% CI = 0.82–0.97, and $P = 0.008$) were protective factors. Multivariate

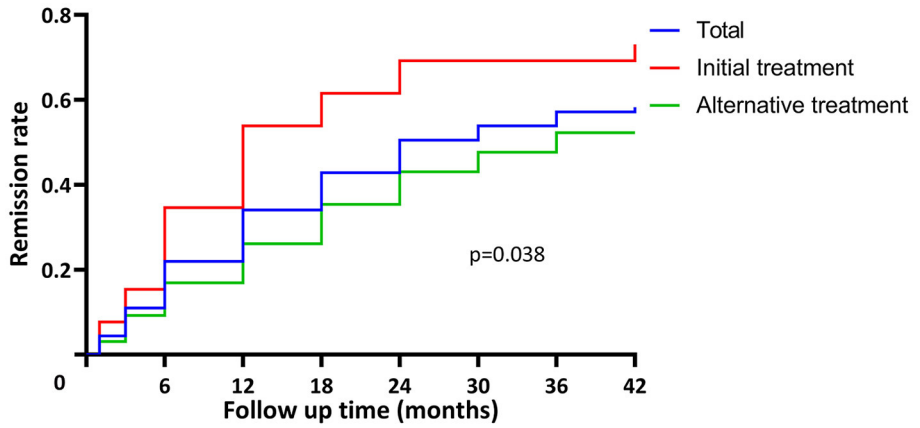

FIGURE 2 | Time to remission in patients with pMN receiving rituximab treatment. The remission rate was 58.2% (53/91) in total. The initial therapy group had higher remission rate compared to the alternative therapy group [19/26 (73.1%) vs. 34/65 (52.3%), $P = 0.038$] and achieved remission sooner [12.0 (6.0, 18.0) vs. 15.0 (6.0, 24.0) months].

TABLE 3 | The risk factors for no-remission of patients with pMN receiving rituximab for initial therapy or alternative therapy (logistic regression).

	Univariate analysis		Multivariate analysis	
	OR (95% CI)	***P*-value**	**OR (95% CI)**	***P*-value**
Initial therapy ($n = 26$)				
Gender (male)	6.65×10^8 (0.00, ∞)	0.999		
Age (years)	1.01 (0.97, 1.06)	0.598		
Urinary protein (g/24 h)	1.08 (0.90, 1.30)	0.424		
Serum albumin (g/L)	1.00 (0.90, 1.10)	0.963		
Anti-PLA2R antibodies (U/mL)	1.00 (0.99, 1.01)	0.380		
Persistent positivity of antibody	45.00 (3.35, 603.99)	**0.004**		
eGFR (mL/min/1.73 m^2)	0.97 (0.95, 1.00)	0.086		
Total dose of rituximab	1.00 (0.99, 1.01)	0.785		
Infusion times of rituximab	1.24 (0.30, 5.15)	0.769		
Alternative therapy ($n = 65$)				
Gender (male)	2.07 (0.35, 12.13)	0.421		
Age (years)	0.96 (0.93, 0.99)	**0.016**	0.96 (0.91, 1.01)	0.115
Round of previous immunotherapy	1.01 (0.66, 1.55)	0.964		
Urinary protein (g/24h)	1.18 (1.06, 1.31)	**0.002**	1.19 (1.01, 1.39)	**0.033**
Serum albumin (g/L)	0.89 (0.82, 0.97)	**0.008**	0.99 (0.87, 1.13)	0.881
Anti-PLA2R antibodies (U/mL)	1.01 (1.00, 1.01)	**0.024**	1.01 (1.00, 1.01)	0.052
Persistent positivity of antibody	11.02 (2.78, 43.75)	**0.001**	5.59 (0.96, 32.46)	0.055
eGFR (mL/min/1.73 m^2)	1.00 (0.98, 1.01)	0.514		
Total dose of rituximab	1.00 (1.00, 1.00)	0.732		
Infusion times of rituximab	1.09 (0.77, 1.55)	0.616		

The bold values represents $P < 0.05$.

logistic regression analysis showed that a higher level of proteinuria (OR = 1.19, 95% CI = 1.01–1.39, and $P = 0.033$) was the independent risk factor to no remission after rituximab treatment (**Table 3**).

Compared to the responders, the non-responders were more likely to be male (97.4 vs. 83.0%, $P = 0.031$), younger [43.5 (26.8, 56.0) vs. 51.0 (33.0, 65.0), $P = 0.025$), and have higher levels of urinary protein [12.0 (9.2, 16.1) vs. 7.6 (4.6, 12.2), $P < 0.001$], lower concentrations of serum albumin [20.2 (16.7, 23.3) vs. 26.5 (19.7, 30.3), $P = 0.005$], and higher levels of anti-PLA2R antibodies [204.0 (53.5, 444.5) vs. 98.0 (47.5, 228.0), $P = 0.004$] at baseline. The univariate logistic regression analysis showed that the higher level of urinary protein (OR 1.18, 95% CI 1.08–1.29, $P < 0.001$), and the persistent positivity of anti-PLA2R antibodies (OR = 15.13, 95% CI = 4.54–50.40, and $P < 0.001$) were risk factors for no remission after rituximab treatment. In contrast, older age (OR = 1.00, 95% CI = 0.95–1.00, and $P = 0.028$) and a higher concentration of serum albumin (OR = 0.91, 95% CI = 0.84–0.97, and $P = 0.007$) were protective factors. Multivariate logistic regression analysis showed that the higher level of urinary protein (OR = 1.22, 95% CI = 1.06–1.40, and $P = 0.006$) and the persistent positivity of anti-PLA2R antibodies (OR = 9.00, 95% CI = 2.18–37.19, and $P = 0.002$) were independent risk factors to rituximab treatment failure, while older age (OR = 0.96, 95% CI = 0.93–1.00, and $P = 0.047$) was an independent protective factor (**Figure 3**).

During the follow-up period, 8/53 (15.1%) patients relapsed after clinical remission (one complete remission and seven partial remission). Among them, three patients were from the initial therapy group and five from the alternative therapy group. After relapsing, one patient achieved partial remission after receiving rituximab therapy again, and the remaining seven patients did not achieve remission in the follow-up period after receiving cyclophosphamide and/or calcineurin inhibitor therapy. Compared to the patients who stayed in remission, relapsed patients presented with lower eGFR [40.1 (19.2, 79.0) vs. 79.5 (46.8, 102.5) mL/min/1.73 m^2, $P = 0.034$] at baseline. Univariate logistic regression analysis showed that higher eGFR

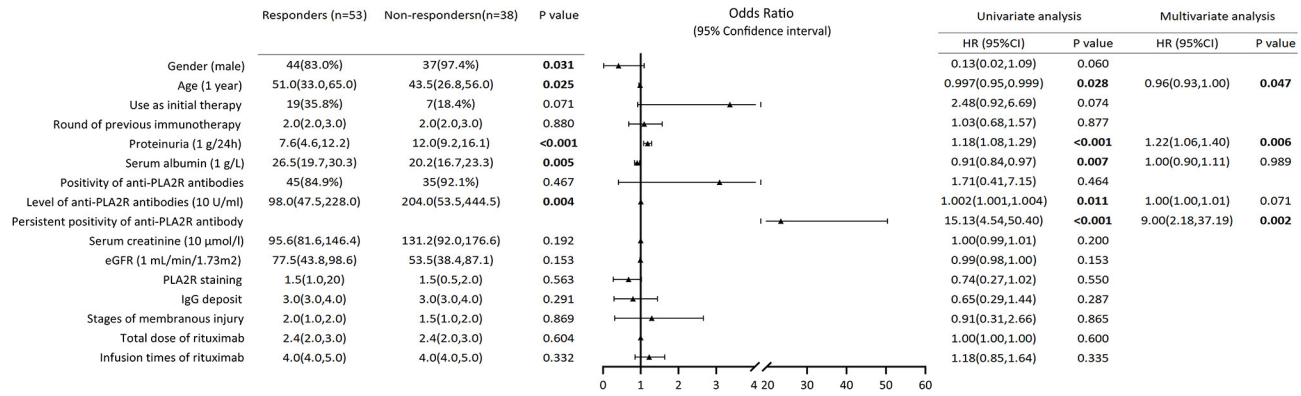

FIGURE 3 | Composite comparisons of clinical features of patients with pMN between responders and non-responders. Compared to the responders, the non-responders were more likely to be young man with higher levels of urinary protein, lower concentrations of serum albumin and higher levels of anti-PLA2R antibodies. The univariate logistic regression analysis showed that the higher level of urinary protein and the persistent positivity of anti-PLA2R antibodies were risk factors for not achieving remission after rituximab treatment. Multivariate logistic regression analysis showed that the higher level of urinary protein and the persistent positivity of anti-PLA2R antibodies were independent risk factors to rituximab treatment failure, while older age was an independent protective factor.

TABLE 4 | The risk factors for relapse of patients with pMN receiving rituximab.

	Univariate analysis	
	OR (95% CI)	***P*-value**
Gender	1.81 (0.30, 10.86)	0.517
Age (years)	1.05 (1.00, 1.11)	0.064
Initial therapy	0.92 (0.19, 4.36)	0.916
Round of previous immunotherapy	1.85 (0.77, 4.48)	0.172
Urinary protein (g/24 h)	1.07 (0.92, 1.24)	0.368
Serum albumin (g/L)	0.93 (0.82, 1.05)	0.240
Higher level of anti-PLA2R antibodies (U/ml)	1.00 (1.00, 1.01)	0.340
Positive antibody at remission	1.44 (0.14, 14.98)	0.760
eGFR (mL/min/1.73 m^2)	0.97 (0.95, 1.00)	**0.045**
Total dose of rituximab	1.00 (0.99, 1.01)	0.744
Infusion times of rituximab	1.03 (0.46, 2.31)	0.943

The bold values represents P < 0.05.

TABLE 5 | The risk factors for kidney dysfunction of patients with pMN receiving rituximab as eGFR decreased >50% or ESRD (Cox regression).

	Univariate analysis	
	OR (95% CI)	***P*-value**
Age (years)	1.03 (1.00, 1.07)	**0.040**
Initial therapy	0.48 (0.12, 1.88)	0.319
Round of previous immunotherapy	0.50 (0.17, 1.51)	0.217
Urinary protein (g/24 h)	1.04 (0.94, 1.16)	0.459
Serum albumin (g/L)	1.04 (0.81, 1.34)	0.734
Higher level of anti-PLA2R antibodies (U/mLs)	1.00 (0.99, 1.01)	0.487
Persistent positivity of antibody	0.94 (0.12, 7.53)	0.952
eGFR (mL/min/1.73 m^2)	0.97 (0.95, 1.00)	**0.020**
Total dose of rituximab	1.00 (0.99, 1.01)	0.910
Infusion times of rituximab	0.88 (0.48, 1.58)	0.654
No remission	0.88 (0.23, 3.31)	0.849
Relapse	51.92 (0.00, 6.36 × 10^6)	0.144

The bold values represents P < 0.05.

(OR = 0.97, 95% CI = 0.95–1.00, $P = 0.045$) was the only protective factor to relapse (**Table 4**).

Kidney Outcomes

During the follow-up period, the median eGFR of all patients decreased 5.6 (−2.9, 14.5) mL/min/1.73 m^2, from the baseline level of 63.9 (39.6, 95.1), to an endpoint of 57.6 (30.0, 86.6) mL/min/1.73 m^2. In the responders, eGFR remained stable compared to the baseline level ($P = 0.750$). In the non-responders, eGFR fell significantly from 53.5 (38.4, 87.1) to 41.5 (17.2, 73.4) mL/min/1.73 m^2 ($P = 0.038$), and six patients progressed to ESRD.

There were 15/91 (16.5%) patients whose eGFR decreased more than 50% from the baseline or progressed to ESRD. Univariate Cox regression analysis indicated older age (OR = 1.03, 95% CI = 1.00–1.07, and $P = 0.040$) to be risk factor and higher eGFR (OR = 0.97, 95% CI = 0.95–1.00, and $P = 0.020$) to be a protective factor to kidney dysfunction outcome (**Table 5**).

Safety and Adverse Effects

Among the 95 patients, 37 (38.9%) patients experienced adverse effects during rituximab treatment (**Table 6**). One patient withdrew from treatment due to severe pulmonary infection, and five patients (two responders and three non-responders) presented with frequent upper respiratory infections (about twice per month) in the first 6 months of rituximab administration. The most frequent, but mild, adverse effects were infusion reactions, including bronchial wheezing, rash, erythema, itching, rhinorrhea, and dysphoria. No malignancy or fatal adverse event was observed in the follow-up period. The adverse events were more frequent in non-responders than in responders (52.6 vs. 28.3%, $P = 0.009$).

TABLE 6 | Adverse events in all patients with pMN receiving rituximab.

Adverse event	Total
Fatal	0
Nonfatal	45
Myelotoxicity	
Anemia	1
Thrombocytopenia	2
Central nervous system events	
Cerebral infarction	2
Transient loss of consciousness	1
Dizziness	5
Respiratory system events	
Pulmonary infection	1
Frequent upper respiratory infection (URI)	5
Dyspnea	2
Cough	1
Digestive system events	
Diarrhea	3
Abdominal pain	1
Nausea	5
Infusion reactions†	14
Others	
Muscular soreness	1
Fever	1
Any events*	
Serious adverse events (Grade ≥ 3)	3
Non-serious adverse events (Grade <3)	34
Total No. of adverse events	45
Patients with adverse events	37

*†Infusion reactions include bronchial wheezing, rash, erythema, itching, rhinorrhea, and dysphoria. *The grade classification standard is WHO Toxicity Grading Scale for Determining the Severity of Adverse Events. The bold values represents P < 0.05.*

DISCUSSION

In this study, we report the findings of rituximab therapy in a cohort of Chinese patients with pMN, and focus on the therapeutic outcomes and side effects of this treatment. We found that rituximab therapy showed good efficacy in 58.2% of all patients with pMN, with a higher rate (73.1%) of clinical remission as an initial therapy and a slightly lower rate (52.3%) as an alternative therapy. The median time to achieving remission was 12.0 months. The persistent positivity of anti-PLA2R antibodies (OR = 9.0) and more severe proteinuria (OR = 1.2) were independent risk factors to treatment failure. Side effects were observed in 38.9% of patients, consisting of mostly mild infusion reactions and several cases of respiratory infections. This retrospective analysis confirmed the therapeutic effects of rituximab therapy in Chinese patients with pMN and highlighted the necessity of antibody clearance for achieving clinical remission and better outcomes.

While the overall response rate of this cohort (58.2%) was lower than the remission rates (55–75%) from other cohorts treated with rituximab, the interval time from drug administration to clinical remission was similar between the current and previous reports (1, 5–9, 12–16). One possible reason may be the different proportions of patients enrolled in different studies. When rituximab was administrated as an initial therapy, patients achieved a higher rate (73.1%) of clinical remission, which was comparable to the remission rate of 69.1% as a first-line therapy reported by Ruggenenti et al. (7), and even better than the remission rate of 60% in the MENTOR study (9) and 64.9% in the GEMRITUX study (8). When administrated as an alternative therapy, rituximab showed a lower rate (52.3%) of clinical remission, which was comparable to the remission rate of up to 50.0% as a second-line therapy reported by Ruggenenti et al. (17). However, in the whole cohort, most (70.5%) patients received rituximab as an alternative therapy, due to the expensive cost and, thus, may explain the lower remission rate in the cohort as a whole. In general, the initial therapy group achieved a higher remission rate [19/26 (73.1%) vs. 34/65 (52.3%)] in shorter time [12.0 (6.0, 18.0) vs. 15.0 (6.0, 24.0) months] with fewer infusions (4.2 ± 0.6 vs. 4.9 ± 1.4). Thus, rituximab should be recommended for use as a first-line therapy for patients with moderate to high-risk pMN, rather than as a remedial therapy.

In this study, compared with the initial therapy group, patients in the alternative group presented with more advanced disease, which may interfere with the comparison of the therapeutic effect of rituximab. Therefore, we performed 1:1 propensity score matching to match urinary protein, albumin, renal function, anti-PLA2R antibodies, and got 17 pairs (34 patients in total). There was no significant difference in the severity of the disease between the two groups for gender, age, urinary protein, serum albumin, anti-PLA2R antibody and eGFR ($P = 0.384$, 0.096, 0.917, 0.673, 0.076, and 0.230, respectively), but there was a significant difference in the remission rate ($P = 0.009$). In the initial therapy group, 15/17 (88.2%) achieved remission, while in the alternative therapy group, only 8/17 (47.1%) achieved remission. Therefore, rituximab is more highly recommended as an initial treatment.

We previously reported the use of rituximab as an alternative therapy in our cohort (12), and most patients were enrolled in the current study, apart from a few patients who received rituximab <4 times. The remission rate increased slightly in recent years (from 41.7 to 52.3%) for several possible reasons. Firstly, all patients in the current study received the standard regimen of four infusions of rituximab (375 mg/m^2) at the first administration and the full dose therapy may be advantageous to better treatment responses. Secondly, the longer follow-up time in the current study (24.0 vs. 12.0 months) may help identify cases of remission that occur after 12 months. It's not possible that "lack of remission" was due to inadequate follow-up time in this study, as there was no significant difference between the complete and partial remission group in follow-up time [30.0 (15.0, 36.0) vs. 24.0 (13.5, 38.3) months, $P = 0.569$].

Anti-PLA2R antibodies have been identified as a pivotal biomarker in pMN clinical practice (1, 5, 18–20), and rituximab is effective at depleting anti-PLA2R antibodies. In the current study, the level of anti-PLA2R antibodies was much higher in non-responders than in responders at both the baseline and endpoint. The MENTOR study revealed that the depletion of anti-PLA2R antibodies occurred more rapidly and at a greater magnitude and duration in the rituximab group than in the

cyclosporine group (9). In this study, 82.1% of patients achieved antibody depletion in 6.0 months, and clinical remission was achieved in 12 months, confirming the efficacy of rituximab in eliminating anti-PLA2R antibodies and achieving clinical remission after antibody depletion. We further found that the persistent positivity of anti-PLA2R antibodies was one of the independent risk factors to no-remission following rituximab therapy, especially in the initial therapy group. For these patients, novel treatments that target memory B cells and long-lived plasma cells might be of consideration.

Severe proteinuria was another independent risk factor to no-remission, especially in the alternative therapy group. In this study, the baseline level of proteinuria was much higher in non-responders. The remission rate was 100% in the patients with proteinuria <4 g/24 h, 67.9% in 4–8 g/24 h, 52.4% in 8–12 g/24 h, and 42.4% in >12 g/24 h ($P = 0.009$). A pharmacokinetic experiment monitoring the serum concentration of rituximab from patients with pMN or rheumatoid arthritis showed that rituximab cleated more quickly in patients with proteinuria compared to those without proteinuria (21). However, rituximab levels did not correlate with treatment response (22, 23). Therefore, the mechanism explaining how severe proteinuria interfering rituximab treatment will require further investigation.

A significant gender bias existed in this study (12.6% female vs. 87.4% male), and may be due to a number reasons. Firstly, the incidence rate of pMN is higher in males than in females. Secondly, male patients require immunosuppressive treatment more often than female patients. Two studies by Cattran (24) reported that female patients are more likely to achieve spontaneous remission, while male patients progress more rapidly, even with comparable proteinuria (24, 25). Among the patients enrolled in this study, female patients had lower levels of proteinuria than male patients [6.19 (3.15, 8.51) vs. 10.52 (6.14, 13.60) g/24 h, $P = 0.034$] at baseline. Additionally, after rituximab treatment, female patients had a higher remission rate (90.0 vs. 54.3%, $P = 0.031$) as well. However, this result may be biased due to the small number of female patients.

Adverse events were observed in 37 (38.9%) patients, 3.2% of which were serious adverse events and 35.8% were non-serious. This was lower than previous studies that reported adverse events in 50–80% of patients and serious adverse events of 0–17% (8, 9, 12, 15). This difference might be due to the retrospective nature of this study, as some adverse events might have been forgotten, left out, or ignored from the medical records. Additionally, in the current and previous studies (9, 26), the most common adverse events were infusion-related reactions. As a biologic product, rituximab may trigger immune-mediated reactions, such as dyspnea, rash, erythema, itching, and others. However, we observed fewer infusion-related adverse events in this study compared to a previous study [14/95 (14.7%) vs. 28/100 (28.0%) (26)]. This may be due to the use of anti-allergic drugs before infusions and the restriction of infusion speed to avoid or ameliorate infusion-related events to some extent. We also found that adverse events were more frequent in non-responders than in responders. This may be due to immune-mediated sensitization syndrome that results from resistance to rituximab (1, 27). For the non-responders with immune-mediate infusion reactions, humanized anti-CD20 antibodies might be an alternative choice to avoid adverse events and drug resistance (28). Compared to steroids and cyclophosphamide therapy, rituximab was safer both in non-serious adverse events and in serious adverse events (26). Compared to cyclosporine, there was no significant difference in the incidence of adverse events from rituximab; however, fewer serious adverse events were observed in the rituximab group (9).

In conclusion, rituximab therapy was effective in the clearance of anti-PLA2R antibodies and for achieving clinical remission in a cohort of Chinse patients with pMN, especially as an initial therapy, with tolerable adverse events.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The current study complies with the Declaration of Helsinki and was approved by the ethics committee of Peking University First Hospital. Written informed consent was obtained for tissue, blood, and urine samples.

AUTHOR CONTRIBUTIONS

ZC and M-hZ: initial idea. ZC, XW, Y-mZ, FW, X-yC, L-qM, F-dZ, GL, and M-hZ: review and comments. SG: figures. SG and ZC: concept and writing. All authors contributed to the article and approved the submitted version.

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